Underexposed features of CHARGE syndrome: immunological, adrenal, and scapular function

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Contents

CHAPTER 1

General introduction

CHARGE syndrome is a complex, highly variable syndrome, and many of its clinical aspects have not yet been intensively studied. With the rapid introduction of new genome-wide genetic techniques, more genetic diagnoses of CHARGE are being made in individuals with otherwise clinically unrecognised expressions of the syndrome, but clinical guidelines are still under development and often lack evidence to support inclusion of newly associated anomalies.

1.1 CHARGE syndrome: a brief history and clinical presentation

CHARGE syndrome (MIM# 214800) is a syndrome presenting with multiple congenital anomalies (Figure 1). The acronym CHARGE stands for **C**oloboma, **H**eart defects, **A**tresia of the choanae, **R**etardation of growth and/or development, **G**enital abnormalities, and **E**ar abnormalities. In the Netherlands, its estimated incidence is 5.9–6.7 per 100,000 live-born children per year [1], and a national multidisciplinary CHARGE outpatients' clinic was established in 2005 to improve care and facilitate research.

The first reports of patients who presented with clinical features indicative for CHARGE syndrome were described by Angelman [3] and Edwards et al [4] in 1961. The authors noticed that coloboma were associated with other congenital anomalies that included, amongst others, heart defects, brain abnormalities, and external ear malformations. This coloboma-oriented association was also described by Hittner et al in 1979 [5]. In the same year, Hall described patients with choanal atresia associated with coloboma, heart defects, developmental delay, hypogenitalism, and small ears [6]. In 1981, Pagon et al recognised that the coloboma-oriented and choanal atresia–oriented associations had overlapping features and proposed the acronym CHARGE to alert clinicians to the need to evaluate patients for the anomalies in the CHARGE association [7].

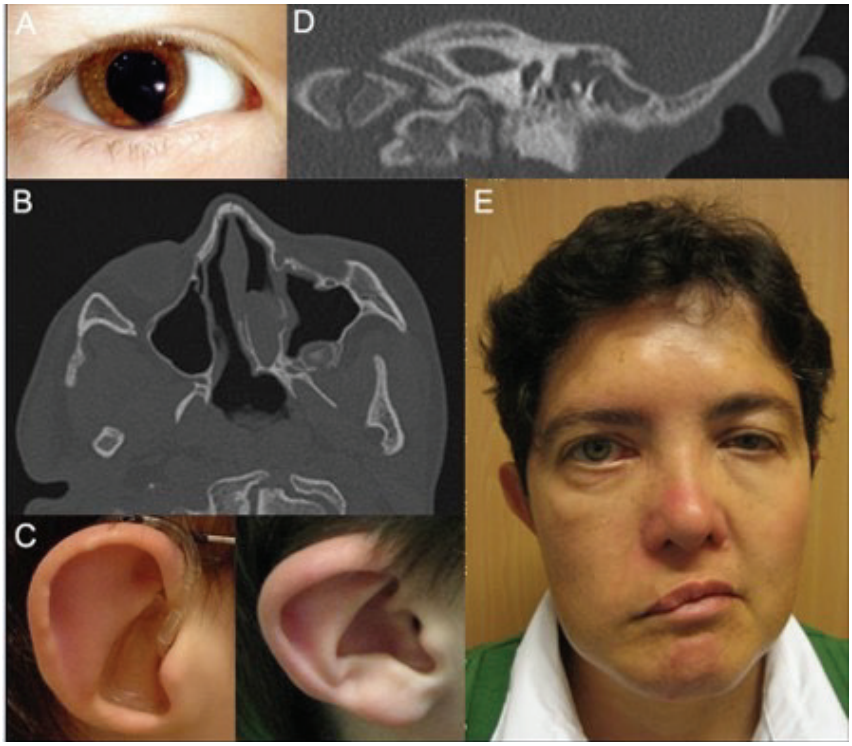

Figure 1. Overview of features occurring in CHARGE syndrome. (A) Coloboma of the iris and/or retina, with or without microphthalmia, often only visible by fundoscopy. (B) Choanal atresia (unilateral in image) or stenosis. (C) Characteristic ear anomaly: cup-shaped ear with triangular conchae and small or absent earlobes. Middle or inner ear malformations may also be present. (D) Semi-circular canal hypoplasia or aplasia. (E) Cranial nerve dysfunction: oculomotor dysfunction (III/VI), less powerful chewing (V), facial palsy (VII) (right-sided seen in image), hearing loss/vestibular problems (VIII), swallowing and feeding problems (IX/X). Informed consent was obtained for publication of the photographs.
Reproduced with permission of the authors from Bergman et al [2].

After CHARGE syndrome had been given the name, more clinical features were suggested, including characteristic facial features, central hypogonadism, renal anomalies, cranial nerve dysfunction, and semi-circular canal anomalies [8,9,10,11]. To accommodate clinicians in recognising the association, in 1998, Blake et al defined diagnostic rules by dividing symptoms into major and minor criteria [9]. Major criteria are features that commonly occur in CHARGE syndrome but are relatively rare in other conditions (Table 1). The minor features occur less frequently or are less specific for CHARGE syndrome. In addition, there are features that are occasionally found and may have consequences for clinical management. These features are thymic or parathyroid hypoplasia; anomalies of the renal tract, hand or spine; abdominal defects; webbed neck; and sloping shoulders. In 2005, Verloes proposed another set of diagnostic criteria that added semi-circular canal defects as a major criterion (Table 1) [12]. Verloes also proposed broadening the diagnosis by allowing the classification of partial and atypical presentations of the syndrome.

Table 1. Clinical diagnostic criteria for CHARGE syndrome

	Major criteria	Minor criteria	Inclusion rule
Blake [9]	Coloboma, microphthalmia	Cardiovascular malformations	*Typical CHARGE* 4 major or 3 major + 3 minor
	Choanal atresia or stenosis	Tracheoesophageal defects	
	Characteristic external ear abnormalities, middle/inner ear malformations, mixed deafness	Cleft lip and/or palate	
		Developmental delay	
	Cranial nerve dysfunction	Growth deficiency	
		Genital hypoplasia, delayed pubertal development	
		Characteristic face	
Verloes [12]	Coloboma	Heart and oesophageal malformations	*Typical CHARGE* 3 major or 2 major + 2 minor *Partial CHARGE* 2 major + 1 minor *Atypical CHARGE* 2 major + 0 minor or 1 major + 3 minor
	Choanal atresia		
	Hypoplastic semi-circular canals	Mental retardation	
		Hypothalamo-hypophyseal dysfunction (growth hormone or gonadotrophin deficiency)	
		Rhombencephalic dysfunction (brainstem/cranial nerve dysfunctions, neurosensory deafness)	
		Abnormal middle or external ear	

1.2 Genetic background

Given the absence of a common pathogenic factor, CHARGE remained an association up until the discovery of haploinsufficiency of the *CHD7* gene (MIM# 608892) as the cause, which allowed CHARGE to finally be classified as a syndrome [13]. The *CHD7* gene is located at chromosome 8 (8q12) and consists of 38 exons, of which the first is non-coding (Figure 2). In 2012, an overview of *CHD7* sequence variants, submicroscopic genomic rearrangements, and translocations was provided via an online-accessible database at www.chd7.org [1]. A total of 528 pathogenic *CHD7* variants have been identified. The majority are intragenic variants. Nonsense (44%) and frameshift (34%) variants are most prevalent, followed by splice site (11%) and missense (8%) variants. Complete and partial deletions/duplications and chromosomal rearrangements are rare (< 1–2%). Variants are distributed along the entire coding region and splice sites of *CHD7*, although pathogenic missense variants are more often found in the highly conserved middle exons of the gene that include functional domains.

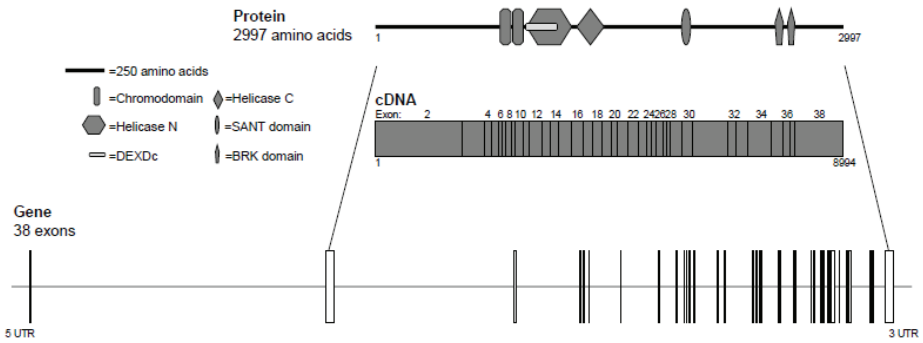

Figure 2. The *CHD7* gene. An overview of *CHD7* with its 38 exons and introns is shown at bottom. The sizes of the exons and introns are drawn to scale. The cDNA of CHD7 consists of 37 exons. The first exon and part of genomic exons 2 and 38 are non-coding (middle). The CHD7 protein consists of 2997 amino acids and has several conserved domains, which are drawn to scale (top). Chromodomain, chromatin organisation 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. Reproduced with permission of the authors from Janssen et al [1] .

Because 5–10% of patients with CHARGE syndrome do not have a haploinsufficiency of CHD7, researchers have looked for variants in other candidate genes, including *PITX2* (MIM# 601542) [14], *SEMA3E* (MIM# 608166) [15], *PAX2* (MIM# 167409) [16], *CHD8* (MIM# 610528) [17], and *SEMA3A* [18]. However, no other gene has been proven to be causative for CHARGE syndrome.

Most patients have a *de novo* variant in *CHD7, a*lthough familial recurrence occurs sporadically. In the rare familial cases with parent to child transmission, the parents are mostly mildly affected and do not fulfil the clinical diagnostic criteria [2]. In most situations, the affected parent carries either a missense variant or a mosaic variant.

1.3 Pathogenesis

The current theory behind the pathogenesis of CHARGE syndrome lies in haploinsufficiency of the *CHD7* gene. During normal embryonic development, CHD7 is highly expressed in tissues related to the congenital anomalies seen in CHARGE syndrome [19,20,21,22,23,24,25]. The CHD7 protein has been shown to regulate the transcription of other genes in a time- and tissue-specific way. It forms complexes with other proteins to ensure specific binding to numerous genes in different cell types [26,27]. In CHARGE syndrome, variants in or deletions of the *CHD7* gene result in a reduced amount of CHD7 protein being available to preserve normal function. Therefore, the regulation of transcription of genes is altered, leading to an abnormal or diseased state [28,26,29,30]. The precise gene targets and complexes for each specific tissue are still being investigated.

1.4 Expanding the phenotypic spectrum

Since CHARGE syndrome can be diagnosed by detecting pathogenic variants of *CHD7,* patients with a milder phenotype who would not meet the clinical diagnostic criteria for CHARGE syndrome may now be diagnosed through their genetic defect [31,32,33,34]. As a consequence, the phenotypic spectrum of CHARGE syndrome has broadened towards the milder or atypical range of the clinical spectrum (Table 2). This effect seems to be even stronger with the use of genome-wide genetic techniques, which have become more reliable, easier to use, and cost-efficient, resulting in the diagnosis of more patients without a prior clinical suspicion of CHARGE syndrome [35].

Table 2. Prevalence of clinical features in patients with CHARGE syndrome before and after *CHD7* analysis was possible (adapted from Bergman et al [2] and van Ravenswaaij and Martin [35]).

Feature	Prevalence (%), Before CHD7 analysis	Prevalence (%), After CHD7 analysis
Major features		
External ear anomaly	96	95
Semi-circular canal anomaly	100	94
Coloboma	77	80
Choanal atresia	61	49
Cranial nerve dysfunction (VII, VIII and others)	86	94
Minor features		
Cleft lip and/or palate	18	40
Feeding difficulties	85	82
Facial palsy	36	58
Anosmia	Unknown	80
Genital hypoplasia	36	68
Congenital heart defect	85	78
Trachea-oesophageal anomaly	18	25
Developmental delay	100	99
Growth retardation	65	55

In addition, the phenotypic spectrum has also expanded in complexity. By studying large groups of patients with a genetically confirmed diagnosis, additional clinical aspects will be found to be more common than previously thought. This was already the case after the association was given the acronym CHARGE, when professionals found additional clinical signs and symptoms resulting in clinical criteria to support the diagnosis [9,12]. Modifications in the clinical diagnostic criteria were and are continuously made [36,37]. In the most recent update, Hale et al [37] proposed

further broadening the set of clinical criteria to include feeding difficulties, brain anomalies, autism, and renal/skeletal/limb anomalies. Importantly, they also include the pathogenic *CHD7* variant as one of the major criteria to account for patients with an atypical or even unrecognisable presentation, which may further expand the list of clinical features with features that were not previously noticed in CHARGE syndrome.

Thus, by using gene-specific (Table 2) or genome-wide molecular diagnostics in patients with non-typical presentation and by studying large cohorts of patients, the phenotypic spectrum of CHARGE syndrome has expanded considerably. Nonetheless, some clinical aspects of the syndrome may still be underdiagnosed even though these can have an impact on the patient's well-being.

1.5 Clinical overlap with other multiple congenital anomaly syndromes

Another way to explore the phenotypic spectrum is to search for features that are common in other multiple congenital anomaly syndromes but not fully explored in CHARGE syndrome. Many other multiple congenital anomaly syndromes share clinical overlap with CHARGE syndrome (see Figure 1 in Chapter 2) [38,39,40]. This overlap can be explained at a molecular level because CHD7 shares the same molecular pathway or pathways with most of the proteins that are affected in the other syndromes [20,28,41,42,43,44]. The most remarkable overlap is with 22q11.2 deletion syndrome (#MIM 192430). Both syndromes share congenital heart defects, cognitive and motor delay, hearing loss, external ear anomalies, cleft lip and palate, growth deficiency, renal anomalies, and immunodeficiency [38,45]. Thus, by exploring the overlap with other multiple congenital anomaly syndromes, new or unrecognised features can be discovered that could be helpful in clinical diagnosis and management (see section 1.7). Furthermore, exploring overlap can generate new insights into disease pathogenesis.

1.6 Consequences of an expanding phenotypic spectrum for clinical management

Because of the phenotypic variability and complexity, it is important to have a comprehensive guideline for clinical surveillance of patients with CHARGE syndrome. General guidelines provide an overview for professionals to understand the syndrome and systematically check for the most prevalent symptoms and signs [46,47,48]. Various groups worldwide have been studying diverse clinical aspects and have formulated an array of guidelines and recommendations on paper. The topics of these publications concern, amongst others, cochlear implantation [49,50], neural imaging [47,51], and puberty [52]. However, we noticed that three clinical aspects had not yet been sufficiently covered by the existing guidelines: immunity to infections, adrenal function, and scapular anomalies. In the following paragraphs I explain why I chose to investigate these three aspects.

1.7 Immunology in CHARGE syndrome

Recurrent infections are well recognised in children with CHARGE syndrome, with infections of the upper airway, sometimes complicated by pneumonia, frequently reported [45,53,54,55,56]. Deviations of the palatal and ear anatomy, as well as cranial nerve dysfunction that affects swallowing, are thought to contribute to heightened susceptibility to infections and to extend the duration of infections by impeding drainage or clearance of infectious debris. However, knowledge about whether immunological abnormalities contribute to the increased frequency and complications of infections is important for optimising the management of care in these patients. T-cell lymphopenia and thymic abnormalities, which could lead to serious infections, have been described in patients with CHARGE syndrome [22,45]. These abnormalities resemble immunological abnormalities seen in 22q11.2 deletion syndrome [45,57]. In contrast to 22q11.2 deletion syndrome, the frequency and exact nature of immunological abnormalities in CHARGE syndrome have so far not been studied prospectively or systematically. The few papers to study immunology in CHARGE syndrome were either retrospective or case studies (see Chapter 2). Due to the lack of a better estimation of the immune function, some authors recommend performing an immunological evaluation in all patients [58,59]. But it is not clear what exactly should be evaluated. Humoral or cellular immunity? Or both? Quantitative or qualitative? Or both?

1.8 Adrenal function in CHARGE syndrome

Another unexplored issue is the adrenal function in patients with CHARGE syndrome. Adrenal insufficiency of hypothalamic/pituitary (central) origin (central adrenal insufficiency, CAI) might play a role in sudden and unexplained death [60], especially during stressful events like infections and surgical procedures when the cortisol level is not sufficient. The prevalence and possible contribution of CAI in sudden death has been explored in children with Prader-Willi syndrome [61,62]. It is important to study CAI in children with CHARGE syndrome because, if CAI is present, mortality may be prevented with the prescription of corticosteroids during stressful events. This recommendation can be included in the current guidelines [46,48], which lack recommendations on adrenal function.

1.9 Scapular anomalies in CHARGE syndrome

Musculoskeletal anomalies, specifically sloping shoulders, webbed neck, and scoliosis, are common features of CHARGE syndrome (Figure 3), although they are not included in the clinical diagnostic criteria of Blake [9] or Verloes [12]. One explanation for this may be that these anomalies are not specific enough. Nonetheless, these anomalies might require additional clinical management if their severity impairs the daily activities of the affected person. We often encounter patients with mobility problems in the shoulder region in the Dutch National multidisciplinary CHARGE

outpatients' clinic [63] and at national and international meetings with patients.

A study that carried out whole-exome sequencing in patients with undiagnosed limb-girdle muscular dystrophies identified a patient with a *CHD7* variant [31]. This patient, who had no other CHARGE features, had been experiencing scapular problems without a known cause for several years. This result, combined with our clinical experiences, prompted us to explore the scapular issues of patients with CHARGE syndrome. It could benefit patient care if scapular issues are included in the medical checklists and guidelines, or even in the clinical diagnostic criteria.

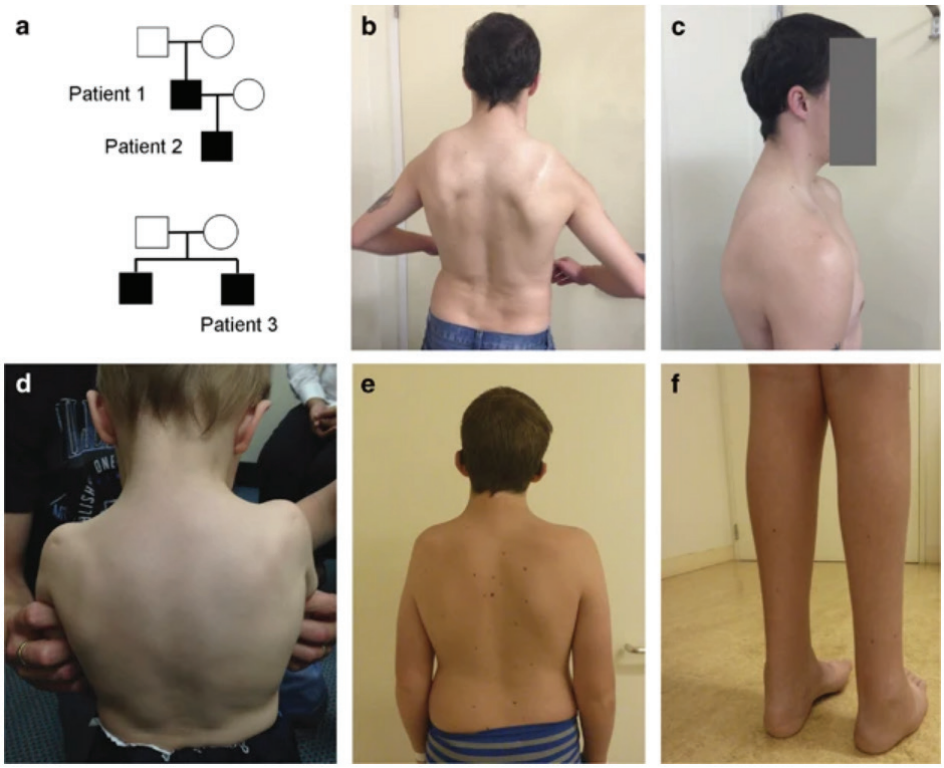

Figure 3. Musculoskeletal anomalies in CHARGE syndrome. (a) Pedigrees illustrating the relationships of Patient 1 and 2 and Patient 3. (b) Posterior view of Patient 1 showing sloping shoulders, dorsal winging, and lateral displacement of the scapulae during arm abduction. (c) Lateral view of Patient 1 showing anterior position of the shoulders, small ears, and mild retrognathia. (d) Posterior view of Patient 2 showing laterally displaced scapulae and bilateral acromial dimples. (e) Patient 3 showing mild prominence of the right scapula. (f) The lower legs of Patient 3 showing calf atrophy and pes cavovarus. Reproduced with permission of the authors from O'Grady et al [64].

1.10 Scope and outline of this thesis

The aim of this thesis is to improve the recognition and clinical management of symptoms in patients with CHARGE syndrome by exploring the phenotypic spectrum, with a focus on immunological aspects and adrenal and musculoskeletal function.

To start, I identified gaps in our knowledge on the immunological aspects in CHARGE syndrome that needed further study by conducting a literature search on immunologic abnormalities in CHARGE syndrome (**Chapter 2**). I also explored the immunological abnormalities in comparable multiple congenital anomaly syndromes to identify common immunological phenotypes and genetic pathways that might regulate the immune system.

My overview showed that knowledge on immunology in CHARGE syndrome is scarce and scattered. So, I performed a systematic study on the prevalence and nature of immune dysfunction in 24 children with CHARGE syndrome using a cross-sectional study design (**Chapter 3**). All patients (or their parents) completed a questionnaire on infectious history. Furthermore, their immune system was extensively assessed through full blood counts, immunoglobulin levels, lymphocyte subpopulations, peripheral B- and T-cell differentiation, T-receptor excision circle analysis, T-cell function, and vaccination responses.

I also noticed in both the overview of literature and in our study on immunology that information on the thymus was scarce. Therefore, I conducted an explorative study to obtain an estimated prevalence of thymic aplasia in CHARGE syndrome (**Chapter 4**) where I used chest radiographs to retrospectively examine the thymus in patients with CHARGE syndrome.

In **Chapter 5**, I examined the prevalence of CAI in two separate cohorts of patients with CHARGE syndrome (a Dutch and an Australian cohort). In the Dutch cohort, a low-dose adrenocorticotropin hormone (ACTH) test (LDAT) was chosen as the primary diagnostic test to detect CAI. Patients with an insufficient cortisol response were retested on a separate occasion with a glucagon stimulation test to confirm the presence of CAI. The Australian cohort was screened using a single measurement of ACTH and cortisol in blood. If suspected, repeat ACTH and cortisol measurements were performed, and if either was abnormal on repeat testing, a standard-dose ACTH test was recommended.

In **Chapter 6**, I describe a case series of patients with CHARGE syndrome who had a musculoskeletal presentation, including hypoplasia of the shoulder and neck muscles. The photographic database of the Dutch National multidisciplinary CHARGE outpatients' clinic was used to collect data on the frequency of musculoskeletal features affecting the shoulder region and limbs.

Chapter 7 provides an overall summary of the thesis and what we have learned from it. I also discuss the implications of our research for individuals with CHARGE syndrome and their clinicians. Furthermore, I explore how knowledge about CHARGE syndrome and clinical care can be improved by researchers and policymakers. Centres of expertise and international reference networks can have an important role in this if they are sufficiently supported and patient participation is structurally implemented.

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CHAPTER 2

CHARGE syndrome: a review of the immunological aspects

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2.1 Abstract

CHARGE syndrome is caused by a dominant variant in the *CHD7* gene. Multiple organ systems can be affected due to haploinsufficiency of *CHD7* during embryonic development. CHARGE syndrome shares many clinical features with 22q11.2 deletion syndrome. Immunological abnormalities have been described, but are generally given little attention in studies on CHARGE syndrome. However, structured information on immunological abnormalities in CHARGE patients is necessary to develop optimal guidelines for diagnosis, treatment and follow-up in these patients. Here we provide an overview of the current literature on immunological abnormalities in CHARGE syndrome. We also explore immunological abnormalities in comparable multiple congenital anomaly syndromes to identify common immunological phenotypes and genetic pathways that might regulate the immune system. Finally, we aim to identify gaps in our knowledge on the immunological aspects in CHARGE syndrome that need further study.

2.2 Introduction

Patients with a combination of coloboma and other multiple congenital abnormalities were first described in 1961 [1,2]. However, the association between coloboma, congenital heart defects and choanal atresia was first recognized by both Hall and by Hittner et al. in 1979 [3,4]. Then in 1981, Pagon et al. [5] proposed the acronym CHARGE, which stands for the following symptoms: Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital abnormalities, and Ear abnormalities (external, middle and inner ear including deafness). The incidence of CHARGE syndrome (MIM# 214800) is estimated at 1 in 15,000 to 17,000 newborns [6] and its clinical diagnosis is based on the criteria proposed by Blake et al. [7] or by Verloes [8]. Since 2004, when the major genetic cause of CHARGE syndrome was identified as a dominant variant in the *CHD7* gene (MIM# 608892), which usually occurs *de novo*, CHARGE syndrome can also be diagnosed by molecular diagnostics. A variant in *CHD7* can be found in over 90% of all children who fulfil the clinical diagnostic criteria [6,9-11]. The *CHD7* gene encodes a member of the chromodomain helicase DNA-binding protein family that regulates the transcription of genes during embryonic development. Haploinsufficiency of *CHD7* affects multiple organ systems, including the heart, the inner ear and the eye. There is no clear genotype-phenotype correlation, but variants leading to a premature stop codon are, in general, associated with a more severe phenotype than variants with a non-truncating effect, i.e. missense variants [12].

Since the genetic cause of CHARGE syndrome was identified, its phenotype has been further explored. In addition to the above symptoms, other common clinical features of CHARGE syndrome are: absent or hypoplastic semicircular canals, cranial nerve dysfunction (including facial nerve palsy), cleft lip and/or palate, anosmia, feeding difficulties and skeletal abnormalities [8,9,13]. Furthermore, deficits in the immune system have been described in CHARGE patients, which might lead to morbidity and even mortality [14].

Here we provide an overview of the current literature on immunological dysfunction in CHARGE syndrome. We also explore what can be learned from the clinical overlap between CHARGE syndrome and other multiple congenital anomaly (MCA) syndromes. Do these syndromes share immunological phenotypes? Last but not least, we try to identify gaps in our knowledge that need further study.

2.3 Methods

A systematic search via the online database PubMed of publications on immunological aspects of CHARGE syndrome was performed (Supplementary 1). All patients with a proven variant in *CHD7*, which affects the normal function of the protein, were included in our review. We also decided to include articles prior to the identification of *CHD7* as the causative gene, in order to have a complete overview. Before *CHD7* was discovered, some patients with CHARGE syndrome were described as having

DiGeorge syndrome (#MIM 188400) or as having DiGeorge syndrome with CHARGE association. For our review, we only selected patients from these papers who fulfilled the diagnostic criteria of Blake et al. [7] and/or Verloes [8], and in whom a deletion of 22q11.2 (the cause of DiGeorge syndrome) had been excluded or was unknown. The titles and abstracts of English-language articles were reviewed for relevance. Relevant articles were studied in detail and their reference lists were scanned for additional publications (Supplementary 2). Information on the thymus and the results of the following immunological tests were used: lymphocyte subset phenotyping (if available, absolute numbers of T-, B- and NK-cells), quantitative immunoglobulin analysis, and T-cell function by response on mitogens (phytohaemagglutinin, PHA). Due to frequently missing absolute values and the differences in reporting laboratory results, especially whether age-related reference values were used or not, we decided to follow the interpretations of the authors instead of using our own cut-off values. For papers that only mentioned laboratory results without interpretation, we used the age-related reference values according to Comans-Bitter et al. [15] for interpretation.

2.4 Results

2.4.1 The collected cohort

All literature on immunology or immune dysfunction in CHARGE syndrome consists of case reports or retrospective studies. We identified 26 publications, comprising 59 patients in total ("our collected cohort"), which fulfilled our inclusion criteria (Table 1). Thirty-six of 59 (61%) patients had a proven variant in *CHD7* with functional effect (Table 1A). Detailed information on the variant in *CHD7* has been reported for 23 of these patients and all variants were known to lead to a premature stop in *CHD7* (data not shown). The remaining 23 of 59 (39%) patients fulfilled the clinical criteria of CHARGE syndrome, but the results of *CHD7* analysis (Table 1B) or the results of both *CHD7* analysis and 22q11.2 deletion testing (Table 1C) were unknown. The median age at which immunological tests (n=64) were performed in our collected cohort was 15 weeks (range 1 day - 8 years). In some patients, immunological tests were performed at different ages.

Table 1. Clinical and immunological findings in patients with CHARGE syndrome, as reported in the literature

A. CHARGE syndrome with proven variant in CHD7with functional effect

Reference	n	Sex	Age	C	H	A	R		G	E			Other and immunological symptoms	Thymus	T-cells	Mitogen response	B-cells	NK-cells	Immunoglobulins
							Growth	Cognition		Dysmorph	HL	SSC							
Sanlaville et al [60]	10[a]	M	21 wg	+	+	+	U	U	-	+	U	+		-	U	U	U	U	U
		M	21 wg	+	+	+	U	U	+	+	U	+		-	U	U	U	U	U
		M	23 wg	+	+	+	U	U	-	+	U	+		N	U	U	U	U	U
		M	23 wg	+	+	+	U	U	+	+	U	+		N	U	U	U	U	U
		M	28 wg	-	+	+	U	U	+	+	U	+		-	U	U	U	U	U
		M	29 wg	-	+	-	U	U	+	+	U	+		N	U	U	U	U	U
		F	29 wg	+	+	+	U	U	+	+	U	+		-	U	U	U	U	U
		M	29 wg	+	+	+	U	U	+	+	U	+		-	U	U	U	U	U
		F	32 wg	+	+	+	U	U	-	+	U	+	TE	-	U	U	U	U	U
		F	36 wg	-	-	+	U	U	-	+	U	+	TE	-	U	U	U	U	U
Writzl et al [29]	2	M	1 d	-	+	+	U	U	+	+	+	+		HP	-	-	N	N	Low IgG, high IgM and IgA
		M	2 d	-	+	U	U	U	+	+	U	U	TE	U	Low	U	N	N	U
Sanka et al [30]	1	F	2 mo	+	+	U	U	U	U	U	+	U	TE	HP	-	Low	N	N	N
Gennery et al [23]	4	F	7 wk	+	+	U	U	+	U	+	U	U		-	-	-	N	N	Low IgA
		M	4 mo	+	+	+	U	U	+	U	U	U	TE	U	-	-	N	N	Low IgG and IgA
		M	2.5 mo	+	+	+	U	U	+	+	U	U	CN, Omenn-like syndrome	-	Low	-	N	N	Low IgA, high IgE
		F	at birth	-	+	U	U	U	U	+	U	U	Omenn-like syndrome	-	-	-	N	N	Low IgA
			3.5 mo												High	U	N	N	N
			5 mo												High	U	N	High	Low IgA
Hoover-Fong et al [31]	1	M	3 wk	-	+	+	U	U	-	+	+	+	TE	-	Low	-	N	N	U
Wincent et al [75]	2	M	1 y	+	+	-	U	-	+	+	U	U	CN, immune deficiency	HP	U	U	U	U	U
		M	1 y	+	+	+	+	+	+	+	+	+	CN	U	Low	U	U	U	U
Chopra et al [32]	3	M	16 d	+	+	U	U	U	U	+	+	U	TE	-	-	U	N	N	N
		F	12 mo	+	+	+	+	+	U	U	+	U	CN	HP	Low	N	N	N	N
		F	4 mo	+	+	U	U	U	U	U	+	U		-	Low	N	N	N	N
Jyonouchi et al [14]	10	U	2 y	U	U	U	U	U	U	U	U	U	Two patients died from, rhinovirus pneumonia and overwhelming sepsis, respectively.	U	Low	U	N	N	N (3 y)
		U	3 mo	U	U	U	U	U	U	U	U	U		U	Low	U	N	N	Low IgG, IgM and IgA
		U	5 wk	U	U	U	U	U	U	U	U	U		U	Low	Low	N	N	Low IgA (3 mo)
		U	5 mo	U	U	U	U	U	U	U	U	U		U	Low	Low	N	N	Low IgG, IgM and IgA
		U	4 y	U	U	U	U	U	U	U	U	U	Two patients with confirmed SCID phenotype	U	N	U	High	N	N
		U	2 mo	U	U	U	U	U	U	U	U	U		U	N	U	N	N	U
		U	1 wk	U	U	U	U	U	U	U	U	U		U	N	U	N	N	U
		U	4 mo	U	U	U	U	U	U	U	U	U		U	N	U	U	U	N
		U	7 y	U	U	U	U	U	U	U	U	U		U	N	U	N	N	Low IgM, absent IgA (8 y)
		U	6 y	U	U	U	U	U	U	U	U	U		U	U	U	U	U	Absent IgA

Kaliakatsos et al [18]	1	M	1 mo	+	+	+	U	U	+	+	U	+	CN, TE, Omenn-like syndrome, recurrent respiratory tract infections, septicaemia	-	Low	-	N	N	N
			7 mo												N	U	N	N	Low IgG, High IgE
Inoue et al [33]	1	M	neonate	+	+	+	U	U	-	+	+	U	CN, TE	-	Low	Low	U	U	N
Assing et al [34]	1	F	1 mo	+	+	-	+	+	-	U	+	U		-	Low	U	N	N	N
			2 mo												Low	U	N	N	N (3 mo)
			6 mo												Low	U	N	High	N (4 mo)
			14 mo												Low	U	N	High	N (13 mo)
			15 mo												Low	U	N	High	U

B. Clinically diagnosed CHARGE syndrome, CHD7 status unknown, but deletion 22q11.2 excluded

Reference	n	Sex	Age	C	H	A	R		G	E			Other and immunological symptoms	Thymus	T-cells	Mitogen response	B-cells	NK-cells	Immunoglobulins
							Growth	Cognition		Dysmorph	HL	SSC							
North et al [16]	1	F	4.5 y	+	+	+	+	+	+	+	+	U	Recurrent otitis media and sinusitis	U	N	U	U	U	Hypogammaglobulinemia with low IgG2
De Lonlay-Debeney et al [19]	5	M	17 d	+	+	+	-	+	-	+	U	U	CN	HP	N	U	U	U	U
		M	19 mo	+	+	+	+	+	+	+	+	U	CN	HP	U	U	U	U	U
		F	4 mo	+	-	+	+	+	-	+	+	U	CN	-	U	U	U	U	U
		M	6 mo	+	+	-	+	+	+	+	+	U	CN	-	-	U	U	U	U
		F	13 d	+	+	-	-	+	-	+	U	U	Sepsis, hepatic abscess	N	Low	U	U	U	U
Markert et al [26,27,35,36], Rice et al [28]	1	M	23 d	+	+	+	+	U	+	+	+	U	CN, TE	-	-	-	N	Low	Low IgA (41 d)
Squires et al [37]	1	M	3 mo	+	+	-	+	U	+	+	U	U	CN, TE	U	Low	Low	U	U	U
Markert et al [36]	1	U	14 d	+	+	+	U	U	+	U	U	U		U	-	Low	N	N	U
			3 mo												Low	Low	U	U	High IgA
Markert et al [26,27], Rice et al [28]	1	M	3 d	+	+	-	U	+	+	U	+	U	CN, TE	-	Low	U	Low	High	Not reliable due to treatment with immunoglobulins
			31 d												Low	Low	N	High	
			44 d												Low	Low	N	High	
			59 d												Low	U	N	N	
Boudny et al [24]	1	F	6 mo	+	+	+	U	U	U	U	+	U	CN, dermatitis, ulcers colon, recurrent respiratory infections	-	Low	-	N	N	Low IgG, IgM and IgA

Theodoropoulos [17]	2	F	30 mo	+	+	+	+	+	U	+	+	U	Recurrent respiratory infections, bronchioloitis, pneumonia, otitis media, urticaria, allergy	U	N	N	N	N	Low IgG2
		M	22 mo	+	+	+	+	U	-	+	+	U	CN, bronchiolitis, sinusitis, conjunctivitis, otitis media	U	Low	N	N	N	N
Markert et al [27], Rice et al [28]	2	U	43 d	+	+	+	U	+	+	U	+	U	CN	U	Low	-	U	U	U
		U	41 d	+	+	+	U	+	U	U	+	U		U	-	-	U	U	U
Markert et al [20,21]	2	M	255 d	U	+	+	+	+	U	+	U	U	CN, dermatitis, infections	-	N	Low	U	U	U
			316 d												N	Low	U	U	U
		M	158 d	U	+	+	+	U	U	+	+	U	CN, dermatitis, sepsis, PCP	-	Low	Low (318 d)	U	U	High IgE
Markert et al [21]	1	U	U	+	+	+	U	U	+	U	+	U	CN, dermatitis	-	Low	Low	U	U	U
Janda et al [22]	1	M	2 mo	+	+	+	+	U	+	+	U	U	TE, recurrent severe infections and septic shocks	-	-	-	U	U	Hypogammaglobulinemia
Lee et al [38]	1	M	42 d	+	+	+	+	+	+	U	+	U	TE	-	U	U	U	U	Low
Chopra et al [32]	1	F	6 d	+	+	U	U	U	U	U	U	U		-	-	U	N	N	N

C. Clinically diagnosed CHARGE syndrome, CHD7 and 22q11.2 deletion status unknown

Reference	n	Sex	Age	C	H	A	R		G	E			Other and immunological symptoms	Thymus	T-cells	Mitogen response	B-cells	NK-cells	Immunoglobulins
							Growth	Cognition		Dysmorph	HL	SSC							
Wood et al [25]	1	M	5 mo	-	+	+	U	U	+	+	U	U	CN, chronic enteric virus infection	HP	Low	Low	U	U	Treatment with immunogammaglobulins
Theodoropoulos [17]	1	M	3 y	+	+	+	+	U	U	+	U	U	CN, pneumonia, otitis media, conjunctivitis	U	Low	-	N	N	Low IgG1

Abbreviations: A, choanal atresia or stenosis, including cleft palate since these anomalies rarely occur together; C, coloboma or microphthalmia; CN, cranial nerve dysfunction; d, day(s); E, ear anomalies; F, female; G, genital anomalies; H, heart anomalies; HL, hearing loss; HP, hypoplasia; M, male; mo, month(s); N, normal; n, number of patients; PCP, *Pneumocysitis carnii* pneumonia; R, retardation in development and/or growth; SCID, severe combined immune deficiency; SSC, semicircular canals anomalies; TE, tracheoesophageal defects; U, unknown; wg, weeks of gestation; wk, week(s); y, year(s); +, present; -, absent (including thymic aplasia).
If available, absolute numbers of T-cells, mitogen response, B-cells and NK-cells were used. Otherwise the interpretations of the authors were followed.
[a] Foetuses.

2.4.2 Immunological abnormalities reported in CHARGE syndrome

Clinical presentation related to immune abnormalities included recurrent otitis media in four patients (6.8%), sinusitis in two patients (3.4%), conjunctivitis in two patients (3.4%), dermatitis in four patients (6.8%), infections of the respiratory tract in six patients (10%), including pneumonia in three (5.1%), and sepsis in five (8.5%) of 59 patients [14,16-22]. Three patients had features resembling Omenn syndrome, a form of Severe Combined Immune Deficiency (SCID), characterized by autoimmune-like features and macular skin rash due to the formation of abnormal, auto-reactive T cells [18,23]. Other severe presentations included a patient with recurrent oral candidiasis, recurrent severe infections, and septic shock due to colonization with multi-resistant species of Pseudomonas aeruginosa, Stenotrophomonas maltophilia, and Acinobacter (Janda et al. [22], Table 1B); another patient with severe general dermatitis and ulcers of the colon (Boudny et al. [24], Table 1B), and a patient with CHARGE association and T-cell deficiency with a chronic viral infection of the gut (Wood et al. [25], Table 1C).

Information on T-cell numbers were reported in 44 of 59 patients and in 24 of 36 patients with a proven variant in *CHD7*. As shown in Table 1, 35 of the 44 (80%) patients with available information on T-cell numbers had low or absent T-cell numbers. Of the 35 patients, one patient had T-cell lymphopenia and a transient B-cell lymphopenia, which normalized with ageing [26-28]. Thymic aplasia was reported in 27 of 59 patients and in 16 of 36 patients with a proven variant in *CHD7*. T-cell lymphopenia in our collected cohort was associated with thymic aplasia or hypoplasia in 21 of 22 (95%) patients [18-36]. T-cell function by response on mitogens was available for 28 of 59 patients and of these 28 patients 24 (86%) had a low or absent response on mitogen. For 23 of 26 (88%) patients, T-cell lymphopenia was concomitant with T-cell dysfunction [14,17,18,20,22-31,33,35-37].

In our collected cohort, B- and/or NK-cell numbers were reported in only 29 of 59 patients and of these 29 patients one had low B-cell numbers, one had high B-cell numbers, one had low NK-cells numbers, and three had high NK-cell numbers. The reported B- and NK-cells numbers of other patients were normal [14,23,26-28,34-36].

Immunoglobulin levels were reported in 33 of 59 patients and in 20 of 36 patients with a proven variant in *CHD7*. Hypogammaglobulineamia was reported in 20 of 33 (61%) patients and measured along with T-cell lymphopenia in 14 of 18 (78%) patients [14,17,22-29,35,36]. Thirteen of 33 (39%) patients with known immunoglobulin levels had IgG deficiency and two of these patients had received immunoglobulin replacement therapy [14,16-18,22-29,38]. Of these 13 patients with IgG deficiency, six had recurrent or chronic infections and one had absent specific antibody responses [14,16-18,22,24,25]. Isolated immunoglobulin deficiencies were rarely reported in CHARGE syndrome. One patient with an isolated IgG2 subclass deficiency [17] and two patients with an isolated IgA deficiency [14] were reported. One patient developed low IgG and elevated IgE while the T-cells normalized [18].

Jyonouchi et al. [14] reported on the largest cohort of patients with CHARGE syndrome (n=25) proven by a variant in *CHD7*, and retrospectively collected their immunological data. However, lymphocyte subset phenotyping and quantitative immunoglobulin analysis were performed in only nine and eight patients, respectively. They concluded that a greater proportion of CHARGE patients had immunological abnormalities than previously thought (lymphopenia in 60% and humoral defects in 16% of patients, respectively). They confirmed the over-representation of T-cell dysfunction and also reported two patients with a SCID phenotype.

In conclusion, T-cell lymphopenia is common (80%) in CHARGE patients and is associated with a reduced T-cell function and hypogammaglobulinaemia, but normal B-cell and NK-cells numbers. Thymic aplasia or hypoplasia might be the underlying cause of T-cell lymphopenia, leading to increased frequencies of infection and other clinical presentations.

According to Jyonouchi et al. [14], immune dysfunction in CHARGE might contribute to the mortality of the syndrome. Two patients died from infectious complications and two patients with a confirmed SCID phenotype died from respiratory failure. Assing et al. [34] reported a patient with CHARGE syndrome who had severely reduced thymic function and a severe lymphopenia, but showed a functional and diverse T-cell receptor repertoire, and a good response to vaccines. An uneventful infection history was reported up to the age of 34 months, but thereafter the clinical status is unclear.

2.4.3 Immunological abnormalities reported in overlapping MCA syndromes

CHARGE syndrome overlaps clinically with other MCA syndromes (see Figure 1) [39]. The most remarkable phenotypic overlap is with 22q11.2 deletion syndrome (#MIM 192430, *TBX1*), including the DiGeorge phenotype, where immunodeficiency is an important symptom. Congenital heart defects, cognitive and motor delay, hearing loss, external ear anomaly, cleft lip and palate, growth deficiency, and renal anomaly are seen in both syndromes [14,39]. The total absence of the thymus, and therefore complete T-cell lymphopenia (DiGeorge phenotype), is rare and is seen in less than 1.5% of patients with 22q11.2 deletion syndrome [40]. Patients with the typical 1.5 or 3.0 Mb deletion of 22q11.2 rarely show the full presentation of the syndrome and only a minority develop opportunistic infections. Much more common is a phenotype with T-cell lymphopenia (67%) [41] combined with mild to moderate functional immunological impairment which improves with age. This T-cell lymphopenia mostly present as frequent viral infections during childhood, with or without secondary bacterial infections, but can also remain clinically unrecognized [40,41]. Due to their abnormal palatal anatomy, which may compromise drainage, most patients are susceptible to upper airway infections, as is also seen in patients with CHARGE syndrome. There are more publications on immunoglobulin

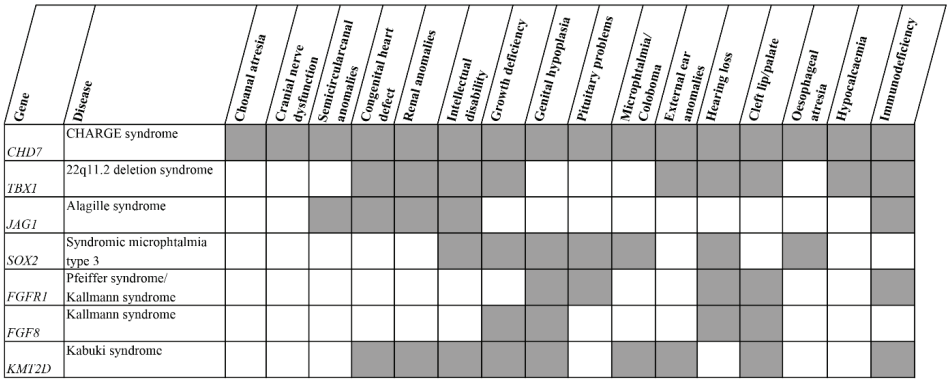

Figure 1. Clinical overlap of CHARGE syndrome with other MCA syndromes. The overlapping clinical features of CHARGE syndrome with other MCA syndromes are shown. All the genes mentioned, or their proteins, have been associated with CHD7. Adapted from Corsten-Janssen et al. [14]

abnormalities in 22q11.2 deletion syndrome compared to CHARGE syndrome [42,43]. Recent studies indicate that the antibody deficiency in 22q11.2 deletion syndrome is likely to be underestimated [44,45]. Patel et al. [44] have reported immunoglobulin deficiency in the largest cohort (n=855) to date and concluded that 6% of the patients over the age of three had hypogammaglobulinaemia, 19% of the total cohort had a low level of IgG, and 2-3% of the patients were receiving immunoglobulin replacement therapy. Björk et al. [45] found that 6 of 26 adult patients (23%) had low immunoglobulin levels. Autoimmune diseases, like rheumatoid diseases and idiopathic thrombocytopenia purpura (ITP), are seen in approximately 10% of patients with 22q11.2 deletion syndrome [46], whereas no autoimmune diseases were seen in the CHARGE syndrome patients, although, 3 of 59 (5%) patients with CHARGE syndrome had an Omenn-like syndrome, which has autoimmune-like features. An explanation of the autoimmune predisposition in 22q11.2 deletion syndrome might be a reduced level of natural regulatory T-cells (nTreg) [47]. These nTregs are important in the maintenance of self-tolerance, i.e. suppression of the immune response against self-antigens. In our collected cohort of CHARGE patients, nTreg levels were mentioned in only two patients: one patient had few nTregs (no value was mentioned) [23] and the other patient had higher proportions of nTregs compared to two adult controls [34]. So CHARGE syndrome shares a large clinical overlap with 22q11.2 deletion syndrome and this seems to be also true for the prevalence of T-cell lymphopenia, leading to susceptibility for viral (and secondary bacterial) infections.

Immunological functions in other overlapping MCA syndromes are less well described. Alagille syndrome (#MIM 118450), caused by variants in *JAG1*, shares abnormalities in the semicircular canals, heart defects, renal anomalies and intellectual disabilities with CHARGE syndrome [39]. Recurrent ear and respiratory tract infections are seen in about a quarter of patients with Alagille syndrome [48]. The underlying immunological dysfunction was described in one paper as a

diminished T-helper 1 response [49].

Patients with Pfeiffer syndrome (#MIM 101600, *FGFR1*) have overlapping features with CHARGE syndrome regarding cleft-lip and/or palate and hearing loss [39]. The immunological function in 12 patients with craniofacial malformation syndromes, including Pfeiffer syndrome, was studied by Scheuerle et al. [50]. Unfortunately, they did not specify the immunological dysfunction per syndrome. However, seven patients were tested for T-cell numbers and T-cell lymphopenia was reported in one patient, decreased T-helper cells in three patients and decreased T-killer cells in three patients. Three patients had additional abnormalities in immunoglobulin counts and in lymphocyte stimulus and response function. Two were brothers, who both had Pfeiffer syndrome.

Kabuki syndrome (#MIM 147920, *KMT2D*) has the following clinical overlap with CHARGE syndrome: cleft palate, mental retardation, short stature, genital hypoplasia, congenital heart defects, abnormalities of the eye and ear, renal abnormalities, scoliosis, and recurrent otitis media in infancy [51,52]. Hoffman et al. [53] evaluated the immune condition of 19 patients with Kabuki syndrome and concluded that hypogammaglobulinemia is a frequent finding (84%). However, they tested lymphocyte subsets in only three patients, who showed no abnormalities in T- and B-cell numbers. Diminished T-cell function by response on PHA and reduced naive T-helper cells were reported in one patient with Kabuki syndrome [54]. Autoimmune disorders, including ITP, are common in children with Kabuki syndrome [53,55].

Thus, CHARGE syndrome and the clinically overlapping 22q11.2 deletion syndrome share an increased prevalence of T-cell dysfunction. Although T-cell dysfunction is only mentioned sporadically in Alagille syndrome, Pfeiffer syndrome, and Kabuki syndrome, the clinical overlap of these syndromes with CHARGE syndrome indicates that their underlying genetic defects may result in shared embryonic pathways leading to T-cell abnormalities.

2.5 Discussion

2.5.1 Does CHD7 share T-cell-related pathways with TBX1, JAGGED1, FGFR1 and MLL2?

The 22q11.2 region contains the *TBX1* gene (#MIM 602054), which has been identified as a candidate gene for most of the phenotypic features seen in the 22q11.2 deletion syndrome [40]. The existence of a shared embryonic pathway or pathways of CHD7 and TBX1 has been studied for heart development [56] and inner ear development [57] in animal models. Both genes are expressed in the pharyngeal arches, of which the third and fourth arches contain the precursors of the thymic stromal cells [58-60]. This might explain the shared underlying pathogenesis of abnormal thymic development and the high proportion of thymic aplasia (44%) in our collected cohort with a proven *CHD7* variant, which can lead to impaired T-cell development. However, it should be noted that the high proportion of thymic aplasia might be due

to reporting bias.

It is also known that impaired T-cell development affects further maturation of the thymic stromal cells resulting in thymic epithelial cells with lack of Aire expression, a transcription factor that regulates the expression of self-antigens. These self-antigens are important for the deletion of autoreactive T-cells [61]. McLean-Tooke et al. [47] suggested that the reduced number of nTregs seen in 22q11.2 deletion syndrome is related to thymic function and structure. So the autoimmune predisposition in 22q11.2 deletion syndrome might be explained by both impaired thymic and T-cell development. In CHARGE syndrome, only three patients were reported to have Omenn-like syndrome, which has autoimmune-like features [18,23]. Well-defined autoimmune diseases have not yet been described in CHARGE syndrome, suggesting that the nTregs physiology might be different in CHARGE syndrome compared to 22q11.2 deletion syndrome. It would therefore be interesting to further study nTregs in CHARGE syndrome.

JAG1 (#MIM 601920) is the underlying causative gene of Alagille syndrome and its protein JAGGED1 is a ligand of the Notch receptor. The Notch signalling pathway regulates cell-fate decisions during ontogeny, including the development of lymphoid cells, and there is accumulating evidence to suggest that Notch signalling is also involved in the maturation of peripheral T-cells [62,63]. In animal models, Jagged1 seems to play a role in the generation of regulatory T-cells [64,65]. CHD7 and JAG1 might be linked indirectly via SOX2 (#MIM 184429), because Chd7 has been identified as a Sox2 transcription cofactor in the regulation of common target genes including Jag1 [66]. Functional affective variants in *SOX2* result in syndromic microphthalmia type 1, a phenotype that shares several features with CHARGE syndrome: microphthalmia, motor disability, neurocognitive delays, sensorineural hearing loss, oesophageal atresia, pituitary defects and gonadotropin deficiency [67]. Immune dysfunction has not been described in syndromic microphthalmia type 1 thus far.

The underlying causative gene for Pfeiffer syndrome is *FGFR1* (#MIM 136350), encoding the fibroblast growth factor receptor 1 (FGFR1). *FGFR1* and *FGF8* (#MIM 600483) are also involved in Kallmann syndrome, which shares features such as anosmia and hypogonadotropic hypogonadism with CHARGE syndrome [68]. A common pathway for CHD7 and fibroblast growth factor 8 (FGF8)/FGFR1 has therefore been suggested [56,69,70]. FGFR1 is expressed in a subset of T-cells and is believed to interact with the T-cell receptor to enhance the activation of T-cells [71].

Kabuki syndrome is caused by variants in *KMT2D* (#MIM 602113), affecting the function of a methyltransferase named MLL2 that is involved in transcriptional regulation. Recently, it has been shown that CHD7 interacts with the same transcriptional proteins as MLL2 [52]. It is unknown whether MLL2 plays a role in the immunological dysfunction in Kabuki syndrome, but it is possible that both MLL2 and CHD7 regulate expression of genes which are involved in the immune system.

CHD7 regulates the expression of genes and shares pathways with other regulatory proteins in embryonic development [39]. The interaction of CHD7 with TBX1 and their role in thymic development has been well established in the literature [58-60]. Evidence for the interaction of CHD7 with JAG1, FGFR1 and MLL2 during embryonic development, especially the development of the immune system, is less strong, but reports are emerging describing possible links in the genetic pathways. Although we only have detailed information on the variant in *CHD7* for 23 patients, the fact that all reported variants lead to a premature stop in *CHD7* is interesting, because truncating *CHD7* variants are in general associated with a more severe phenotype [12].

2.5.2 Are certain phenotypic features more common in our collected cohort?

In Table 2 we compare the clinical features of 36 patients in our collected cohort for whom a variant in *CHD7* with functional effect had been proven (Table 1A) with two other cohorts described by Bergman et al. [13]. These two cohorts consist of 280 patients with a proven variant in *CHD7* from Bergman et al. [13] and 254 patients from Zentner et al. [72]. Most features show similar frequencies in all three cohorts. We see a higher frequency in our collected cohort for heart defects (96% versus 76% and 77%), choanal atresia (85% versus 55% and 38%), and tracheoesophageal anomaly (53% versus 29% and 19%). Facial palsy (100%) and growth retardation (100%) also seem to occur more frequently in our collected cohort, but we note that we only have clinical information on six and three patients, respectively. It should also be noted that two cases [29] were included in all three cohorts. Since the differences found might be due to the predominance of truncating variants in our cohort, Table 3 shows the phenotypic comparison of patients carrying variants leading to a premature stop in *CHD7* in our collected cohort (n=23) and in that of Bergman et al. [12] (n=315). The frequencies between the two cohorts are more comparable than the frequencies shown in Table 2. However, Table 3 shows that our collected cohort still had a higher frequency for heart defects (95.7% versus 82.5%), choanal anomaly (84.2% versus 60.4%), and tracheoesophageal anomaly (50.0% versus 33.6%). This is interesting because heart defects, choanal atresia, and tracheoesophageal anomalies are all midline defects, including abnormal thymus development.

Table 2. Phenotypic comparison of our collected cohort with a proven variant in *CHD7* compared to cohorts with a proven variant in *CHD7* from the literature

Feature	Our collected CHD7-positive cohort (n=36)	CHD7-positive cohort from Bergman et al [13] (n=280)	CHD7-positive cohort from Zentner et al [72] (n=254)[a]
Coloboma	19/26[b]	189/234	190/253
	73% (53-81%)[c]	81% (68-84%)	75%
Heart defect	25/26	191/252	193/250
	96% (69-97%)	76% (68-78%)	77%
Choanal atresia	17/20[d]	99/179	95/247
	85% (47-92%)	55%	38%
Cleft lip and/or palate	No isolated cleft lip	79/163	79/242
		48% (28-70%)	33%
Growth retardation	3/3	35/94	101/141
	100% (8-100%)	37% (13-79%)	72%
Developmental delay	Developmental delay	Delayed motor milestones	Developmental delay
	4/5	147/149	107/141
	80% (11-97%)	99% (53-99%)	76%
		Intellectual disability	
		108/134	
		74% (39-91%)	
Genital hypoplasia	13/20	118/145	116/187
	65% (36-81%)	81% (42-90%)	62%
External ear anomaly	21/21	224/231	214/235
	100% (58-100%)	97% (80-98%)	91%
Semicircular canal anomaly	14/14	110/117	94/96
	100% (39-100%)	94% (39-98%)	98%
Cranial nerve dysfunction	12/12	173/174	Unknown
	100% (33-100%)	99% (62-100%)	
Facial palsy	6/6	80/121	72/187
	100% (17-100%)	66% (29-85%)	39%
Tracheooesophageal anomaly	9/17	42/146	35/185
	53% (25-78%)	29% (15-63%)	19%

Adapted from Bergman et al [13] and Zentner et al [72]. [a]This cohort partially overlaps with the cohort of Bergman et al.[13] because the phenotypes of 64 of the patients in that study had been published previously. [b]Frequencies are represented as the number of patients with a particular feature/the total number of patients that were tested for that particular feature. [c]The range of percentages presented between brackets was calculated as: (positive/total)x100% - (positive +unknown/total)x100%. [d]Cleft palate is included in choanal atresia since these anomalies rarely occur together.

Table 3. Phenotypic comparison of patients carrying a variant in *CHD7* leading to a premature stop in *CHD7*

Feature	Our collected cohort (n=23)[a]	Cohort of Bergman et al [12] (n=315)[b]
Coloboma	16/23 (69.6%)	199/229[c] (86.9%)
Heart defect	22/23 (95.7%)	212/257 (82.5%)
Choanal atresia	16/19[d] (84.2%)	110/182 (60.4%)
Cleft lip and/or palate	No isolated cleft lip	80/144 (55,6%)
External ear anomaly	20/20 (100%)	217/221 (98.2%)
Semicircular canal anomaly	14/14 (100%)	121/121 (100%)
Cranial nerve dysfunction	5/5 (100%)	119/131 (90.8%)
Tracheoesophageal anomaly	8/16 (50.0%)	43/128 (33.6%)

The patients come from our collected cohort and the cohort of Bergman *et al* [12].

[a]23 truncating mutations: 2 deletions, 8 frameshift mutations, 13 nonsense mutations.

[b]315 truncating mutations: 5 deletions, 139 frameshift mutations; 171 nonsense mutations.

[c]Due to lacking clinical data, the number of patients is lower than the total number of patients.

[d]Cleft palate is included in choanal atresia since these anomalies rarely occur together.

2.5.3 Suggested future studies

As shown in Table 1, the publications on immunology in CHARGE syndrome are scarce and most studies describe only one or a few patients, with data collected retrospectively. The differences in reporting the laboratory results, e.g. absolute versus relative values, and the use of different reference values, whether age-related or not, makes it difficult to draw conclusions. A limitation of our approach is that reporting bias might play a role, since most publications are case reports. Furthermore, the median age is quite young in our collected cohort. Since the T-cell number may well improve with age, as has been shown in 22q11.2 deletion syndrome [73], our collected cohort might represent the more severe phenotypes.

We found lymphopenia in 80% of our collected cohort, which is in contrast to the 60% found in the study of Jyonouchi et al. [14]. Our higher percentage might reflect reporting bias. Another contrast is the reported prevalence of immunoglobulin abnormalities in 22q11.2 deletion syndrome of 6% in recent studies [44,45] and 40% in older reports [42,43], with the prevalence of 61% in our collected cohort. However, we only had information on immunoglobulin levels from 33 of 59 (56%) patients. Nonetheless, the percentages that we found indicate that immunological dysfunction in CHARGE syndrome might play a greater role in the phenotype than previously thought. There is a need to broaden our knowledge on the frequency and exact nature of immune abnormalities in CHARGE syndrome, including information on B- and NK-cells. Since the pathophysiology of the immunological dysfunction in 22q11.2 deletion syndrome has been extensively studied, it is worthwhile to explore whether the same pathophysiology also applies to CHARGE syndrome.

Anatomical deviations, such as palatal defects, contribute to the susceptibility to infections and their duration by impeding drainage or clearance of infectious debris. It is important to know whether immunological issues complicate the severity of infections in order to optimize care management in patients. This is especially true for children with CHARGE syndrome, who have other co-morbidities (such as heart defects and tracheoesophageal defects) that require operative procedures, since these also constitute risk factors for infections.

Because immunological problems might contribute to the morbidity and even the mortality of patients with CHARGE syndrome, timely diagnosis of an immune dysfunction is relevant. Information on the type of immune abnormality will provide clues for future studies that could develop guidelines to protect these children from excess morbidity (and maybe mortality) due to infections. Such guidelines could include clinical follow-up strategies, prescribing prophylactic antibiotics, or even a procedure to restore T-cell lymphopenia by replacing thymic tissue with a thymic transplant [40,74].

To better understand the frequency and nature of immune dysfunction in CHARGE syndrome, a prospective study is needed to systematically collect immunological data on more patients with CHARGE syndrome, including those without overt symptoms of immunological defects. Correlating immunological laboratory results with clinical data will yield more insight into the immune dysfunction. In addition, new genome-wide sequencing techniques can be applied to identify patients with atypical CHARGE syndrome and further expand the phenotypic spectrum of CHARGE syndrome, including its immunological features.

2.6 Conclusion

Immunological dysfunction, which predominantly affects T-cell function, has occasionally been described in patients with CHARGE syndrome. A prospective study, with systematically collected immunological and clinical data, is needed to explore the frequency and nature of their immune dysfunction. It would help identify clinical management issues in this infection-prone group of patients.

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Supplementary information

Supplement 1. PubMed advanced search strategy

#1 “charge syndrome”[All Fields]
#2 “charge association”[All Fields]
#3 “chd7”[All Fields]
#4 (#1 OR #2 OR #3)
#5 immune[All Fields]
#6 (“immunology”[Subheading] OR “immunology”[All Fields] OR “allergy and immunology”[MeSH Terms] OR (“allergy”[All Fields] AND “immunology”[All Fields]) OR “allergy and immunology”[All Fields])
#7 (“t-lymphocytes”[MeSH Terms] OR “t-lymphocytes”[All Fields] OR “t cell”[All Fields])
#8 (“b-lymphocytes”[MeSH Terms] OR “b-lymphocytes”[All Fields] OR “b cell”[All Fields])
#9 humoral[All Fields]
#10 (#5 OR #6 OR #7 OR #8 OR #9)
#11 (#4 AND #10)

Supplement 2. PRISMA 2009 Flow Diagram

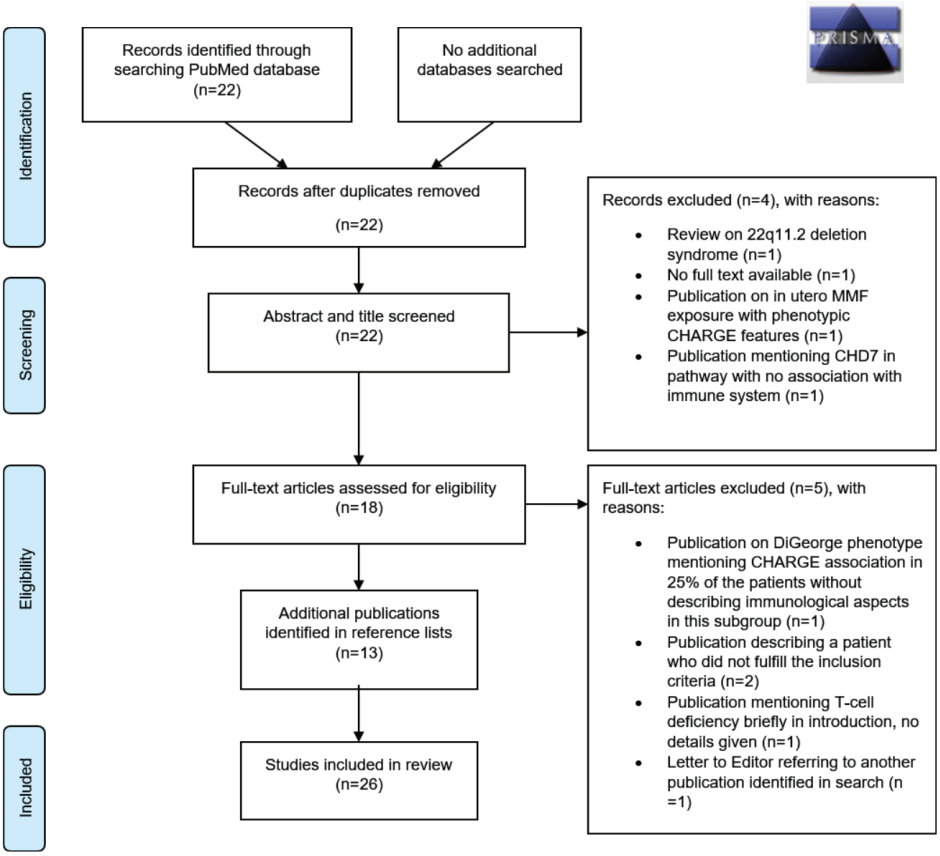

Figure 2. Supplement 2. The PRISMA 2009 Flow Diagram reflects the strategy used to include and exclude studies for this review. From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097. For more information, visit www.prisma-statement.org

CHAPTER 3

Immune dysfunction in children with CHARGE syndrome: a cross-sectional study

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3.1 Abstract

CHARGE syndrome is a variable, multiple congenital malformation syndrome. Patients with CHARGE syndrome have frequent infections that are presumed to be due to anatomical anomalies of the craniofacial region and upper airway, and cranial nerve problems resulting in swallowing difficulties and aspiration. The possible contribution of immunological abnormalities to these infections has not been systematically studied even though immune deficiencies have been described in patients with 22q11.2 deletion syndrome, a condition which shares remarkable clinical overlap with CHARGE syndrome. We assessed the frequency and nature of immune dysfunction in 24 children with genetically proven CHARGE syndrome. All patients, or their parents, completed a questionnaire on infectious history. Their immune system was extensively assessed through full blood counts, immunoglobulin levels, lymphocyte subpopulations, peripheral B- and T-cell differentiation, T-receptor excision circle (TREC) analysis, T-cell function, and vaccination responses. All CHARGE patients had a history of infections (often frequent), mainly otitis media and pneumonia, leading to frequent use of antibiotics and to hospital admissions. Decreased T-cell numbers were found in 12 (50%) patients, presumably caused by insufficient thymic output since TREC amounts were also diminished in CHARGE patients. Despite normal peripheral B-cell differentiation and immunoglobulin production in all patients, 83% of patients had insufficient antibody titers to one or more early childhood vaccinations. Based on our results, we recommend immunological evaluation of CHARGE patients with recurrent infections.

3.2 Introduction

CHARGE syndrome (MIM# 214800) is a rare, multiple congenital anomaly syndrome with an estimated birth prevalence of 1 in 15,000 to 17,000 newborns [1]. The clinical diagnosis is made using criteria proposed by Blake et al. [2] or Verloes [3]. The syndrome is caused by a dominant loss-of-function mutation in, or a deletion of, the *CHD7* gene (#MIM 608892), which usually occurs *de novo* and can be found in over 90% of all children who meet the clinical diagnostic criteria. The encoding protein of *CHD7* is a member of the chromodomain helicase DNA-binding protein family that regulates the transcription of genes during embryonic development. Because of the regulating function of CHD7, haploinsufficiency of *CHD7* affects multiple organ systems, which explains the broad clinical variability seen in CHARGE syndrome. No clear genotype-phenotype correlations have been found, although variants leading to a premature stop codon are, in general, associated with a more severe phenotype than variants with a non-truncating effect (i.e. missense variants) [4].

Since Pagon et al. [5] proposed the acronym CHARGE (Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital abnormalities, and Ear abnormalities), new clinical features have been added to CHARGE syndrome that include cranial nerve dysfunction, absent or hypoplastic semicircular canals, anosmia, cleft lip and/or palate, and skeletal abnormalities [3,6,7]. In addition, patients with CHARGE syndrome have frequent infections including recurrent otitis media, sinusitis, and infections of the respiratory tract, which lead to morbidity and even mortality [8,9]. Deviations of the palatal and ear anatomy, as well as cranial nerve dysfunction affecting swallowing, contribute to these infections. However, the contribution of abnormalities in the immune system may be of importance because T-cell lymphopenia and thymic abnormalities have been described in individual patients with CHARGE syndrome, and these abnormalities resemble immune abnormalities seen in 22q11.2 deletion syndrome (#MIM 192430) [9]. In contrast to 22q11.2 deletion syndrome, the frequency and exact nature of the immunological abnormalities in CHARGE syndrome have so far not been studied either prospectively or systematically. In this respect, knowledge is needed to develop guidelines to optimize the care of children with CHARGE syndrome. Our aim in this study was to systematically explore the prevalence and nature of immune dysfunction in children with CHARGE syndrome.

3.3 Patients and Methods

3.3.1 Patients

Children with genetically confirmed CHARGE syndrome were recruited through the Dutch Expert Clinic for CHARGE syndrome between September 2013 and June 2014. Mutations in *CHD7* were classified as truncating (*i.e.* nonsense, frameshift, or deletion) or non-truncating (*i.e.* missense or splice-site). Healthy children, mainly siblings of CHARGE patients, were included as age-matched controls for the T-cell

function assay and as a control group for the T-cell receptor excision circle (TREC) analysis. Exclusion criteria were age below 20 months or above 18 years or active infection and/or immunosuppressive therapy (e.g. steroids) at the time of the blood tests. Further exclusion criteria for healthy controls were ear-nose-throat problems in the previous two years defined as adenoidectomy, placement of tympanostomy tubes, or otitis media with effusion. Potential healthy controls with primary immune deficiencies or autoimmune disease were also excluded.

Patients, or their parents, filled in a Dutch questionnaire on infectious history (available from the authors upon request). Questions were based on international guidelines and protocols for identifying primary immune deficiency [10,11]. Additional medical information was extracted from the patient files and the database of the Dutch Expertise Centre for CHARGE syndrome. Because the thymus has not been routinely examined in CHARGE patients, information on the thymus could only be retracted from cardiac surgery reports, where available. The study was approved by the Medical Ethical Review Committee of the University Medical Centre Groningen and written informed consent was obtained from all patients, controls and/or their parents.

3.3.2 Immunologic assays

Peripheral blood was obtained from all patients and healthy controls for immunological assessment. All the immunological assays we performed have been validated and are used in routine patient diagnostics. For all assays, age-matched reference values are available [12-15], except for the T-cell subpopulations of patients under the age of 5 years, TRECs, and T-cell function assay [16]. Healthy controls were used to establish age-matched reference values for the T-cell function assay and as the control group for TREC analysis. Lymphocyte populations and peripheral B- and T-cell subpopulations were also analysed in the healthy controls. Results of all assays, except for the TRECs, were assessed on the basis of the age-matched reference values.

Full blood counts were measured with an automated cell counter (Sysmex XN10/20, Kobe, Japan); serum immunoglobulin (Ig) G, IgG subclasses 1-4, IgA, and IgM were nephelometrically analysed using BNII system (Siemens AG, Munich, Germany); and serum total IgE was measured by fluoro-enzyme-immuno-assay (Phadia, Uppsala, Sweden). These assays were performed according to the manufacturer's protocol.

IgG-specific antibodies to diphtheria toxoid and tetanus toxin were analysed at the National Institute for Public Health and the Environment (RIVM, Netherlands) using a Luminex-technology-based multiplex immunoassay developed in-house [17,18]. A protective concentration of antibody to both diphtheria and tetanus was defined as ≥0.10 IU/ml. IgG-specific antibodies to *Haemophilus influenzae* type b and to 13 types of pneumococcal polysaccharides were analysed at the laboratory of the Antonius Hospital (Nieuwegein, Netherlands). Enzyme-linked immunosorbent assay (ELISA, Binding Site, San Diego, CA, USA) was used to analyse IgG-specific antibodies to *H. influenzae* type b and a concentration of >1.0 mg/l was considered protective. IgG-specific antibodies to pneumococcal polysaccharides were analysed by multiplex

assay [19,20]. An adequate response to pneumococcal polysaccharides was defined as an absolute level of >0.35 µg/ml in > 50% of the serotypes.

Multicolour flow cytometric phenotyping of the lymphocyte populations was performed using a FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analysed using FACSCanto Clinical Software version 2.4 and FACSDiva software version 7.0 (Becton Dickinson). Absolute numbers of CD3+, CD4+, and CD8+ T-cells; CD19+ B-cells; and CD16+/56+ NK-cells were measured using the MultiTest TruCount method with MultiTest reagents to CD45/3/4/8/16+56/19 (Becton Dickinson). The lyse-no-wash preparation method was performed as prescribed by the manufacturer.

Peripheral B- and T-cell differentiation was assessed by multicolour flow cytometric phenotyping of the peripheral B- and T-cell subpopulations based on the methods described by Driessen et al. [14]. The monoclonal antibodies and the gating strategy for the B- and T-cell subpopulations are discussed in S1 Appendix. Absolute numbers of the B- and T-cell subpopulations were calculated using their relative numbers and the absolute number of CD19+ B-cells or CD4+ and CD8+ T-cells. Absolute numbers of the B- and T-cell subpopulations were then compared to age-matched reference values [14,15]. Because of the heterogeneity of the peripheral T-cells, T-cell subpopulations were only considered to be decreased if both the absolute and relative numbers were lower than the age-matched reference values.

TRECs, which can be used as a reflection of thymic T-cell output [21], were assessed by the methods proposed by Hazenberg et al. [22] and Chan and Puck [23]. In brief, DNA was extracted from dry blood spots using generation DNA elution solution method (Qiagen, Hilden, Germany). Subsequently, real-time quantitative PCR for TRECs was performed with Albumin as control for DNA input. The amount of TRECs per µg DNA was calculated.

T-cell function was assessed by stimulating whole blood with five different stimuli and measuring T-cell activation (percentage CD69+ T-cells) and cytokine production (TNF-α, IFN-γ, IL-2, IL-4) as described by us earlier [16] and further described in S2 Appendix. T-cell activation and intracellular production of cytokines were determined within the CD4+ and CD8+ T-cells. The interpretation of these T-cell function tests was done by the medical immunologist (AJAL) who has extensive experience with it in our immunologic laboratory.

3.3.3 Statistical analysis

We used descriptive statistics to provide summary results on individual outcomes. Fisher's exact test was used to compare two groups of dichotomous outcomes. Student's t test was used to compare the TRECs results of the patients with the healthy control group. In addition, the effect of age on the TRECs results was analysed with linear regression. A two-sided *p*-value smaller than 0.05 was considered as significant. The statistical software programme IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.) was used for statistical analysis. To construct graphs, GraphPad Prism for Windows, Version 5.04 (La Jolla, CA, USA) was used.

Table 1. Clinical characteristics of 24 patients with CHARGE syndrome

Patient	Age[1]	Sex	*CHD7* Mutation		Clinical CHARGE syndrome[2]	Infectious history			ENT procedures		Atopy	Cardiac surgery
						UAI	Pneumonia	Other	A/T	tubes		
CHD01	14.9	F	5222G>C	mis	typical	+	-	-	-	-	-	-
CHD02	8.3	M	1480C>T	non	typical	+[4]	-	++[4]	+	+	-	-
CHD04	3.8	F	8077-1G>A	splice	atypical	+	-	-	-	+	-	-
CHD05	13.8	M	2442+5G>C	splice	typical	+	-	+	-	-	-	-
CHD06	16.9	M	2442+5G>C	splice	atypical	+	-	+	+	-	-	-
CHD08	11.6	F	5405-17G>A	splice	atypical	+	-	-	-	+	-	-
CHD09	3.2	M	5405-17G>A	splice	typical	+[4]	-	-	-	+	all	-
CHD10	2.3	F	5428C>T	non	typical	-	++[3]	-	-	-	-	+
CHD11	13.5	M	8016G>A	non	typical	+[3]	+[3]	-	-	+	-	-
CHD12	5.9	F	5944_5989dup	fs	typical	+	+[3]	-	+	-	all	-
CHD13	5.8	M	7160C>A	non	typical	+[3]	-	-	-	+	-	-
CHD14	6.6	M	5241-5244del	fs	atypical	+[3]	-	++[3]	+	+	-	+
CHD15	14.4	F	5833C>T	non	typical	+[5]	-	-	+	-	all	+
CHD16	15.5	M	7650_7651del	fs	atypical	-	++[4]	-	-	-	-	-
CHD17	2.9	M	964delTT	fs	typical	+[5]	-	++[3]	-	-	ecz	-
CHD18	1.9	F	4542dup	fs	typical	-	++[4]	++[3]	-	-	-	+
CHD19	14.9	M	5405-17G>A	splice	typical	+	-	-	+	+	all	-
CHD20	8.4	M	3514_3515del	fs	typical	+	-	++[3]	-	-	-	-
CHD21	14.9	F	5181C>G	non	typical	+[3]	-	-	-	+	all; ecz	-
CHD22	3.1	F	Deletion 8q12.1q12.3	del	typical	++[3]	-	-	+	+	-	-
CHD23	8.1	M	4731delA	fs	typical	+[3]	-	-	-	-	all	-
CHD25	4.1	M	4783C>T	non	typical	-	++[4]	-	-	-	-	-
CHD26	11.8	M	5051-15T>A	splice	typical	+	-	-	-	+	-	+
CHD27	10.7	F	2572C>T	non	typical	+	++[3]	+[3]	-	+	asthma	+

-, none; +, yes; ++, yes, including hospital admission
[1] Age in years at time of evaluation
[2] Based on the criteria by Blake et al. [2] and/or Verloes [3]
[3] Antibiotics given
[4] Antibiotics given in addition to prophylactic antibiotics
[5] Prophylatic antibiotics given
all, allergy; A/T, adenectomy and/or tonsillectomy; del, deletion; ecz, eczema; F, female; fs, frameshift; M, male; mis, missense; n, number; non, nonsense; splice, splice site; tubes, tympanostomy tubes; UAI, upper airway infection, including otitis media.

3.4 Results

3.4.1 Clinical characteristics

We initially included 27 patients and 14 healthy controls in the study. Two of the patients were later excluded because blood sampling was unsuccessful, and one patient and two healthy controls withdrew from the study. The clinical characteristics of the remaining 24 patients are presented in Table 1. The median ages of patients and controls were 8.3 (range 1.9-16.9) and 11.5 (range 5.5-17.3) years, respectively. Two-thirds of the mutations in *CHD7* in our cohort are known to lead to a premature stop in *CHD7* (truncating mutations). All but five patients fulfilled the clinical criteria for typical CHARGE syndrome as defined by Blake et al. [2] and/or Verloes [3].

All 24 patients had a history of infections (often frequent). Twenty (83%) patients had experienced upper airway infections, including otitis media in 16 (67%) patients. Seven (29%) patients had had pneumonia and eight (33%) patients had a history of other infections, including dermatomucosal infections (n=5), gastroenteritis (n=1), and pyelonephritis (n=2). Ten (42%) patients had needed hospital admission for their infections, predominantly for pneumonia (n=5). In addition, 12 (50%) patients needed placement of tympanostomy tubes because of recurrent otitis media. Antibiotics had been ever given to 17 (71%) patients and seven (29%) patients had received prophylactic antibiotics for recurrent upper airway infections or pneumonia. None of the patients had had life-threatening infections like sepsis or meningitis. Candidiasis was only seen with concomitant antibiotic use. Atopic disorders (n=8, 33%) were mentioned as allergy (n=6, 25%), eczema (n=2, 8%) and asthma (n=1, 4%). None of the patients had an autoimmune disease.

In summary, all CHARGE patients had a history of infections. Otitis media and pneumonia were the most prevalent, with prophylactic antibiotics given to 29% of patients. Eighteen patients (75%) needed hospital admission for reasons related to infectious diseases.

3.4.2 Full blood count

Haemoglobin levels, numbers of erythrocytes, thrombocytes, and leukocytes (including neutrophils, lymphocytes, basophils, eosinophils, and monocytes) were normal in all 24 patients.

3.4.3 Humoral immunity

Humoral immunity was evaluated by determining immunoglobulin levels and absolute numbers of peripheral B-cells and B-cell subpopulations. The levels of immunoglobulins and immunoglobulin subclasses are shown in Table 2 and were normal in all 24 patients, except for one who had a marginally decreased IgA level of 0.50 g/l (normal 0.54 g/l).

Table 2. Immunoglobulin levels per CHARGE patient

Patient	Immunoglobulin levels[1]							
	IgG	IgA	IgM	IgG1	IgG2	IgG3	IgG4	IgE
CHD01	9.8 (5.2-15.6)	1.3 (0.54-3.6)	1.6 (0.13-2.4)	7.4 (3.7-12.8)	2.0 (0.85-6.1)	0.6 (0.13-1.63)	0.5 (0.023-2.3)	13 (<100)
CHD02	8.2 (5.2-15.6)	1.1 (0.54-3.6)	0.6 (0.13-2.4)	6.3 (3.7-12.8)	1.5 (0.85-6.1)	0.3 (0.13-1.63)	0.2 (0.023-2.3)	29 (<50)
CHD04	9.2 (4.3-13.4)	1.2 (0.19-2.2)	1.3 (0.21-1.8)	7.3 (3.2-12.8)	1.5 (0.52-3.4)	0.4 (0.13-1.33)	0.2 (0.012-1.58)	**20 (<10)**
CHD05	6.1 (5.2-15.6)	**0.5 (0.54-3.6)**	0.9 (0.13-2.4)	5.4 (3.7-12.8)	1.3 (0.85-6.1)	0.3 (0.13-1.63)	0.2 (0.023-2.3)	84 (<100)
CHD06	8.0 (7.0-16.0)	0.7 (0.70-4.0)	0.9 (0.40-2.3)	5.9 (3.7-12.8))	2.1 (0.85-6.1)	0.7 (0.13-1.63)	0.1 (0.023-2.3)	**420 (<100)**
CHD08	10.1 (5.2-15.6)	1.2 (0.54-3.6)	0.6 (0.13-2.4)	7.6 (3.7-12.8)	1.8 (0.85-6.1)	0.9 (0.13-1.63)	1.3 (0.023-2.3)	12 (<100)
CHD09	7.2 (4.3-13.4)	0.4 (0.19-2.2)	1.0 (0.21-1.8)	6.0 (3.2-10.0)	1.3 (0.52-3.4)	0.2 (0.13-1.33)	0.5 (0.012-1.58)	<2.0 (<10)
CHD10	7.0 (4.3-13.4)	0.5 (0.19-2.2)	1.1 (0.21-1.8)	5.8 (3.2-10.0)	1.6 (0.52-3.4)	0.3(0.13-1.33)	0.8 (0.012-1.58)	**15 (<10)**
CHD11	9.9 (5.2-15.6)	2.5 (0.54-3.6)	0.7 (0.13-2.4)	5.9 (3.7-12.8)	3.5 (0.85-6.1)	0.3 (0.13-1.63)	0.5 (0.023-2.3)	13 (<100)
CHD12	9.7 (4.3-13.4)	1.2 (0.19-2.2)	1.1 (0.21-1.8)	5.6 (3.2-10.0)	3.0 (0.52-3.4)	0.4 (0.13-1.33)	**2.2 (0.012-1.58)**	145 (<50)
CHD13	6.8 (4.3-13.4)	0.6 (0.19-2.2)	0.7 (0.21-1.8)	6.0 (3.2-10.0)	0.8 (0.52-3.4)	0.3 (0.13-1.33)	<0.1 (0.012-1.58)	24 (<25)
CHD14	7.0 (5.2-15.6)	1.2 (0.54-3.6)	1.3 (0.13-2.4)	5.4 (3.7-12.8)	2.1 (0.85-6.1)	0.3 (0.13-1.63)	0.3 (0.023-2.3)	7.5 (<25)
CHD15	12.3 (5.2-15.6)	2.1 (0.54-3.6)	1.2 (0.13-2.4)	8.1 (3.7-12.8)	3.4 (0.85-6.1)	0.4 (0.13-1.63)	**3.0 (0.023-2.3)**	70 (<100)
CHD16	9.4 (5.2-15.6)	1.1 (0.54-3.6)	0.5 (0.13-2.4)	7.0 (3.7-12.8)	1.8 (0.85-6.1)	0.3 (0.13-1.63)	0.8 (0.023-2.3)	22 (<100)
CHD17	11.2 (4.3-13.4)	0.8 (0.19-2.2)	1.0 (0.21-1.8)	9.2 (3.7-12.8)	1.2 (0.52-3.4)	0.4 (0.13-1.33)	0.3 (0.012-1.58)	**70 (<10)**
CHD18	8.3 (2.6-15.2)	0.7 (0.16-1.1)	0.9 (0.10-1.2)	7.3 (2.0-8.5)	1.3 (0.34-2.6)	0.5 (0.15-1.13)	<0.1 (0.011-0.79)	2.9 (<10)
CHD19	12.8 (5.2-15.6)	0.9 (0.54-3.6)	1.5 (0.13-2.4)	8.0 (3.7-12.8)	3.1 (0.85-6.1)	0.7 (0.13-1.63)	0.2 (0.023-2.3)	7.1 (<100)
CHD20	11.0 (5.2-15.6)	1.1 (0.54-3.6)	1.5 (0.13-2.4)	9.2 (3.7-12.8)	1.4 (0.85-6.1)	0.5 (0.13-1.63)	0.5 (0.023-2.3)	27 (<50)
CHD21	11.4 (5.2-15.6)	3.1 (0.54-3.6)	1.8 (0.13-2.4)	8.3 (3.7-12.8)	3.0 (0.85-6.1)	0.5 (0.13-1.63)	2.0 (0.023-2.3)	**1231 (<100)**
CHD22	6.1 (4.3-13.4)	0.5 (0.19-2.2)	0.9 (0.21-1.8)	5.2 (3.2-10.0)	1.2 (0.52-3.4)	0.2 (0.13-1.33)	<0.1 (0.012-1.58)	**190 (<10)**
CHD23	6.7 (5.2-15.6)	1.3 (0.54-3.6)	1.0 (0.13-2.4)	5.6 (3.7-12.8)	1.7 (0.85-6.1)	0.3 (0.13-1.63)	<0.1 (0.023-2.3)	26 (<50)
CHD25	12.0 (4.3-13.4)	1.4 (0.19-2.2)	0.8 (0.21-1.8)	9.2 (3.2-10.0)	1.3 (0.52-3.4)	0.4 (0.13-1.33)	0.4 (0.012-1.58)	**160 (<25)**
CHD26	12.7 (5.2-15.6)	2.3 (0.54-3.6)	2.2 (0.13-2.4)	10.6 (3.7-12.8)	0.9 (0.85-6.1)	0.5 (0.13-1.63)	0.2 (0.023-2.3)	56 (<100)
CHD27	13.3 (5.2-15.6)	1.7 (0.54-3.6)	2.3 (0.13-2.3)	10.0 (3.7-12.8)	3.3 (0.85-6.1)	1.1 (0.13-1.63)	1.0 (0.023-2.3)	**138 (<100)**

[1] Immunoglobulin concentration in g/l, except for IgE (IE/ml). Age-matched reference values are shown in brackets [12]. Values below or above the age-matched reference values are shown in **bold**.

Table 3. Peripheral B-cells, memory B-cells and IgM expression on class-switched memory B-cells per CHARGE patient.

Patient	B-cells[1] CD45+ CD19+	Memory B-cells[1] CD27+ IgD-	Class-switched memory B-cells[2] CD27+ IgD- IgM-/CD27+IgD-IgG+	IgM-only memory B-cells[2] CD27+ IgD- IgG- IgM+	IgM expression on class-switched memory B-cells CD27+ IgD- IgM-/CD27+IgD-IgG+
CHD01	397 (200-500)	23 (10-76)	22	1	IgM expression[3]
CHD02	420 (300-700)	18 (13-100)	18	1	N
CHD04	696 (400-1500)	52 (20-149)	46	5	IgM expression[3]
CHD05	430 (200-500)	26 (10-76)	24	2	IgM expression[3]
CHD06	360 (100-400)	19 (12-122)	19	1	IgM expression[3]
CHD08	282 (200-500)	18 (10-76)	18	0	N
CHD09	656 (400-1500)	31 (20-149)	26	5	N
CHD10	1287 (400-1500)	44 (20-149)	41	3	N
CHD11	488 (200-500)	38 (10-76)	37	1	IgM expression[3]
CHD12	562 (300-700)	76 (13-100)	74	2	N
CHD13	542 (300-700)	43 (13-100)	41	3	N
CHD14	409 (300-700)	15 (13-100)	14	1	N
CHD15	**173 (200-500)**	16 (10-76)	15	0	IgM expression[3]
CHD16	263 (200-500)	**9 (10-76)**	8	1	N
CHD17	534 (400-1500)	**19 (20-149)**	17	1	N
CHD18	**748 (900-2500)**	**8 (9-114)**	8	1	N
CHD19	273 (200-500)	15 (10-76)	14	1	IgM expression[3]
CHD20	350 (300-700)	45 (13-100)	36	9	N
CHD21	376 (200-500)	36 (10-76)	34	2	N
CHD22	1108 (400-1500)	45 (20-149)	44	1	IgM expression[3]
CHD23	337 (300-700)	23 (13-100)	21	1	N
CHD25	509 (400-1500)	**15 (20-149)**	12	2	N
CHD26	**179 (200-500)**	14 (10-76)	12	2	N
CHD27	281 (200-500)	10 (10-76)	7	3	N

[1] Absolute numbers in cells/µl. Age-matched reference values are shown in brackets [13,14]. Values below the age-matched reference values are shown in **bold**.

[2] No age-matched reference values available

[3] Class-switched memory B-cells, as shown by strong IgG expression, also retaining IgM expression.

N, normal (no expression of IgM on class-switched memory B-cells).

Absolute numbers of peripheral B-cells and B-cell subpopulations, in which relevant results were found, are shown in Table 3. Three (13%) patients had low numbers of B-cells. The absolute number of memory B-cells, consisting mainly of class-switched memory B cells and a small amount of IgM-only B-cells, is only slightly lower than the reference value in 4 of 24 (17%) CHARGE patients. Absolute numbers of all other peripheral B-cell subpopulations (transitional, naive mature, marginal zone-like, plasmablasts and CD21low B-cells) were normal or only slightly decreased in all CHARGE patients (see S3 Table).

Interestingly, in eight patients (33%), the memory B-cells, which clearly had undergone class-switch recombination as shown by strong IgG expression, retained IgM expression (Table 3 and Figure 1).

Thus, immunoglobulin production and peripheral B-cell differentiation were normal in our CHARGE patients, except for the abnormal expression of IgM on class-switched memory B-cells in a third of the patients.

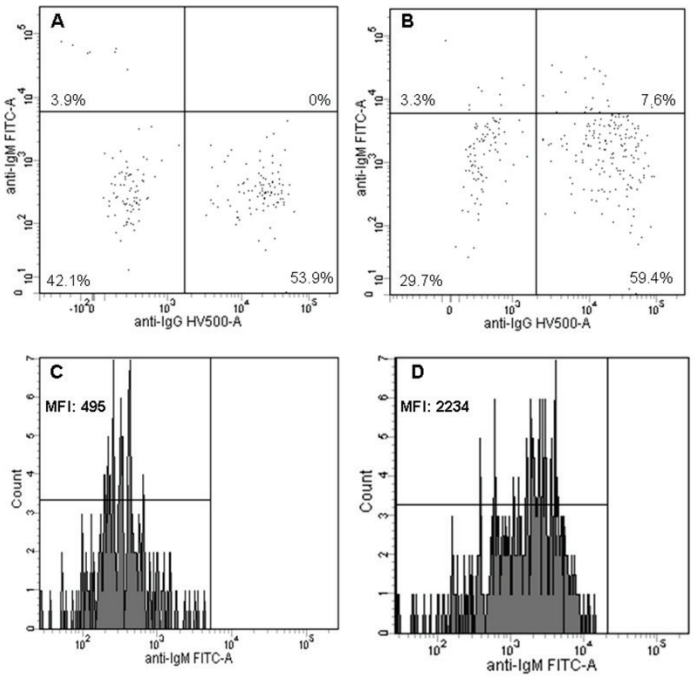

Figure 1. IgM and IgG expression on memory B-cells. IgM and IgG expression on memory B-cells (CD27+IgD-) of a CHARGE patient (B, D) and a simultaneously analyzed healthy control (A, C). A, B) IgG and IgM expression are used to differentiate between class-switched memory B-cells (CD27+IgD-IgM-/CD27+IgD-IgG+) and IgM-only memory B-cells (CD27+IgD-IgG-IgM++). Memory B-cells with high IgM expression and no expression of IgG are considered to be IgM-only memory B-cells (upper-left quadrant), all other memory B-cells are considered to be class-switched memory B-cells. C, D) Part of the CHARGE patients show abnormal expression of IgM on class-switched memory B-cells compared to healthy controls. MFI, mean fluorescence intensity.

3.4.4 Cellular immunity

Cellular immunity was evaluated by determining absolute numbers of peripheral NK-cells, T-cells and T-cell subpopulations, TRECs, and T-cell function. In Table 4, the results of peripheral NK-cells, T-cells and naive T-cells are shown. Two of 24 (8%) patients had low numbers of NK-cells. Overall, 12 of 24 (50%) patients had low peripheral T-cell numbers (CD3+, CD4+ and/or CD8+ T-cells), of which five (21%) had low numbers of CD3+ T-cells, five (21%) had low numbers of CD4+ T-cells, and eleven (46%) had low numbers of CD8+ T-cells. Compared to the healthy control group, decreased CD8+ T-cell numbers were found more often in CHARGE patients (p=0.031). Decreased numbers of CD4+ T-cells also occurred more often in CHARGE patients than in the healthy control group, but this difference was not significant (p=0.146).

In the T-cell subpopulations, we primarily saw deviations in the number of naive mature T-cells. The absolute or relative numbers of other T-cell subpopulations (central memory, effector memory, terminally differentiated, activated, CD4+ regulatory, αβ, γδ and double negative αβ T-cells) were not abnormal in a relevant way (S4 Table). For seven patients, T-cell subpopulations could not be interpreted because age-matched reference values were not available. Of the other seventeen patients, five (29%) and eight (47%) patients had low numbers of naive mature CD4+ T-cells and naive mature CD8+ T-cells, respectively. Most patients with low numbers of naive mature CD4+ or CD8+ T-cells also had low numbers of total CD4+ T or CD8+ T-cells. Decreased numbers of naive mature CD4+ T-cells (p=0.059) and naive mature CD8+ T-cells (p=0.009) occurred more often in CHARGE patients than in the healthy control group.

Table 4. Peripheral NK-cells, T-cells and naive T-cells per CHARGE patient

Patient	NK-cells[1]	CD3+ T-cells[1]	CD4+ T-cells[1]	Naive mature CD4+ T-cells[2]	Naive mature CD4+ T-cells[2]	CD8+ T-cells[1]	Naive mature CD8+ T-cells[2]	Naive mature CD8+ T-cells[2]
	CD16+56+	CD45+ CD3+	CD3+ CD4+	CD45RO- CCR7+ CD27+ CD28+	CD45RO- CCR7+ CD27+ CD28+	CD3+ CD8+	CD45RO- CCR7+ CD27+ CD28+	CD45RO- CCR7+ CD27+ CD28+
CHD01	261 (100-700)	1628 (1000-2000)	884 (500-1300)	527 (277-796)	59.6% (42.4-66.3)	578 (300-800)	396 (205-465)	68.5% (48.8-72.9)
CHD02	584 (100-600)	1755 (1100-2800)	1209 (500-1800)	800 (515-913)	66.2% (60.2-74.6)	446 (400-1200)	261 (369-578)	58.6% (49.1-78.8)
CHD04	117 (100-700)	2010 (1400-3600)	1322 (700-2000)	1000 (n/a)	75.7% (n/a)	500 (500-1400)	405 (n/a)	81.1% (n/a)
CHD05	**88 (100-700)**	1641 (1000-2000)	1001 (500-1300)	629 (277-796)	62.8% (42.4-66.3)	452 (300-800)	273 (205-465)	60.3% (48.8-72.9)
CHD06	110 (100-400)	1110 (700-1900)	610 (400-1300)	338 (277-796)	55.4% (42.4-66.3)	250 (200-700)	147 (205-465)	58.6% (48.8-72.9)
CHD08	230 (100-700)	1285 (1000-2000)	732 (500-1300)	394 (277-796)	53.8% (42.4-66.3)	392 (300-800)	229 (205-465)	58.4% (48.8-72.9)
CHD09	467 (100-700)	1458 (1400-3600)	862 (700-2000)	553 (n/a)	64.2% (n/a)	**417 (500-1400)**	325 (n/a)	77.9% (n/a)
CHD10	957 (100-700)	2546 (1400-3600)	1488 (700-2000)	1019 (n/a)	68.5% (n/a)	813 (500-1400)	236 (n/a)	29.1% (n/a)
CHD11	234 (100-700)	1282 (1000-2000)	845 (500-1300)	488 (277-796)	57.8% (42.4-66.3)	340 (300-800)	198 (205-465)	58.4% (48.8-72.9)
CHD12	182 (100-600)	1374 (1100-2800)	915 (500-1800)	**390 (515-913)**	**42.7% (60.2-74.6)**	**357 (400-1200)**	**115 (369-578)**	**32.2% (49.1-78.8)**
CHD13	372 (100-600)	2192 (1100-2800)	1269 (500-1800)	814 (515-913)	64.2% (60.2-74.6)	652 (400-1200)	**247 (369-578)**	**37.9% (49.1-78.8)**
CHD14	330 (100-600)	**899 (1100-2800)**	**458 (500-1800)**	**215 (515-913)**	**47.0% (60.2-74.6)**	**297 (400-1200)**	**87 (369-578)**	**29.3% (49.1-78.8)**
CHD15	172 (100-700)	1110 (1000-2000)	787 (500-1300)	490 (277-796)	62.3% (42.4-66.3)	**250 (300-800)**	138 (205-465)	55.5% (48.8-72.9)
CHD16	184 (100-700)	1042 (1000-2000)	**308 (500-1300)**	**111 (277-796)**	**36.2% (42.4-66.3)**	649 (300-800)	**115 (205-465)**	**17.7% (48.8-72.9)**
CHD17	118 (100-700)	1622 (1400-3600)	894 (700-2000)	691 (n/a)	77.3% (n/a)	520 (500-1400)	79 (n/a)	71.8% (n/a)
CHD18	219 (100-1100)	**1483 (2200-5500)**	**1008 (1100-3600)**	725 (n/a)	72.0% (n/a)	**373 (500-1800)**	310 (n/a)	83.2% (n/a)
CHD19	303 (100-700)	**887 (1000-2000)**	507 (500-1300)	250 (277-796)	49.3% (42.4-66.3)	**230 (300-800)**	**94 (205-465)**	**41.0% (48.8-72.9)**
CHD20	580 (100-600)	**940 (1100-2800)**	**400 (500-1800)**	**138 (515-913)**	**34.5% (60.2-74.6)**	**290 (400-1200)**	**73 (369-578)**	**25.1% (49.1-78.8)**
CHD21	187 (100-700)	1455 (1000-2000)	917 (500-1300)	654 (277-796)	71.4% (42.4-66.3)	417 (300-800)	310 (205-465)	74.6% (48.8-72.9)
CHD22	354 (100-700)	1431 (1400-3600)	1035 (700-2000)	602 (n/a)	58.1% (n/a)	**182 (500-1400)**	102 (n/a)	56.2% (n/a)
CHD23	192 (100-600)	1239 (1100-2800)	734 (500-1800)	495 (515-913)	67.5% (60.2-74.6)	**365 (400-1200)**	265 (369-578)	72.8% (49.1-78.8)
CHD25	**98 (100-700)**	1404 (1400-3600)	896 (700-2000)	625 (n/a)	69.7% (n/a)	**422 (500-1400)**	315 (n/a)	74.7% (n/a)
CHD26	656 (100-700)	1261 (1000-2000)	775 (500-1300)	511 (277-796)	65.9% (42.4-66.3)	342 (300-800)	**143 (205-465)**	**41.9% (48.8-72.9)**
CHD27	452 (100-700)	**606 (1000-2000)**	**368 (500-1300)**	**109 (277-796)**	**29.5% (42.4-66.3)**	**169 (300-800)**	**7 (205-465)**	**4.1% (48.8-72.9)**

[1] Absolute numbers in cells/µl. Age-matched reference values are shown in brackets [13,15]. Values below the age-matched reference values are shown in **bold**.

[2] Absolute numbers in cells/µl and relative numbers in percentages of naive mature CD4+ or CD8+ T-cells. Age-matched reference values are shown in brackets [15]. If both the absolute and the relative numbers are below the age-matched reference values, values are shown in **bold**.

n/a, age-matched reference value not available

Low numbers of naive mature and total T-cells can be caused by congenital thymic aplasia or hypoplasia, both of which have been described in patients with CHARGE syndrome [9]. However, low numbers of naive mature and total T-cells may also be caused by thymectomy during cardiac surgery due to congenital heart defects in CHARGE patients. Information on the thymus was available for six patients who had undergone cardiac surgery (Table 1). Two patients (CHD18 and 27) had thymectomy during cardiac surgery and had low peripheral T-cells. One patient (CHD 26) had only a partial thymectomy and had normal T-cell numbers (except for low naive CD8+ T-cells). In one patient (CHD14) thymus aplasia was confirmed during surgery, with resulting low T-cell numbers. The thymus was not mentioned in the operation reports for the other two patients (CHD10 and 15). Of the eighteen patients without cardiac surgery, eight patients (CHD09, 12, 16, 19, 20, 22, 23 and 25; 44%) had low peripheral T-cell numbers (CD3+, CD4+ and/or CD8+ T-cells).

TREC analysis could be performed in 22 patients with CHARGE syndrome. The mean TRECs/µg DNA was 998 (SD 535), which is significantly lower than the TRECs in the healthy control group (Figure 2, mean 1688, SD 814, p=0.005). To evaluate the effect of age on the amount of TRECs, a linear regression analyses was performed and showed no significant effect.

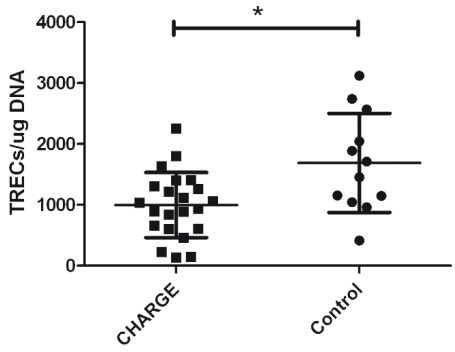

Figure 2. T-cell receptor excision circle (TREC) analysis. Numbers of TRECs in patients with CHARGE syndrome (n=22) compared to healthy controls (n=12). Error bars indicate means and standard deviations, *p=0.005.

Overall, the results of all 24 patients showed sufficient T-cell activation and intracellular cytokine production after stimulation with different mitogenic and antigenic stimuli (S5 Table). Of note, 11 (46%) patients had increased T-cell responses to one or more stimuli, compared to the age-matched reference values established on the results of our 12 healthy controls. Eight of these patients had decreased percentages of naive mature T-cells and increased percentages of effector memory or terminally differentiated T-cells, which might be an explanation of the increased responses.

In summary, 50% of patients had low peripheral T-cell numbers, mainly caused by low numbers of naive mature T-cells, which is consistent with the decreased numbers of TRECs. Although the numbers of T-cells are decreased, deficiencies in T-cell function were not found with the assay we performed.

3.4.5 Vaccination responses

All patients were vaccinated according to the Dutch or Belgian National Immunization Programmes [24,25], with the exception of one patient who had not been vaccinated at all (Table 5). Levels of IgG-specific antibodies to tetanus toxin were normal in all vaccinated patients. Eight of 22 (36%; assessment was unsuccessful in one patient) vaccinated patients had insufficient levels of IgG-specific antibodies to diphtheria and 15 of 23 (65%) vaccinated patients had insufficient levels of IgG-specific antibodies to *H. influenzae* type b. The vaccination response to pneumococcal polysaccharides could only be interpreted in 11 patients, since vaccination to pneumococcal polysaccharides was introduced into the Dutch vaccination programme in 2006. Three of 11 (27%) patients had insufficient antibodies to pneumococcal polysaccharides. Of 23 vaccinated patients, 19 (83%) had insufficient levels of IgG-specific antibodies to one or more of the vaccines we tested, while only four patients had sufficient levels of IgG-specific antibodies to all of the vaccines tested. Overall, reduced responses to one or more vaccinations, given in early childhood, are prevalent in patients with CHARGE syndrome.

Table 5. IgG-specific vaccine-induced antibody responses per CHARGE patient.[1]

Patient[2]	Diphtheria[3]	Tetanus[3]	Hib[4]	PPS[5]												
				PPS1	PPS3	PPS4	PPS5	PPS6A	PPS6B	PPS7F	PPS9V	PPS14	PPS18C	PPS19A	PPS19F	PPS23F
CHD01	**0.090**	2.06	**0.55**	0.09	0.34	0.06	0.06	0.42	**0.16**	**0.13**	**0.06**	**0.14**	3.2	8.1	7.6	1.7
CHD02	**0.089**	0.147	**0.35**	**0.33**	2.5	**0.28**	**0.07**	**0.06**	**0.09**	0.69	**0.13**	**0.07**	0.43	1.9	19	**0.27**
CHD04	**0.027**	0.155	**0.47**	**0.04**	**0.06**	**0.25**	**0.02**	**0.10**	**0.23**	**0.03**	0.56	0.54	0.59	0.51	6.8	9.8
CHD05	**0.070**	0.880	1.1	**0.11**	2.4	**0.04**	1	**0.09**	**0.16**	**0.13**	**0.05**	1.1	0.75	1.5	3.9	**0.04**
CHD06	**0.060**	7.00	**0.88**	**0.33**	14	**0.09**	**0.29**	**0.28**	3.8	0.37	**0.09**	>37	1.8	9.3	15	**0.05**
CHD08	0.100	7.00	**0.94**	**0.06**	**0.1**	**0.03**	**0.21**	**0.04**	**0.03**	**0.12**	**0.03**	0.86	**0.03**	1.8	15	**0.04**
CHD09	**0.066**	0.195	**0.70**	0.10	**0.09**	0.46	**0.12**	>23	4.4	0.54	**0.08**	**0.07**	**0.12**	**0.17**	0.80	3.9
CHD10	0.403	1.81	1.4	1.0	**0.23**	2.2	1.5	1.2	3.1	6.8	0.83	1.1	>17	**0.20**	>75	2.4
CHD11	**0.098**	1.35	>9.0	0.46	0.49	**0.03**	**0.11**	**0.25**	**0.26**	0.88	1.1	4.5	2.2	**0.15**	2.5	16
CHD12	1.85	1.64	**0.64**	**0.13**	4.0	>3.0	**0.070**	**0.11**	**0.26**	0.95	0.55	0.44	1.2	**0.25**	2.3	10
CHD13	0.790	9.49	**0.27**	**0.08**	**0.13**	**0.13**	**0.06**	1.6	3.9	**0.06**	0.53	9.9	1.3	5.7	>75	1.4
CHD14	1.47	4.35	**0.27**	**0.060**	1.8	**0.28**	**0.020**	2.6	3.7	>16	1.1	0.69	**0.19**	1.6	29	>21
CHD15	0.520	2.08	**0.61**	0.88	11	1.4	1.5	3.5	11	**0.26**	**0.18**	**0.24**	0.37	1.2	11	1.1
CHD16	**0.042**	0.101	>9.0	>8.1	**0.31**	1.6	2.1	**0.11**	**0.12**	1.0	**0.27**	9.1	**0.19**	**0.05**	1.9	**0.04**
CHD17	U	U	**0.32**	3.2	**0.16**	**0.11**	1.3	**0.04**	**0.21**	0.6	1.2	0.93	**0.21**	**0.05**	0.51	>21
CHD18	7.05	10.8	>9.0	0.7	n/a	**0.24**	0.53	n/a	0.58	1	**0.14**	2.1	**0.27**	n/a	1.3	0.54
CHD19	420	1.24	3.6	**0.32**	11	**0.15**	4.6	**0.06**	**0.03**	**0.31**	**0.06**	**0.18**	>17	0.96	5.2	**0.04**
CHD20	**0.020**	0.827	**0.30**	**0.06**	**0.19**	**0.11**	**0.02**	**0.04**	**0.06**	**0.03**	0.35	**0.08**	**0.03**	5.2	41	**0.04**
CHD21	0.190	0.405	**0.33**	**0.33**	**0.15**	**0.17**	1.0	**0.18**	0.52	**0.23**	4.1	1.8	**0.16**	0.66	4.7	3.0
CHD22	0.506	0.438	4.5	1.7	**0.070**	0.51	0.87	0.050	0.80	1.3	2.2	**0.16**	3.8	2.3	70	0.42
CHD23	1.43	1.74	**0.70**	**0.09**	0.91	**0.25**	**0.030**	1.2	6.7	1.4	0.41	0.62	5.4	2.7	59	**0.27**
CHD25	0.160	0.168	**0.72**	**0.05**	**0.09**	**0.09**	**0.01**	0.84	2.0	**0.03**	**0.23**	0.58	**0.05**	**0.08**	1.0	0.64
CHD26	0.311	6.20	**0.49**	1.1	2.1	**0.080**	**0.060**	**0.040**	**0.040**	**0.060**	0.41	4.7	1.6	0.70	7.7	**0.040**
CHD27	0.733	5.82	7.8	**0.20**	7.2	0.50	**0.28**	**0.23**	0.42	**0.30**	**0.15**	0.99	4.5	0.81	1.0	1.0

[1] All patients were vaccinated according to the Dutch or Belgian National Immunization Programmes [24,25], with the exception of patient CHD02 who had not been vaccinated. All vaccine titers were obtained without booster or recheck.
[2] Patients who were not vaccinated for pneumococcal polysaccharides are shown in *italic.*
[3] Concentration of antibodies to diphtheria or tetanus in IU/ml. A concentration ≥0.10 IU/ml is considered protective. Insufficient responses (<0.10 IU/ml) are shown in **bold**.
[4] Concentration of antibodies to *Haemophilus influenzae* type b in mg/l. A concentration >1.0 mg/l is considered protective. Insufficient responses (<1.0 mg/l) are shown in **bold**.
[5] Concentration of antibodies to pneumococcal polysaccharides in µg/ml. An adequate response to pneumococcal polysaccharides was defined as an absolute level >0.35 µg/ml in >50% of serotypes. Insufficient responses (<0.35 µg/ml) are shown in **bold**.
Hib, *Haemophilus influenzae* type b; PPS, pneumococcal polysaccharides serotype; U, unknown

3.5 Discussion

This is the first study to systematically and extensively explore the immune system of CHARGE patients. In our cohort of CHARGE patients, all had a history of infections (often frequent), specifically upper airway infections that often led to hospital admissions and the use of prophylactic antibiotics. Anomalies in the upper airway (atresia of choanae, abnormal outer and inner ear anatomy) contribute to patient susceptibility to infections and extend infection duration by impeding drainage or clearance of infectious debris. It is, however important, to know whether immunological abnormalities contribute to the frequency and complicate the severity of infections in order to optimize the management of care in these patients.

No abnormalities were found in routine diagnostics (full blood count), but with detailed immunologic assays, we found T-cell lymphopenia in 50% of patients, mainly caused by low numbers of naive mature T-cells. Our finding is comparable with the results of a retrospective study by Jyonouchi et al. [8], who found overall T-cell lymphopenia in four of nine (44%) CHARGE patients.

Notably, we found low T-cell numbers in 44% of patients who had not undergone cardiac surgery and therefore should have an "intact" thymus. Congenital dysmorphology or dysfunction of the thymus might be the underlying cause of T-cell lymphopenia and this is supported by our finding of diminished TREC numbers in the patients. Little is known about thymic abnormalities in CHARGE patients from the literature. Thymic anomalies have been reported in foetuses with confirmed *CHD7* mutations [26] and were reported in 16 of 36 (44%) patients with a proven mutation in *CHD7* [9]. Unfortunately, there is no specific information on the thymus for 18 of 24 patients in our CHARGE cohort.

Evidence for the role of *CHD7* and *TBX1*, the causative gene of 22q11.2 deletion syndrome, has been shown in the embryonic development of the thymus in animal models. Both genes are expressed in the pharyngeal arches which contain precursors of thymic stromal cells [27,28]. Bi-directional molecular interaction between thymic epthelial cells and T-cell progenitor cells is critical for the complete morphological and functional maturation of both cell compartments [27]. Thus, abnormal thymic development presumably not only affects the level of T-cell output, but could also affect the function of T-cells. For example, the T-cell receptor repertoire [29] and the development of natural regulatory T-cells [30] have been shown to be affected in patients with 22q11.2 deletion syndrome. Although we found normal T-cell responses with our T-cell function assay, we cannot exclude subtle dysfunctions in more complex T-cell function, such as the delicate interaction between T-cells and B-cells.

Peripheral B-cell and NK-cell numbers were normal in almost all patients, comparable to former reports [9]. However, hypogammaglobulinaemia was found in 61% of CHARGE patients in former case reports [9], while in our study the immunoglobulin levels were normal in all patients. Publication bias needs to be taken in consideration for the higher percentages found in case reports. Actually, our

results were more comparable with those from 22q11.2 deletion syndrome, where hypogammaglobulinaemia was found in only 6% of a large cohort of 855 patients [31]. Although peripheral B-cell differentiation was normal, a third of the CHARGE patients had class-switched memory B-cell retaining IgM expression. We had not anticipated on this finding in our methods by including isotype controls, which limits the interpretation of these data. Nonetheless, these cells may indicate impaired class-switch recombination and memory B-cell formation in CHARGE patients. To our knowledge, these phenotypically abnormal class-switched memory B-cells have not been reported before, but lower numbers of class-switched memory B-cells have been found in adults with 22q11.2 deletion syndrome [32]. We can speculate that the formation of fully functional class-switched memory B-cells in CHARGE patients is impeded due to insufficient T-cell help during the class-switch recombination process, leading to diminished production of specific antibodies. However, our data is insufficient to fully support this tentative hypothesis which links our findings in the peripheral T-cell populations with the humoral abnormalities. More research is needed on memory B-cell formation and function in CHARGE patients.

Specific antibodies to one or more vaccines given in childhood were insufficient in 83% of patients, specifically to diphtheria and *H. influenzae* type b vaccines. In the literature, vaccination responses in CHARGE syndrome are only described in case reports and in one retrospective study. Reduced responses to diphtheria (n=3), tetanus (n=4), *H. influenzae* type b (n=2), and pneumococcal polysaccharides (n=1) have been reported [8,33,34]. Although protective levels of specific antibodies decrease over time, this waning seems to occur at an earlier age in patients with CHARGE syndrome (median age 14.7 years) compared with the general population (30-40 years of age) [35,36].

We studied the largest cohort of well-defined CHARGE patients with confirmed *CHD7* mutations so far but, of course, statistical analysis on only 24 patients limits the interpretation of our results. What we can state is that immunological abnormalities are often seen in patients with CHARGE syndrome. We hypothesize that abnormal thymic development leads to diminished numbers of T-cells that may also be impaired in more subtle functions as activating B-cells to differentiate into fully functional class-switched memory B-cells. Incomplete class-switched memory B-cell formation can be an explanation for the insufficient responses to vaccines in our CHARGE patients due to poor humoral memory.

The high prevalence of immunological abnormalities combined with the frequent occurrence of infections demonstrates the need for more research in a larger cohort to extend the analysis of correlations between clinical data and immunological laboratory results, and to confirm some of our immunological findings. With such data, evidence-based guidelines can be developed for the timely diagnosis of immune dysfunctions based on clinical symptoms, which will help protect these children from excess morbidity and mortality due to infections. Nonetheless, based on the results of this study, we would recommend performing specialised immunologic assays (B- and T-cell numbers and vaccination responses) in patients with persistent infections

who need prophylactic antibiotics, since the immune abnormalities we found will not be apparent with routine diagnostics. Considering the high prevalence of reduced antibody responses, it may be worthwhile to give these patients booster vaccinations and recheck the antibody responses. Firstly because *H. influenza* and pneumococcal infections are highly prevalent in otitis media [37], and secondly to determine if CHARGE patients have a primary lack of response to some vaccines or if they have poor humoral memory.

3.6 Acknowledgements

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Supplementary information

S1 Appendix. Monoclonal antibodies and gating strategy for the B- and T-cell subpopulations.

The following monoclonal antibodies were used: CD19/CD3-PerCP-Cy5.5 (SJ25C1/SK3); CD27/CD25-APC (L128/2A3), CD38/CD8-APC-H7 (HB7/SK1), IgD/CD27/CD127/TCRγδ-PE (IA6-2/L128/HIL-7R-M21/11F2); IgM/CD45R0/TCRαβ-FITC (Polyclonal/UCHL1/WT31); IgG-BV510 (G18-145), CD21/CD4-Horizon V450 (B-ly4/RPA-T4); CD24/CD28/HLA-DR-PE-c7 (ML5/CD28.2/L243); and CCR7-Alexa Fluor 647 (150503). All reagents were purchased from Becton Dickinson, except for IgM-FITC (IQProducts, Groningen , Netherlands). Within the B-cells (CD19+), transitional (CD27- CD24++ CD38+), naive mature (CD27- IgD+), marginal zone-like (CD27+ IgD+), memory (CD27+ IgD-), class-switched memory (CD27+ IgD- IgM-/CD27+IgD-IgG+), IgM-only memory (CD27+ IgD- IgG- IgM++), CD27- memory (CD27- IgD-), plasmablasts (CD27++ IgM- CD24- CD38++), and CD21low (CD21low CD38-) B-cell subpopulations were distinguished. Within the CD4+ and CD8+ T cells, naïve (CD45R0- CCR7+ CD27+ CD28+), central memory (CD45R0+ CCR7+), effector memory (CD45R0+ CCR7-), terminally differentiated (CD45R0- CCR7-/CD45R0- CCR7+ CD28-), and activated (HLA-DR+) T-cell subpopulations were distinguished. Furthermore, CD4+ regulatory (CD25+ CD127-), double negative αβ (CD3+ CD4- CD8- TCRαβ+), and **γδ** (CD3+ TCRγδ+) T-cells were distinguished.

S2 Appendix. Stimulation, fluorescent barcoding, and monoclonal antibodies in the T-cell function assay.

Heparinised whole blood was stimulated with either 10 μg/ml purified protein derivative (PPD; Statens serum institute, Copenhagen, Denmark), 5 μg/ml Staphylococcal enterotoxin B (SEB; Sigma, Deisenhofen, Germany), 100 μl anti-CD3 (in house stock of WT32, 1:10 diluted), 15 Lf/ml Tetanus toxoid (TT; Statens serum institute), or 5 μg/ml phytohemagglutinin (PHA; Remel, Lenexa, KS, USA). Unstimulated blood was used as negative control. To all samples, except PHA and SEB stimulated samples, 2 μl CD28/CD49d (1 mg/ml) was added. After 2 hours of incubation at 37ºC, 2 μl Brefaldin A (1 mg/ml) was added, and the samples were incubated for another 18-22 hours. After incubation and lysation, samples were stained with different concentrations of Pacific Orange and/or Pacific Blue for fluorescent barcoding (Invitrogen Carlsbad, CA, USA). Subsequently, samples were stained with the following monoclonal antibodies: CD3-FITC (UCHT1), CD4-PE-Cy7 (SK3), CD8-PerCP (SK1), CD69-APC-Cy7 (FN50), IFN-γ-PE (B27), TNF-α-APC (Mab11), IL-2-PE (MQ1-17H12), and IL-4-APC (8D4-8). IFN-γ-PE and IL-2-PE were stained in separate tubes. The same applies for TNF-α-APC and IL-4-APC. All reagents were purchased from Becton Dickinson.

S3 Table. Peripheral B-cell subpopulations per CHARGE patient.

Patient	Transitional B-cells[1] CD27- CD24++ CD38+	Naive mature B-cells[1] CD27- IgD+	Marginal zone-like B-cells[1] CD27+ IgD+	CD27- memory[1] CD27- IgD-	Plasmablasts[1] CD27++ IgM- CD24- CD38++	CD21low[2] CD21low CD38-
CHD01	59 (4-108)	251 (87-390)	47 (7-90)	6 (n/a)	1 (0.5-20)	3.4% (<10%)
CHD02	50 (11-77)	284 (111-486)	42 (15-88)	13 (n/a)	0 (1-15)	3.5% (<10%)
CHD04	116 (24-333)	401 (170-1691)	72 (16-226)	19 (n/a)	5 (n/a)	5.0% (<10%)
CHD05	40 (4-108)	289 (87-390)	55 (7-90)	10 (n/a)	0 (0.5-20)	4.7% (<10%)
CHD06	23 (3-50)	278 (57-447)	25 (9-88)	7 (n/a)	0 (1-23)	3.1% (<10%)
CHD08	55 (4-108)	170 (87-390)	20 (7-90)	4 (n/a)	1 (0.5-20)	1.8% (<10%)
CHD09	109 (24-333)	423 (170-1691)	49 (16-226)	16 (n/a)	1 (n/a)	3.9% (<10%)
CHD10	239 (24-333)	859 (170-1691)	75 (16-226)	17 (n/a)	5 (n/a)	2.7% (<10%)
CHD11	122 (4-108)	247 (87-390)	44 (7-90)	9 (n/a)	6 (0.5-20)	2.8% (<10%)
CHD12	83 (11-77)	306 (111-486)	56 (15-88)	22 (n/a)	6 (1-15)	7.2% (<10%)
CHD13	65 (11-77)	293 (111-486)	98 (15-88)	12 (n/a)	5 (1-15)	6.6% (<10%)
CHD14	64 (11-77)	263 (111-486)	41 (15-88)	9 (n/a)	3 (1-15)	5.0% (<10%)
CHD15	15 (4-108)	105 (87-390)	16 (7-90)	3 (n/a)	6 (0.5-20)	3.6% (<10%)
CHD16	20 (4-108)	210 (87-390)	14 (7-90)	4 (n/a)	1 (0.5-20)	3.1% (<10%)
CHD17	142 (24-333)	279 (170-1691)	56 (16-226)	10 (n/a)	3 (n/a)	4.4% (<10%)
CHD18	211 (38-551)	441 (322-1991)	38 (23-195)	4 (n/a)	1 (n/a)	3.8% (<10%)
CHD19	65 (4-108)	152 (87-390)	19 (7-90)	4 (n/a)	2 (0.5-20)	6.7% (<10%)
CHD20	27 (11-77)	166 (111-486)	75 (15-88)	20 (n/a)	2 (1-15)	9.4% (<10%)
CHD21	43 (4-108)	227 (87-390)	44 (7-90)	12 (n/a)	2 (0.5-20)	3.6% (<10%)
CHD22	177 (24-333)	700 (170-1691)	127 (16-226)	14 (n/a)	4 (n/a)	4.7% (<10%)
CHD23	64 (11-77)	183 (111-486)	30 (15-88)	12 (n/a)	4 (1-15)	7.2% (<10%)
CHD25	118 (24-333)	311 (170-1691)	44 (16-226)	4 (n/a)	2 (n/a)	2.7% (<10%)
CHD26	33 (4-108)	105 (87-390)	10 (7-90)	6 (n/a)	1 (0.5-20)	8.9% (<10%)
CHD27	67 (4-108)	172 (87-390)	18 (7-90)	5 (n/a)	0 (0.5-20)	9.6% (<10%)

[1] Absolute numbers in cell/µL. Age-matched reference values are shown in brackets [14]. Values below the age-matched reference values are shown in **bold**.

[2] Relative numbers in percentages within B-cells. Age-matched reference values are shown in brackets. Values above the age-matched reference values are shown in **bold**.

n/a, age-matched reference value not available

S4 Table. Peripheral T-cells subpopulations per CHARGE patient.

Patient	Central memory CD4+ T-cells[1] *CD45R0+ CCR7+*	Central memory CD4+ T-cells[2] *CD45R0+ CCR7+*	Effector memory CD4+ T-cells[1] *CD45R0+ CCR7-*	Effector memory CD4+ T-cells[2] *CD45R0+ CCR7-*	Terminally differentiated CD4+ T-cells[1] *CD45R0- CCR7-/ CD45R0- CCR7+ CD28-*	Terminally differentiated CD4+ T-cells[2] *CD45R0- CCR7-/ CD45R0- CCR7+ CD28-*	Activated CD4+ T-cells[2] *HLA-DR+*
CHD01	218 (127-270)	24.7% (13.7-31.9)	106 (83-206)	12.0% (11.8-26.2)	28 (14-76)	3.2% (1.4-7.8)	3.0% (<5%)
CHD02	227 (151-249)	18.8% (14.2-22.8)	135 (82-166)	11.2% (7.4-17.2)	45 (16-67)	3.7% (1.4-5.4)	4.5% (<5%)
CHD04	239 (n/a)	18.1% (n/a)	57 (n/a)	4.3% (n/a)	22 (n/a)	1.7% (n/a)	2.0% (<5%)
CHD05	238 (127-270)	23.8% (13.7-31.9)	115 (83-206)	**11.5% (11.8-26.2)**	18 (14-76)	1.8% (1.4-7.8)	3.9% (<5%)
CHD06	127 (127-270)	20.8% (13.7-31.9)	114 (83-206)	18.7% (11.8-26.2)	31 (14-76)	5.0% (1.4-7.8)	**6.6% (<5%)**
CHD08	182 (127-270)	24.9% (13.7-31.9)	143 (83-206)	19.5% (11.8-26.2)	**12 (14-76)**	1.7% (1.4-7.8)	**6.3% (<5%)**
CHD09	234 (n/a)	27.2% (n/a)	52 (n/a)	6.0% (n/a)	22 (n/a)	2.5% (n/a)	3.1% (<5%)
CHD10	277 (n/a)	18.6% (n/a)	113 (n/a)	7.6% (n/a)	70 (n/a)	4.7% (n/a)	3.0% (<5%)
CHD11	193 (127-270)	22.8% (13.7-31.9)	145 (83-206)	17.2% (11.8-26.2)	19 (14-76)	2.2% (1.4-7.8)	4.1% (<5%)
CHD12	**259 (151-249)**	**28.3% (14.2-22.8)**	**237 (82-166)**	**25.9% (7.4-17.2)**	28 (16-67)	3.1% (1.4-5.4)	**6.0% (<5%)**
CHD13	**294 (151-249)**	**23.2% (14.2-22.8)**	138 (82-166)	10.9% (7.4-17.2)	19 (16-67)	1.5% (1.4-5.4)	2.3% (<5%)
CHD14	**123 (151-249)**	**26.8% (14.2-22.8)**	90 (82-166)	**19.7% (7.4-17.2)**	28 (16-67)	**6.1% (1.4-5.4)**	4.5% (<5%)
CHD15	182 (127-270)	23.1% (13.7-31.9)	95 (83-206)	12.1% (11.8-26.2)	19 (14-76)	2.4% (1.4-7.8)	3.0% (<5%)
CHD16	**107 (127-270)**	**34.7% (13.7-31.9)**	**79 (83-206)**	25.6% (11.8-26.2)	**10 (14-76)**	3.4% (1.4-7.8)	**7.3% (<5%)**
CHD17	149 (n/a)	16.7% (n/a)	34 (n/a)	3.8% (n/a)	18 (n/a)	2.0% (n/a)	2.7% (<5%)
CHD18	195 (n/a)	19.4% (n/a)	64 (n/a)	6.4% (n/a)	21 (n/a)	2.1% (n/a)	3.0% (<5%)
CHD19	159 (127-270)	31.3% (13.7-31.9)	93 (83-206)	18.3% (11.8-26.2)	**6 (14-76)**	**1.1% (1.4-7.8)**	**6.4% (<5%)**
CHD20	151 (151-249)	**37.8% (14.2-22.8)**	99 (82-166)	**24.7% (7.4-17.2)**	**11 (16-67)**	2.8% (1.4-5.4)	**5.2% (<5%)**
CHD21	176 (127-270)	19.2% (13.7-31.9)	**59 (83-206)**	**6.4% (11.8-26.2)**	25 (14-76)	2.7% (1.4-7.8)	2.2% (<5%)
CHD22	276 (n/a)	26.6% (n/a)	121 (n/a)	11.7% (n/a)	24 (n/a)	2.3% (n/a)	**6.3% (<5%)**
CHD23	165 (151-249)	22.5% (14.2-22.8)	**59 (82-166)**	8.0% (7.4-17.2)	**14 (16-67)**	1.9% (1.4-5.4)	2.9% (<5%)
CHD25	210 (n/a)	23.4% (n/a)	47 (n/a)	5.3% (n/a)	13 (n/a)	1.5% (n/a)	1.6% (<5%)
CHD26	**102 (127-270)**	**13.2% (13.7-31.9)**	**66 (83-206)**	**8.5% (11.8-26.2)**	**92 (14-76)**	**11.9% (1.4-7.8)**	2.2% (<5%)
CHD27	147 (127-270)	**40.0% (13.7-31.9)**	87 (83-206)	23.7% (11.8-26.2)	23 (14-76)	6.2% (1.4-7.8)	4.2% (<5%)

Patient	Central memory CD8+ T-cells[1] *CD45R0+ CCR7+*	Central memory CD8+ T-cells[2] *CD45R0+ CCR7+*	Effector memory CD8+ T-cells[1] *CD45R0+ CCR7-*	Effector memory CD8+ T-cells[2] *CD45R0+ CCR7-*	Terminally differentiated CD8+ T-cells[1] *CD45R0- CCR7-/ CD45R0- CCR7+ CD28-*	Terminally differentiated CD8+ T-cells[2] *CD45R0- CCR7-/ CD45R0- CCR7+ CD28-*	Activated CD8+ T-cells[2] *HLA-DR+*
CHD01	15 (11-60)	2.6% (1.9-11.4)	72 (35-111)	12.5% (5.3-18.6)	94 (68-207)	16.2% (13.0-30.4)	4.3% (<11%)
CHD02	21 (6-63)	4.7% (0.9-9.3)	82 (26-114)	**18.5% (3.8-13.8)**	**81 (82-246)**	18.2% (11.0-32.6)	**16.0% (<11%)**
CHD04	10 (n/a)	2.0% (n/a)	28 (n/a)	5.7% (n/a)	56 (n/a)	11.3% (n/a)	2.0% (<11%)
CHD05	**10 (11-60)**	2.3% (1.9-11.4)	96 (35-111)	**21.2% (5.3-18.6)**	73 (68-207)	16.2% (13.0-30.4)	5.2% (<11%)
CHD06	**10 (11-60)**	4.0% (1.9-11.4)	65 (35-111)	**25.8% (5.3-18.6)**	**29 (68-207)**	11.5% (13.0-30.4)	**11.8% (<11%)**
CHD08	11 (11-60)	2.8% (1.9-11.4)	80 (35-111)	**20.4% (5.3-18.6)**	72 (68-207)	18.3% (13.0-30.4)	6.8% (<11%)
CHD09	9 (n/a)	2.1% (n/a)	28 (n/a)	6.8% (n/a)	55 (n/a)	13.1% (n/a)	2.5% (<11%)
CHD10	13 (n/a)	1.6% (n/a)	89 (n/a)	11.0% (n/a)	469 (n/a)	57.7% (n/a)	7.0% (<11%)
CHD11	18 (11-60)	5.2% (1.9-11.4)	73 (35-111)	**21.5% (5.3-18.6)**	**51 (68-207)**	15.0% (13.0-30.4)	6.4% (<11%)
CHD12	15 (6-63)	4.2% (0.9-9.3)	**121 (26-114)**	**33.9% (3.8-13.8)**	105 (82-246)	29.5% (11.0-32.6)	8.6% (<11%)
CHD13	33 (6-63)	5.0% (0.9-9.3)	**216 (26-114)**	**33.1% (3.8-13.8)**	156 (82-246)	24.0% (11.0-32.6)	**12.0% (<11%)**
CHD14	14 (6-63)	4.6% (0.9-9.3)	73 (26-114)	**24.8% (3.8-13.8)**	121 (82-246)	**40.9% (11.0-32.6)**	**18.4% (<11%)**
CHD15	11 (11-60)	4.4% (1.9-11.4)	74 (35-111)	**29.7% (5.3-18.6)**	**26 (68-207)**	**10.5% (13.0-30.4)**	4.8% (<11%)
CHD16	**7 (11-60)**	**1.1% (1.9-11.4)**	**136 (35-111)**	**21.0% (5.3-18.6)**	**389 (68-207)**	**60.0% (13.0-30.4)**	10.6% (<11%)
CHD17	8 (n/a)	1.9% (n/a)	89 (n/a)	12.4% (n/a)	16 (n/a)	14.0% (n/a)	7.0% (<11%)
CHD18	6 (n/a)	1.7% (n/a)	17 (n/a)	4.6% (n/a)	39 (n/a)	10.4% (n/a)	4.5% (<11%)
CHD19	**10 (11-60)**	4.3% (1.9-11.4)	106 (35-111)	**46.3% (5.3-18.6)**	**19 (68-207)**	**8.4% (13.0-30.4)**	**18.8% (<11%)**
CHD20	9 (6-63)	3.0% (0.9-9.3)	**123 (26-114)**	**42.5% (3.8-13.8)**	85 (82-246)	29.4% (11.0-32.6)	7.5% (<11%)
CHD21	**7 (11-60)**	**1.6% (1.9-11.4)**	**26 (35-111)**	6.3% (5.3-18.6)	72 (68-207)	17.4% (13.0-30.4)	2.1% (<11%)
CHD22	5 (n/a)	2.6% (n/a)	29 (n/a)	15.9% (n/a)	46 (n/a)	25.0% (n/a)	10.2% (<11%)
CHD23	13 (6-63)	3.5% (0.9-9.3)	52 (26-114)	**14.4% (3.8-13.8)**	**33 (82-246)**	**9.2% (11.0-32.6)**	4.4% (<11%)
CHD25	14 (n/a)	3.4% (n/a)	40 (n/a)	9.4% (n/a)	52 (n/a)	12.4% (n/a)	1.9% (<11%)
CHD26	12 (11-60)	3.4% (1.9-11.4)	76 (35-111)	**22.1% (5.3-18.6)**	110 (68-207)	**32.3% (13.0-30.4)**	4.2% (<11%)
CHD27	**2 (11-60)**	**1.2% (1.9-11.4)**	83 (35-111)	**48.9% (5.3-18.6)**	77 (68-207)	**45.7% (13.0-30.4)**	5.7% (<11%)

Patient	CD4+ regulatory T-cells[2] *CD25+ CD127-*	αβ T-cells[2] *CD3+ TCRαβ+*	γδ T-cells[2] *CD3+ TCRγδ+*	Double negative αβ T-cells[2] *CD3+ CD4- CD8- TCRαβ+*
CHD01	8.6% (3-10%)	89.2% (81-98%)	10.4% (1-18%)	2.3% (<2.5%)
CHD02	4.0% (3-10%)	95.6% (81-98%)	4.0% (1-18%)	2.2% (<2.5%)
CHD04	8.4% (3-10%)	92.1% (81-98%)	7.5% (1-18%)	1.7% (<2.5%)
CHD05	6.6% (3-10%)	84.1% (81-98%)	15.1% (1-18%)	2.0% (<2.5%)
CHD06	8.3% (3-10%)	**67.5% (81-98%)**	**32.0% (1-18%)**	1.8% (<2.5%)
CHD08	5.8% (3-10%)	88.8% (81-98%)	10.9% (1-18%)	**3.0% (<2.5%)**
CHD09	7.1% (3-10%)	91.7% (81-98%)	7.3% (1-18%)	**3.2% (<2.5%)**
CHD10	8.0% (3-10%)	96.2% (81-98%)	2.7% (1-18%)	**5.4% (<2.5%)**
CHD11	9.0% (3-10%)	92.7% (81-98%)	6.9% (1-18%)	1.6% (<2.5%)
CHD12	10.0% (3-10%)	93.5% (81-98%)	5.5% (1-18%)	1.6% (<2.5%)
CHD13	6.3% (3-10%)	87.6% (81-98%)	11.9% (1-18%)	1.2% (<2.5%)
CHD14	7.8% (3-10%)	84.2% (81-98%)	15.7% (1-18%)	1.6% (<2.5%)
CHD15	8.0% (3-10%)	93.4% (81-98%)	6.3% (1-18%)	1.4% (<2.5%)
CHD16	4.3% (3-10%)	90.6% (81-98%)	8.3% (1-18%)	**4.5% (<2.5%)**
CHD17	7.3% (3-10%)	83.9% (81-98%)	15.9% (1-18%)	1.3% (<2.5%)
CHD18	5.4% (3-10%)	95.2% (81-98%)	4.2% (1-18%)	**4.5% (<2.5%)**
CHD19	7.7% (3-10%)	84.1% (81-98%)	14.7% (1-18%)	1.7% (<2.5%)
CHD20	9.4% (3-10%)	**73.6% (81-98%)**	**26.1% (1-18%)**	2.2% (<2.5%)
CHD21	6.5% (3-10%)	90.1% (81-98%)	8.5% (1-18%)	**2.6% (<2.5%)**
CHD22	7.0% (3-10%)	90.1% (81-98%)	9.7% (1-18%)	2.4% (<2.5%)
CHD23	8.5% (3-10%)	91.7% (81-98%)	7.4% (1-18%)	**2.5% (<2.5%)**
CHD25	6.1% (3-10%)	95.8% (81-98%)	3.2% (1-18%)	**2.7% (<2.5%)**
CHD26	8.0% (3-10%)	86.7% (81-98%)	12.5% (1-18%)	1.4% (<2.5%)
CHD27	7.4% (3-10%)	82.8% (81-98%)	16.4% (1-18%)	2.0% (<2.5%)

[1] Absolute numbers in cell/µL. Age-matched reference values are shown in brackets [15]. Values below or above the age-matched reference values are shown in **bold**.

[2] Relative numbers in percentages of T-cell subpopulations within CD3+, CD4+ or CD8+ T-cells. Age-matched reference values are shown in brackets [15]. Values below or above the age-matched reference values are shown in **bold**.

n/a, age-matched reference value not available

S5 Table. T-cell function assay per CHARGE patient.

CD4+ T-cells		CHD01	CHD02	CHD04	CHD05	CHD06	CHD08	CHD09	CHD10*	CHD11	CHD12*	CHD13*	CHD14*	CHD15	CHD16*	Reference values§
Blanco	CD69†	0,9	1,2	1,0	1,3	1,1	1,0	**2,8**	1,5	1,0	1,3	0,8	1,0	0,9	1,4	0,8-1,5
	IFN-γ‡	1,0	0,2	**0,1**	0,6	0,8	0,6	**1,4**	0,3	0,8	0,4	1,0	0,8	0,9	0,6	0,2-1,0
	TNF-α‡	1,2	0,9	0,9	1,5	**1,9**	1,0	1,0	**0,7**	0,9	**0,7**	1,1	**0,5**	1,1	1,0	0,9-1,5
	IL-2‡	**1,1**	0,4	0,5	0,5	0,4	0,9	0,9	0,7	0,9	0,6	0,6	**0,3**	0,9	0,8	0,4-0,9
	IL-4‡	3,0	2,9	**1,8**	3,3	3,2	4,6	**10,2**	**6,9**	3,2	**2,1**	3,5	3,4	3,4	5,2	2,3-5,4
PHA	CD69†	50,1	36,7	**24,8**	57,1	**62,3**	37,7	**32,2**	**58,4**	**25,3**	46,7	43,7	58,3	**74,6**	**58,6**	34,7-58,3
	IFN-γ‡	4,6	1,9	**0,5**	5,3	4,3	5,1	2,8	4,5	3,1	2,5	1,2	4,6	**7,2**	**15,4**	1,0-6,9
	TNF-α‡	30,2	13,6	**8,8**	23,0	27,8	31,2	15,2	25,1	17,9	23,6	14,9	29,3	**43,9**	**42,6**	12,0-32,5
	IL-2‡	16,8	5,9	**2,4**	11,8	14,5	16,5	6,7	11,9	8,4	9,3	7,5	16,1	**21,3**	**20,7**	4,0-17,3
	IL-4‡	6,9	**1,4**	1,7	2,6	3,7	2,1	8,7	8,6	3,1	2,7	**0,4**	3,3	5,3	5,0	1,5-13,7
anti-CD3	CD69†	**19,4**	3,8	8,2	6,3	12,4	3,3	2,8	**17,0**	**0,8**	**15,5**	12,3	**21,8**	**14,6**	10,5	2,8-12,8
	IFN-γ‡	0,0	0,1	0,0	0,0	0,0	0,0	**0,6**	0,0	0,0	0,0	0,0	**0,6**	0,0	**1,2**	0,0-0,5
	TNF-α‡	**3,6**	**0,5**	1,6	2,7	3,4	**3,6**	**0,5**	1,0	**0,1**	2,6	2,3	**7,7**	**5,2**	2,7	0,7-3,5
	IL-2‡	**0,5**	**0,6**	0,3	0,1	0,2	**1,0**	0,0	0,3	0,0	**0,6**	0,0	**2,5**	0,3	**1,2**	0,0-0,4
	IL-4‡	1,1	0,2	0,0	0,0	0,1	1,1	0,1	0,0	1,2	1,5	0,0	0,6	**2,0**	0,0	0,0-1,5
SEB	CD69†	**26,5**	21,6	21,6	**9,0**	20,4	**4,2**	19,5	**24,8**	**5,3**	21,7	20,6	**27,4**	**32,5**	**23,3**	12,7-22,5
	IFN-γ‡	**4,9**	3,7	**1,0**	**1,4**	3,9	**1,6**	2,5	2,6	2,5	4,3	2,2	**6,5**	**5,0**	**9,8**	1,8-4,7
	TNF-α‡	**19,9**	**17,2**	**16,6**	**5,1**	14,4	**5,1**	**16,4**	12,6	**6,2**	**17,6**	14,9	**16,7**	**27,6**	**19,1**	8,8-15,8
	IL-2‡	**14,5**	**13,8**	9,2	**3,3**	10,5	**4,0**	9,2	9,5	**4,2**	**13,5**	11,2	**14,5**	**19,1**	**15,1**	5,6-11,9
	IL-4‡	**3,8**	1,9	1,0	0,0	0,4	0,0	**4,9**	2,5	1,6	**3,3**	1,1	**2,6**	1,4	2,0	0,0-2,5
TT	CD69†	**0,1**	2,2	**0,1**	**0,0**	**0,0**	0,3	**0,0**	**9,5**	0,2	**5,5**	0,8	**5,4**	0,3	0,4	0,2-2,4
	IFN-γ‡	0,0	0,0	0,0	0,0	0,0	0,1	0,0	**0,7**	0,0	**0,2**	0,0	**0,4**	0,0	0,0	0,0-0,1
	TNF-α‡	0,0	0,2	0,2	0,0	0,0	0,0	**0,6**	**1,3**	0,0	**0,6**	0,0	**0,4**	0,0	0,0	0,0-0,3
	IL-2‡	0,0	0,0	0,0	0,0	0,0	0,2	0,0	**1,3**	0,0	**0,5**	0,1	**0,3**	**0,3**	**0,6**	0,0-0,2
	IL-4‡	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	**0,2**	0,0	0,0	**1,0**	0,0	0,0-0,1
PPD	CD69†	0,1	**2,9**	0,7	0,5	0,6	0,4	**0,0**	**7,3**	0,4	0,4	0,7	**3,9**	0,4	1,5	0,1-1,9
	IFN-γ‡	0,0	0,0	0,0	0,3	0,0	0,0	0,2	0,0	0,0	0,0	0,0	0,0	0,3	0,0	0,0-0,4
	TNF-α‡	0,0	0,0	0,2	0,2	**0,8**	0,0	**0,5**	0,2	0,0	0,0	0,0	0,3	0,1	0,0	0,0-0,4
	IL-2‡	0,0	**0,1**	0,0	**0,1**	0,0	0,0	0,0	**0,4**	0,0	0,0	**0,1**	0,0	0,0	**0,2**	0,0-0,0
	IL-4‡	0,5	0,0	0,0	0,2	**1,1**	0,5	0,2	0,0	0,5	0,0	0,0	0,7	0,3	0,0	0,0-1,0

CD4+ T-cells		CHD17	CHD18	CHD19*	CHD20*	CHD21	CHD22	CHD23	CHD25	CHD26	CHD27*	Reference values§
Blanco	CD69†	1,5	0,9	0,8	1,1	0,9	1,1	1,8	1,0	1,0	0,9	0,8-1,5
	IFN-γ‡	0,4	0,4	0,4	0,2	0,5	0,3	2,8	0,8	0,4	0,5	0,2-1,0
	TNF-α‡	1,4	0,8	0,9	0,8	0,9	0,6	1,1	0,9	0,9	0,6	0,9-1,5
	IL-2‡	0,7	0,3	1,0	0,2	0,4	0,3	0,9	0,6	0,8	0,6	0,4-0,9
	IL-4‡	6,8	3,2	4,5	5,1	1,8	2,3	9,1	8,2	4,3	1,6	2,3-5,4
PHA	CD69†	27,5	86,1	40,5	73,2	53,3	43,0	39,6	49,9	37,2	52,7	34,7-58,3
	IFN-γ‡	0,6	1,8	6,4	7,7	3,2	1,8	2,9	3,0	1,9	7,0	1,0-6,9
	TNF-α‡	9,0	57,3	35,3	37,1	22,7	7,2	24,5	24,7	16,4	41,3	12,0-32,5
	IL-2‡	2,8	26,8	17,6	20,5	11,0	4,8	9,7	12,2	6,9	23,8	4,0-17,3
	IL-4‡	1,7	7,6	4,3	3,8	4,2	0,4	14,3	7,5	2,3	4,3	1,5-13,7
anti-CD3	CD69†	3,2	5,8	5,3	19,2	12,0	5,8	10,0	2,6	5,0	10,1	2,8-12,8
	IFN-γ‡	0,0	0,0	1,7	0,0	0,1	0,1	0,9	0,6	0,1	0,0	0,0-0,5
	TNF-α‡	0,5	0,4	6,9	2,7	2,6	0,1	2,7	0,5	1,5	3,0	0,7-3,5
	IL-2‡	0,0	0,0	4,2	0,3	0,7	0,1	0,7	0,6	0,4	0,8	0,0-0,4
	IL-4‡	0,5	0,0	5,2	1,9	1,2	0,1	2,3	0,0	0,0	0,8	0,0-1,5
SEB	CD69†	17,3	40,8	17,1	13,5	11,1	26,9	11,9	25,9	7,9	25,1	12,7-22,5
	IFN-γ‡	1,1	2,7	5,7	2,8	1,6	1,8	1,3	2,4	1,0	7,5	1,8-4,7
	TNF-α‡	11,2	25,6	18,5	8,3	7,1	11,4	9,9	18,0	4,7	20,8	8,8-15,8
	IL-2‡	6,0	15,3	12,2	8,0	4,0	9,3	6,1	11,8	3,3	18,4	5,6-11,9
	IL-4‡	0,6	2,5	1,2	0,6	1,8	1,1	3,6	3,0	0,0	4,9	0,0-2,5
TT	CD69†	1,1	1,2	0,4	0,2	0,1	0,8	0,2	0,0	2,1	2,1	0,2-2,4
	IFN-γ‡	0,0	0,1	0,3	0,5	0,1	0,0	0,0	0,0	0,1	0,0	0,0-0,1
	TNF-α‡	0,0	0,2	0,4	0,0	0,0	0,1	0,2	0,1	0,0	0,1	0,0-0,3
	IL-2‡	0,0	0,2	0,3	0,4	0,0	0,1	0,3	0,1	0,3	0,1	0,0-0,2
	IL-4‡	0,8	0,0	0,0	0,0	0,6	0,0	0,0	0,0	0,0	0,0	0,0-0,1
PPD	CD69†	1,0	0,3	0,1	0,6	0,4	4,7	0,0	0,0	0,5	13,5	0,1-1,9
	IFN-γ‡	0,2	0,0	0,0	0,0	0,2	0,0	0,0	0,0	0,1	0,2	0,0-0,4
	TNF-α‡	0,0	0,0	0,6	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0-0,4
	IL-2‡	0,0	0,1	0,4	0,1	0,0	0,1	0,0	0,1	0,2	0,5	0,0-0,0
	IL-4‡	0,0	0,3	0,0	0,1	0,6	0,0	0,0	0,0	0,0	0,1	0,0-1,0

CD8+ T-cells		CHD01	CHD02	CHD04	CHD05	CHD06	CHD08	CHD09	CHD10*	CHD11	CHD12*	CHD13*	CHD14*	CHD15	CHD16*	Reference values§
Blanco	CD69†	1,1	0,9	0,7	1,0	1,3	1,0	**3,3**	1,0	1,0	0,9	0,9	0,9	0,9	1,0	0,7-1,3
	IFN-γ‡	0,7	0,2	0,2	0,8	0,7	0,7	**1,5**	0,4	1,0	0,4	0,6	0,5	0,8	0,5	0,2-1,0
	TNF-α‡	0,9	**0,7**	**0,5**	1,2	**2,0**	0,8	0,9	1,0	0,9	**0,3**	1,2	0,6	1,0	1,3	0,8-1,6
	IL-2‡	0,9	0,4	0,6	0,4	0,6	0,8	0,7	0,8	**1,0**	0,5	**1,0**	0,3	0,8	0,3	0,4-0,9
	IL-4‡	2,3	1,5	1,6	2,4	2,8	2,8	**10,4**	3,8	2,3	1,8	3,2	2,8	2,8	2,4	1,5-5,3
PHA	CD69†	44,3	33,5	**30,4**	45,0	**50,8**	**24,9**	**28,6**	**47,6**	**23,1**	37,2	**50,1**	40,5	**56,3**	41,6	30,8-46,9
	IFN-γ‡	**13,1**	**11,3**	4,4	4,0	6,4	5,8	**3,2**	**36,4**	5,3	**18,6**	**19,5**	10,1	8,5	**30,0**	3,6-11,0
	TNF-α‡	**16,9**	13,9	6,3	7,6	13,9	7,8	**3,4**	**43,3**	8,4	**27,0**	**23,6**	**17,1**	12,5	**38,1**	5,6-15,3
	IL-2‡	**6,7**	3,6	**1,9**	3,0	4,3	**5,8**	**0,7**	2,8	4,1	3,5	**6,3**	**8,1**	4,6	4,7	2,0-5,4
	IL-4‡	2,9	1,0	0,8	1,6	1,0	**0,3**	4,6	2,2	0,8	**0,3**	**0,0**	**0,5**	1,4	1,9	0,6-6,1
anti-CD3	CD69†	**16,3**	7,3	8,5	8,5	**17,6**	**2,7**	6,0	**16,4**	**1,4**	10,8	**18,2**	**16,7**	**14,5**	7,7	4,3-13,1
	IFN-γ‡	1,7	1,7	0,6	0,0	1,1	0,3	0,2	**5,3**	0,0	0,7	**2,9**	0,3	1,0	**3,0**	0,0-1,9
	TNF-α‡	2,5	3,7	1,5	1,7	3,3	0,5	0,6	**11,6**	**0,0**	2,2	**5,5**	3,8	1,9	**5,8**	0,3-4,3
	IL-2‡	0,1	0,6	0,6	0,4	0,6	0,6	0,1	0,7	0,0	0,4	0,6	**1,5**	0,2	0,2	0,0-1,2
	IL-4‡	0,1	0,0	0,1	0,0	0,0	0,1	0,0	0,0	0,9	0,4	0,0	0,0	0,6	0,3	0,0-1,1
SEB	CD69†	**22,5**	15,9	17,0	**8,2**	19,8	**5,0**	16,5	15,9	**5,3**	11,9	17,3	**28,4**	**22,6**	20,9	10,6-21,6
	IFN-γ‡	**7,1**	**8,4**	2,2	**1,2**	5,5	4,2	2,7	**7,6**	2,3	5,7	**7,0**	**11,8**	**6,6**	**16,1**	2,2-5,9
	TNF-α‡	**9,2**	**9,6**	3,7	**2,3**	**7,7**	4,0	3,3	**9,4**	**2,7**	**7,5**	**9,2**	**14,3**	**10,5**	**21,5**	3,1-7,1
	IL-2‡	**5,9**	**6,0**	2,7	**0,9**	3,6	3,6	**1,4**	1,9	**1,3**	3,1	**4,4**	**7,3**	**6,5**	3,9	1,7-4,2
	IL-4‡	0,7	0,6	0,7	0,2	0,0	0,1	**2,9**	0,1	0,4	0,4	0,0	0,7	0,5	0,2	0,0-1,0
TT	CD69†	0,2	**1,7**	0,3	0,1	**0,0**	0,1	**0,0**	**6,3**	0,3	**3,9**	0,4	**6,4**	0,2	**0,0**	0,1-1,4
	IFN-γ‡	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,4	0,0	0,0	0,0	0,0	0,1	0,0	0,0-0,4
	TNF-α‡	0,0	0,0	0,1	0,0	0,0	0,0	**1,3**	**0,5**	0,0	0,2	0,2	0,1	0,0	0,0	0,0-0,2
	IL-2‡	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,1	0,0	0,2	0,0	0,0	0,0	0,0	0,0-0,2
	IL-4‡	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	**0,1**	0,0	0,0	0,0	**0,7**	0,0	0,0-0,0
PPD	CD69†	**0,1**	**3,0**	0,5	0,6	0,8	**0,0**	0,4	**5,1**	0,6	0,0	0,6	**4,4**	**0,1**	0,8	0,2-1,4
	IFN-γ‡	0,0	0,0	0,1	0,0	0,0	0,3	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,1	0,0-0,8
	TNF-α‡	0,1	0,0	0,1	0,2	**0,7**	0,0	**0,8**	**0,5**	0,0	0,0	0,0	0,0	0,0	**0,4**	0,0-0,2
	IL-2‡	0,0	0,0	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,2	0,0	0,1	0,0-0,2
	IL-4‡	0,0	0,3	0,0	0,1	**0,8**	0,0	0,0	0,0	0,4	0,0	0,0	**1,0**	**0,6**	0,0	0,0-0,4

CD8+ T-cells		CHD17	CHD18	CHD19*	CHD20*	CHD21	CHD22	CHD23	CHD25	CHD26	CHD27*	Reference values§
Blanco	CD69†	1,3	0,9	0,8	0,9	0,8	0,9	**2,3**	1,1	0,9	0,9	0,7-1,3
	IFN-γ‡	0,9	0,7	0,6	0,3	0,8	0,5	0,5	1,0	1,0	0,4	0,2-1,0
	TNF-α‡	**2,3**	**0,6**	1,1	1,1	1,0	**0,5**	1,3	1,1	1,3	1,0	0,8-1,6
	IL-2‡	0,8	**1,0**	0,7	0,8	0,5	0,5	0,8	0,6	0,7	0,7	0,4-0,9
	IL-4‡	4,9	2,7	3,2	2,9	2,1	1,8	**7,5**	**6,1**	2,2	1,2	1,5-5,3
PHA	CD69†	**30,7**	**80,4**	31,9	**56,0**	46,3	**52,7**	**30,7**	46,9	**26,0**	**27,1**	30,8-46,9
	IFN-γ‡	**2,9**	3,7	**23,3**	**18,0**	4,5	**7,3**	7,0	**11,2**	6,9	**16,9**	3,6-11,0
	TNF-α‡	**5,5**	10,9	**24,6**	**21,1**	5,8	7,8	9,3	10,6	12,8	**26,5**	5,6-15,3
	IL-2‡	1,1	2,4	**5,5**	**5,6**	3,1	2,2	2,4	**5,5**	2,4	**7,3**	2,0-5,4
	IL-4‡	**0,3**	3,8	2,9	**0,0**	0,9	**0,3**	**8,0**	1,8	1,1	0,8	0,6-6,1
anti-CD3	CD69†	**3,9**	**16,5**	**2,9**	**14,4**	6,5	**14,5**	6,3	6,4	8,7	7,2	4,3-13,1
	IFN-γ‡	0,1	0,0	0,5	0,8	0,8	0,0	1,2	1,1	1,1	0,8	0,0-1,9
	TNF-α‡	0,4	0,4	0,9	1,6	0,3	0,5	0,5	0,7	2,4	3,6	0,3-4,3
	IL-2‡	0,4	0,0	0,2	0,4	0,6	0,0	0,2	0,6	0,8	0,8	0,0-1,2
	IL-4‡	0,1	0,0	**4,2**	0,3	0,0	0,3	**1,4**	0,0	0,5	0,3	0,0-1,1
SEB	CD69†	19,3	**35,1**	11,8	10,7	11,1	**25,5**	**8,5**	**22,1**	**6,5**	11,1	10,6-21,6
	IFN-γ‡	3,4	2,5	**10,9**	**6,6**	**2,0**	4,7	3,6	5,0	**1,7**	**8,1**	2,2-5,9
	TNF-α‡	3,8	3,9	**13,7**	4,8	**1,9**	4,7	3,5	5,9	**2,6**	**9,5**	3,1-7,1
	IL-2‡	2,1	1,8	**4,9**	3,3	1,8	2,1	1,8	3,8	**1,4**	**4,3**	1,7-4,2
	IL-4‡	0,0	0,7	**1,1**	0,1	0,3	0,1	**1,1**	0,2	0,0	0,1	0,0-1,0
TT	CD69†	0,7	**1,5**	0,3	0,1	0,1	0,9	**0,0**	**0,0**	**3,2**	1,4	0,1-1,4
	IFN-γ‡	0,0	0,0	0,0	0,0	0,1	0,0	0,0	0,0	0,0	**0,7**	0,0-0,4
	TNF-α‡	0,0	0,0	0,2	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0-0,2
	IL-2‡	0,1	0,1	0,1	0,0	0,0	0,0	0,0	0,2	**0,5**	0,0	0,0-0,2
	IL-4‡	**0,4**	0,0	0,0	0,0	0,0	**0,3**	0,0	0,0	0,0	0,0	0,0-0,0
PPD	CD69†	**1,5**	0,7	**0,0**	0,9	0,3	**6,2**	**0,0**	**0,0**	1,1	**9,1**	0,2-1,4
	IFN-γ‡	0,0	0,0	0,0	0,0	0,3	0,0	0,0	0,2	0,1	0,4	0,0-0,8
	TNF-α‡	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0-0,2
	IL-2‡	**0,7**	0,2	0,0	0,0	0,0	0,0	0,0	0,0	**0,7**	**0,6**	0,0-0,2
	IL-4‡	0,0	0,2	0,0	0,3	0,3	0,4	0,0	0,0	0,0	0,0	0,0-0,4

* Patients with decreased percentages of naive mature T-cells and increased percentages of effector memory or terminally differentiated T-cells.

† Percentage of CD69+ cells within CD4+ or CD8+ T-cells. Percentage of background T-cell activation (blanco) is substracted from percentages of T-cell activation after different stimuli. Values below or above the age-matched reference values are shown in **bold**.

‡ Percentage of cytokine-positive CD4+ or CD8+ T-cells. Values below or above age-matched reference values are shown in **bold**.

§ Age-matched reference values were established with the results of 12 healthy controls

PHA, phytohemagglutinin; PPD, purified protein derivative; SEB, Staphylococcal enterotoxin B; TT, Tetanus toxoid

S6 Dataset. Data of the T-cell receptor excision circle analysis

Group	Age (years)	TRECs/ug DNA	Group	Age (years)	TRECs/ug DNA
Control	11,4	2044,88	CHARGE	16,9	1119,07
Control	5,5	1889,85	CHARGE	13,8	2255,99
Control	8,3	2569,46	CHARGE	5,8	1408,91
Control	15,3	960,13	CHARGE	14,9	1803,81
Control	13,1	416,51	CHARGE	14,4	1309,00
Control	5,8	1044,68	CHARGE	2,9	1638,44
Control	11,9	1148,11	CHARGE	3,8	1063,65
Control	10,1	2740,85	CHARGE	11,6	891,96
Control	7,6	3120,32	CHARGE	13,5	899,89
Control	17,3	1155,32	CHARGE	1,9	1218,62
Control	11,6	1454,13	CHARGE	14,9	842,72
Control	11,9	1710,06	CHARGE	15,5	136,36
			CHARGE	2,3	230,33
			CHARGE	4,1	605,57
			CHARGE	8,4	150,07
			CHARGE	8,3	608,93
			CHARGE	6,6	n/a
			CHARGE	3,2	1404,11
			CHARGE	8,1	460,20
			CHARGE	5,9	666,34
			CHARGE	10,7	n/a
			CHARGE	11,8	934,59
			CHARGE	3,1	1039,58
			CHARGE	14,9	1260,01

CHAPTER 4

An explorative study to assess thymus presence in CHARGE syndrome

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4.1 Abstract

Background: We previously found a role for thymus-matured lymphocytes in immune dysfunction in CHARGE syndrome, a rare genetic disorder caused by haploinsufficiency of the *CHD7* gene. The incidence of thymic anomalies in CHARGE syndrome, however, remains unknown. Our aim in this study was to explore the prevalence of thymic aplasia in children with CHARGE syndrome and examine its correlation with *CHD7* mutation type, the presence of congenital heart defects, T-cell lymphopenia and hypocalcaemia.

Methods: Chest radiographs of 37 patients with a *CHD7* mutation made before age four years and prior to cardiac surgery were examined for the presence of a thymic shadow by two radiologists. Surgery reports and patient files were screened for information on the thymus, type of *CHD7* mutation, congenital heart defects, T-cell lymphopenia, naive mature CD4+ and CD8+ T-cells and hypocalcaemia.

Results: Six of the 37 patients (16%) had a thymic shadow on their radiographs. The thymus was described as being present in the surgery reports of six other patients, but this was in conflict with their chest radiographs. Based on the combined results from chest radiographs and surgery reports, there were no significant correlations between the presence of the thymus and the type of *CHD7* mutation, nor with the presence of congenital heart defects, T-cell lymphopenia, naive mature CD4+ and CD8+ T-cells and hypocalcaemia.

Conclusions: We were not able to estimate the prevalence of thymic aplasia in children with CHARGE syndrome because chest radiographs proved inadequate for this purpose. Prospective studies with other methods including age-related controls are needed.

4.2 Introduction

CHARGE syndrome (OMIM# 214800) is a highly variable genetic disorder with an incidence of 1 in 15,000-17,000 live births [1]. The genetic cause of CHARGE syndrome is haploinsufficiency of the *CHD7* gene (OMIM# 608892) [2], which is expressed during the embryonic development of various organs. This haploinsufficiency leads to the variable phenotypic features that compose the acronym CHARGE: Coloboma, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital abnormalities, and Ear anomalies including deafness. Although, no clear genotype-phenotype correlations have been found, *CHD7* mutations leading to a premature stop codon (i.e. truncating mutations) are, in general, associated with a more severe phenotype [3].

CHD7 expression has been found in neural crest cells of the pharyngeal arches, from which the great vessels, the heart, the thymus and the parathyroid glands develop [4-7]. Heart defects are well described in CHARGE syndrome [8], but little is known about thymic abnormalities even though immunological abnormalities have been described incidentally in CHARGE syndrome [9-11]. We recently reported that 12/24 (50%) children with a *CHD7* mutation had decreased T-cell numbers [12], with one explanation for our findings being decreased thymic output due to thymic hypoplasia or aplasia. Remarkably, very little information about the thymus was available for these children, although thymic aplasia has been reported in fetuses and individuals with CHARGE syndrome [13,14]. Recently, defects of thymic organogenesis are observed in *chd7*-knockdown and knockout zebrafish embryos [7]. In a recent review we collected information that indicated thymic aplasia in 16/23 (70%) patients with a *CHD7* mutation for whom information about their thymus was available [15]. We also had information on T-cells available for 9/16 (56%) and found an association with T-cell lymphopenia in all nine. However, our review was predominantly based on case reports in which immunological observations were published. In the majority of clinical papers describing CHARGE syndrome, little is said about immune function apart from an increased susceptibility to upper airway infections. This could mean that the relatively high percentages of thymic abnormalities in our review on immunological aspects in CHARGE syndrome may have been due to a reporting bias. Thus, the real incidence of thymic aplasia in CHARGE syndrome remains unknown.

In this retrospective study, we aimed to better estimate the incidence of thymic aplasia in children with CHARGE syndrome by evaluating their chest radiographs. Furthermore, we explored the correlation between thymic aplasia and type of *CHD7* mutation, the presence of congenital heart defects and the presence of T-cell lymphopenia, including low counts of naive mature CD4+ and CD8+ T-cells. We also explored the correlation between thymic aplasia and hypocalcaemia, since hypocalcaemia has been reported in patients with CHARGE syndrome [11,16].

4.3 Methods

4.3.1 Patients

The Dutch Expert Clinic for CHARGE syndrome at the University Medical Centre Groningen (UMCG) maintains a detailed clinical database of 135 patients with CHARGE syndrome. For this study, we selected patients who had genetically confirmed CHARGE syndrome and for whom at least one chest radiograph was made before age four years and prior to cardiac surgery. In the literature, the reported upper age limit for when the thymus can be examined on chest radiographs varies from age two [17,18] up to six years [19,20]. Since there is no consensus on the age limit, we chose an age of four years as our cut-off. To ensure that only congenital thymic aplasia would be explored, radiographs made after cardiac surgery were excluded because the thymus is frequently all or partially removed during surgery to provide a better view of the cardiac anatomy.

Because of its observational nature, this study was exempted from ethical review by the UMCG's Medical Ethical Review Committee. All patients or their legal representatives gave consent for use of their patient data in CHARGE syndrome research and for requesting information from other hospitals.

4.3.2 Data collection

We first identified eligible chest radiographs of patients with CHARGE syndrome in our own hospital. If no eligible chest radiographs were available, a request was sent to other hospitals. We extracted information on the thymus and calcium levels from patient files for those patients for whom a chest radiograph was available. Cardiac surgery reports, in which the procedure and detailed observations made during surgery were reported by the surgeon, were screened for a description of the thymus. We extracted data on the type of *CHD7* mutation, the presence of congenital heart defects and the presence of T-cell lymphopenia from our in-house CHARGE syndrome database. The type of *CHD7* mutation was classified into truncating mutations (nonsense, frame shift, and whole-gene deletions) and non-truncating mutations (missense and splice site). T-cell lymphopenia was defined if reported in the patient file or if the absolute CD3+, CD4+ and/or CD8+ T-cell numbers were decreased compared to age-matched reference values. Thirteen patients had participated in our immunology study in which their naive mature CD4+ and CD8+ T-cell (CD45RO- CCR7+ CD27+ CD28+) counts were assessed [12]. Because age-matched reference values are not available for children younger than five years, interpretation of the results was only possible for six patients.

4.3.3 Evaluation of chest radiographs

Two pediatric radiologists evaluated the chest radiographs together for the presence of a thymic shadow, and their assessments had to agree to be included in our study. When there were multiple chest radiographs for a patient, we examined a maximum of three and used the radiograph made at the youngest age first. To reduce the chance

of evaluating radiographs during the same illness, a minimal time interval of one month between multiple radiographs of one patient was chosen. Chest radiographs selected for evaluation were listed on a score chart for the radiologists (see S1 File).

4.3.4 Statistical analysis

Descriptive statistics were used to provide summary results. The thymus was considered to be present when it was visible on at least one of the patient's chest radiographs. The correlation between thymic aplasia and the other categorical variables (type of *CHD7* mutation, presence of congenital heart defects, T-cell lymphopenia, low naive mature CD4+ or CD8+ T-cell counts, and hypocalcaemia) were explored by Fisher's exact test. A two-sided *p*-value less than 0.05 was considered significant. The software program IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.) was used for statistical analysis.

4.4 Results

Genetically confirmed CHARGE syndrome with a known *CHD7* mutation was present in 107 of the 135 patients in our database. We had chest radiographs meeting our criteria for 37/107 (35%) patients (18 males, 19 females; Figure 1 and Table 1). For 19 patients we had only one radiograph, for 12 patients we had two, and for six patients we had at least three. In total, we had 61 chest radiographs to examine. The age at which the chest radiographs were made ranged from day of birth to 3 years 4 months, with a median age of 2 months.

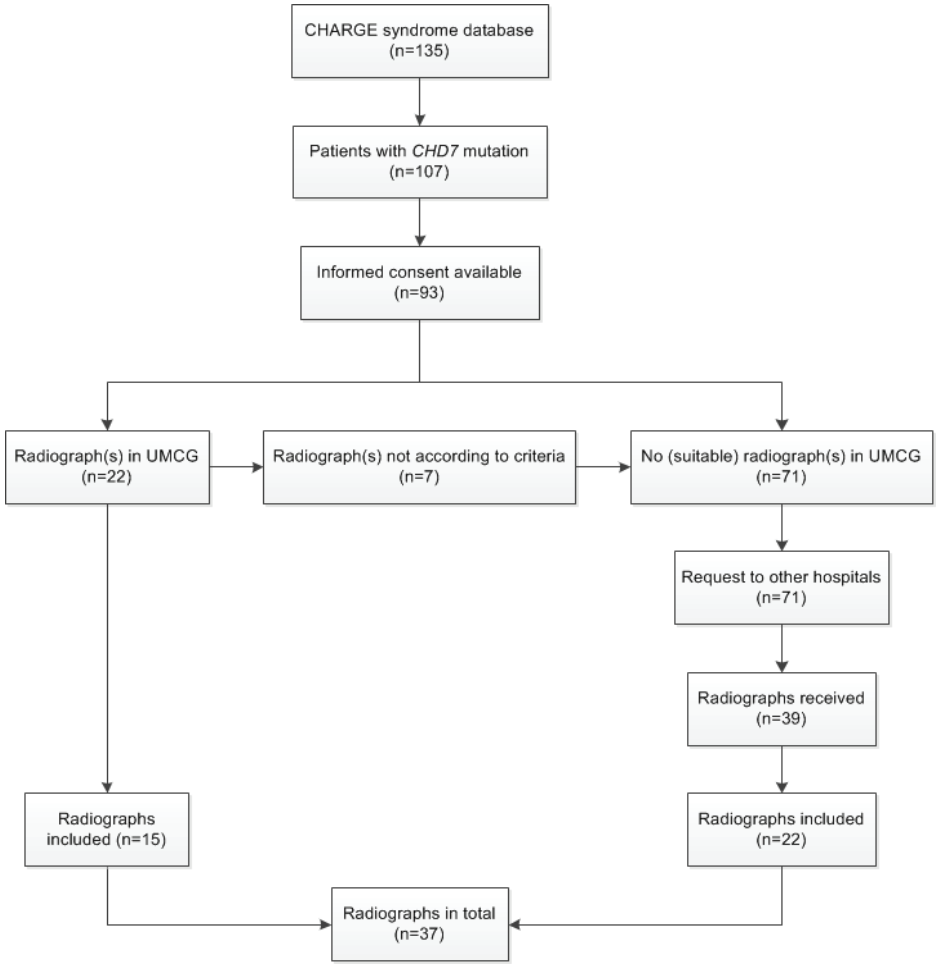

Figure 1. Flow diagram for inclusion of patients and chest radiographs. n, number of patients.

Table 1. Characteristics of 37 patients and their chest radiographs, examined retrospectively

ID	Sex	*CHD7* mutation			Age at radiograph (year;month)			Thymic shadow	Cardiology		Surgery report: presence of thymus	T-cell lymphopenia	Low naive mature T-cell counts		Hypo-calcaemia
					1st	2nd	3rd		CHD	Surgery			CD4+	CD8+	
1	F	8077-1G>A	splice	N-T	0;4			+	+	-		-			
2	M	964delTT	fs	T	0;0			+	+	-		-			
3	M	7160C>A	non	T	0;1			+	+	-		-	-	+	
4	M	8630_8634del	fs	T	0;0			+	+	-					+
5	F	3340A>T	splice	N-T	0;2	0;6		+	-	-					-
6	F	5434G>C	mis	N-T	0;0	0;2		+	-	-					
7	M	5241-5244del	fs	T	0;0			-*	+	+	+ (sternotomy)	+	+	+	+
8	F	4542dup	fs	T	0;0			-*	+	+	+ (sternotomy)	+			
9	F	2572C>T	non	T	0;1			-*	+	+	+ (sternotomy)	+	+	+	-
10	F	7879C>T	non	T	0;0			-*	+	+	+ (sternotomy)				-
11	M	1480C>T	non	T	0;1	0;7		-*	+	+	+ (sternotomy)				-
12	F	del8q12.1q12.3	del	T	0;6			-*	+	+	+ (sternotomy)				-
13	F	7824T>A	non	T	0;0	0;7		-†	+	-					
14	M	6857dup	fs	T	0;0	0;6	1;6	-†	-	-					+
15	F	5833C>T	non	T	0;2	0;6		-	+	+	n/d (thoracotomy)	+	-	-	-
16	M	3173T>A	non	T	0;0	0;1		-	+	+	n/d (thoracotomy)				-
17	F	4393C>T	non	T	0;0	0;3		-	+	+	n/d (sternotomy)				-
18	M	6018dupA	fs	T	0;0	1;10		-	+	+	n/d (thoracotomy)				-
19	F	6157C>T	non	T	0;0			-	+	+	n/d (sternotomy)				-
20	M	8077-2A>G	splice	N-T	0;10			-	+	+	n/d (thoracotomy)				-
21	F	5428C>T	non	T	0;0			-	+	+	n/a	-			-
22	M	934C>T	non	T	0;0	0;1		-	+	+	n/a				
23	M	5405-17G>A	splice	N-T	0;0			-	+	-		+			-
24	F	del8q12.1q12.3	del	T	2;3			-	+	-		+			
25	M	1828dupG	fs	T	0;0	0;4		-	+	-		-			
26	F	7344_7345del	fs	T	0;0			-	+	-					-
27	M	1480C>T	non	T	0;0			-	+	-					-
28	F	7106del	fs	T	0;0	0;4	3;4	-	+	-					
29	F	7252C>T	non	T	0;4	1;4		-	+	-					-

Table 1 (Continued)

ID	Sex	*CHD7* mutation			Age at radiograph (year;month) 1st	2nd	3rd	Thymic shadow	Cardiology CHD	Surgery	Surgery report: presence of thymus	T-cell lymphopenia	Low naive mature T-cell counts CD4+	CD8+	Hypo-calcaemia
30	F	1953dupA	fs	T	0;6			-	+	-					-
31	M	4731delA	fs	T	0;0			-	-	-		+	-	-	
32	M	4783C>T	non	T	0;0	0;7	1;7	-	-	-		+			-
33	F	5944_5989dup	fs	T	0;2	1;7	2;10	-	-	-		+	+	+	
34	M	1820_1821insTTGT	fs	T	0;0	0;4		-	-	-					
35	M	6835del	fs	T	0;0	0;5	1;9	-	-	-					
36	M	911_912dup	fs	T	0;0	0;7	1;1	-	-	-					-
37	F	4644+5G>A	splice	N-T	0;1			-	-	-					

Abbreviations: CHD, congenital heart defect; del, deletion; F, female; fs, frameshift; ID, identity number of patient; M, male; mis, missense; n/a, no surgery report available; n/d, thymus not described in surgery report; non, nonsense; N-T, non-truncating; splice, splice site; T, truncating; +, yes; -, no; blank, unknown or not applicable. Ages at chest radiography with a thymic shadow are in bold. *Absent thymic shadow does not correspond with the presence of the thymus as described in the surgery report. †Thymic aplasia reported in patient's file.

A thymic shadow was visible on seven chest radiographs in 6/37 (16%) patients (Table 1, patients 1-6, and Figure 2). Of the 18 patients for whom two or three radiographs were available, discordancy was seen in only one patient (patient 6). Fourteen patients had had cardiac surgery and a surgery report was available for 12 of them. The thymus was only mentioned in the surgery reports of six patients (patients 7-12) who underwent sternotomy, but none of them had a visible thymic shadow on their chest radiographs. The size of the thymus was mentioned in only two reports: it was described as normal in patient 10 and as quite small in patient 7. The thymus was not mentioned in the surgery reports for patients 15-20. Of these, however, four were thoracotomies (patients 15, 16, 18 and 20), which means that the thymus was probably not within the field of operation. Additionally, there was information on the thymus in two patient files. The thymus could not be detected by ultrasound (US) in patient 13 and thymic aplasia based on T-cell dysfunction was described in patient 14. These two patients did not have a thymic shadow on their chest radiographs. If we combine the results of the chest radiographs with the surgical information, the presence of a thymus was reported or was seen in 12/37 patients (32%).

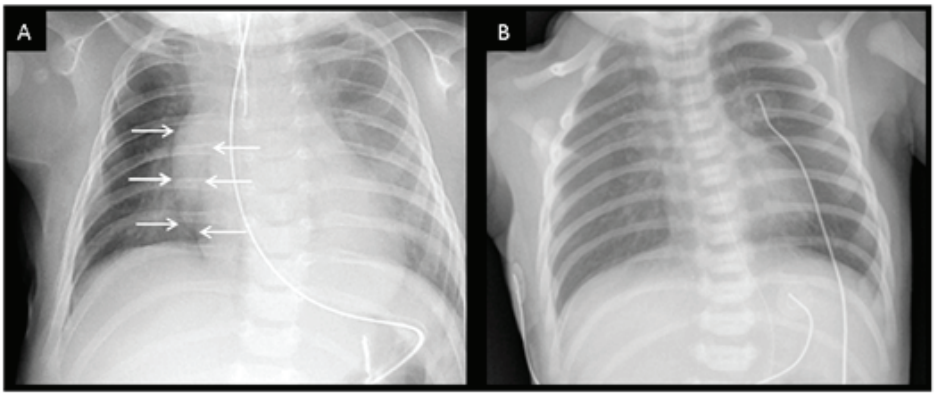

Figure 2. Comparison of two chest radiographs with and without thymic shadow. (A) A thymic shadow seen in patient 3 (1 month old). The thymic shadow (arrows) is present in the right cardiomediastinal contour. (B) An absent thymic shadow in patient 16 (1 month old). No thymic shadow in the cardiomediastinal contour is evident on the radiographic image.

Based on the combined results of chest radiographs and surgery reports, we found no significant differences in the presence of a thymus between patients categorized by type of *CHD7* mutation (*p*-value 0.37), the presence of a congenital heart defect (*p*-value 0.45), of T-cell lymphopenia (*p*-value 0.58), low naive mature CD4+ T-cell (*p*-value 1.00) or CD8+ T-cell counts (*p*-value 0.40), or hypocalcaemia (*p*-value 0.23).

4.5 Discussion

This is the first study to systematically investigate thymic aplasia in a relatively large group of 37 patients with CHARGE syndrome postnatally. By retrospectively evaluating chest radiographs and surgery reports, we explored how often the thymus could be identified in individuals with CHARGE syndrome. Our results may provide more insight into the pathophysiology of the immunological abnormalities in CHARGE syndrome that are increasingly being reported [12,16]. The consequences of thymic aplasia is T-cell deficiency and impaired antibody synthesis. Children with thymic aplasia have a seriously increased susceptibility to infections and are only allowed to receive irradiated blood products. In children with CHARGE syndrome, it is important to have information on thymic dysfunction because they are often in a vulnerable neonatal condition that requires several surgical procedures, such as correction of a choanal atresia or a congenital heart defect, at a very young age. A similar situation is known to occur for patients with 22q11.2 deletion syndrome, which shares considerable overlap in phenotypic characteristics with CHARGE syndrome, including malformations related to the pharyngeal arches [21]. The reported prevalence of thymic aplasia in 22q11.2 deletion syndrome varies between 17-47% [22,23], based on retrospective data obtained from surgery or necropsy reports. In 0.5-1% of individuals with 22q11.2 deletion syndrome, there are no circulating T-cells detectable, a condition known as DiGeorge syndrome, which is associated with life-threatening infections [24,25].

In this study we show that the thymus was indeed seen on chest radiographs in 6/37 (16%) CHARGE patients and that the thymus had been reported as present in surgery reports for another six patients, making a total of 12/37 (32%) patients with a thymus identified as present. We searched the literature for other cohorts to compare, but publications on the radiographic evaluation of the thymus in children are scarce. Meyers et al [26] described the thymus size of a cohort of children diagnosed with an HIV-1 infection, or who were born to HIV-1 infected mothers but subsequently found to be HIV-uninfected. Radiographs performed before age 1 year were included. Ten out of 58 HIV-infected children, but none of the 38 HIV-uninfected children, had an abnormally small thymus. Remarkably, they could establish the presence of the thymus by chest radiography in all children in their cohort. This clearly contrasts with our results in patients with CHARGE syndrome. We could identify a thymus shadow in 6/36 patients with a chest radiograph made before age 1 year.

However, the observation that the thymus was present during surgery despite the fact that it was not visible on chest radiographs in six patients underscores the inadequacy of current radiographic methods for this diagnostic purpose. The inadequate sensitivity of chest radiography for detecting the thymus has been reported earlier [27]. Since an absent thymic shadow on a chest radiograph does not exclude the presence of a thymus, we could not draw conclusions on the estimated prevalence of thymic aplasia and its correlations with type of *CHD7* mutation, the presence of congenital heart defect, T-cell lymphopenia or hypocalcaemia. Thus our preliminary results should be confirmed by more robust imaging techniques like US, computed

tomography (CT) and magnetic resonance imaging (MRI) [17,20], as well as systematic description by cardiac surgeons on the presence or absence of thymic tissue.

The absence of a thymic shadow on chest radiographs may have various explanations. Firstly, it may be due to the natural involution of the thymus. While we allowed for an upper age limit of 4 years for examination of radiographs, we actually managed to study chest radiographs made before age 1 year for all but one child (patient 24). Involution by age does not therefore seem to be a viable explanation for an absent thymic shadow in our study. Secondly, the size of the thymus can be influenced by external factors such as stress and disease [17], and its size might recover after the external stress factor has been resolved [28]. Therefore, the size and visibility of the thymus on chest radiographs depends on the child's clinical condition at the time the radiograph was made. This could explain the conflicting results of an absent thymic shadow in patients who were confirmed to have a thymus during thoracic surgery. Thirdly, technical artifacts such as limited resolution or overprojection may result in false-negative findings. We chose retrospective evaluation of chest radiographs as our method because it is relatively simple, inexpensive and non-invasive, and radiographs are often available. Data from other more reliable methods for examining the thymus appeared to be scarce in our study population.

We realize the methodological limitations of this explorative study. Selection bias might have played a role in the relatively high rate of an absent thymic shadow since chest radiographs were included retrospectively. Although we could not further elucidate the incidence of thymic aplasia in patients with CHARGE syndrome, we confirmed that chest radiography has minimal diagnostic value in determining thymic aplasia. This is in itself an important observation, since chest radiographs are still used to determine thymic aplasia in general clinical practice [29]. Given these insights, we strongly recommend that prospective studies should be conducted on the presence/absence of the thymus using suitable imaging methods and preferably in newborns, e.g. during their routine diagnostic work-up. In addition, we suggest that researchers include a control group in such studies, because there are no reference values for thymus imaging in young children. We recently proposed that observation of the thymus during cardiac surgery be included in a new surveillance check-list for individuals with CHARGE syndrome [30]. This will improve awareness of this aspect of CHARGE syndrome and may benefit individuals who suffer from recurrent infections.

4.6 Acknowledgments

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Supplementary information

Score chart used for examining chest radiographs. This chart has been anonymized and shortened for illustration.

Thymus study CHARGE syndrome: score chart for radiologic evaluation

Instructions

Please fill in your answer in the outlined boxes.

Evaluate the radiographs as indicated by the date on which it is made.

If multiple radiographs were made on one date, evaluation of one radiograph is sufficient.

If necessary, other radiographs can be evaluated. Please evaluate only radiographs as indicated at Extra information to avoid radiopragphs made after the age of 4 years or after cardiac surgery.

Last name	Date of birth	Patient ID	Date radiograph 1	Thymic shadow radiograph 1: Yes/No	Date radiograph 2	Thymic shadow radiograph 2: Yes/No	Date radiograph 3	Thymic shadow radiograph 3: Yes/No	Extra information
Patient 1	xx-xx-xxxx		10-4-2010						
Patient 2	xx-xx-xxxx		16-11-2005						evaluate radiographs before 14-7-2006
Patient 3	xx-xx-xxxx		15-1-2007		6-4-2007				
Patient 4	xx-xx-xxxx		15-3-2006						
Patient 5	xx-xx-xxxx		12-12-2014		28-4-2015				
Patient 6	xx-xx-xxxx		21-8-2008		15-1-2010		6-4-2011		
Patient 7	xx-xx-xxxx		17-2-2006		29-6-2006		13-6-2009		
Patient 8	xx-xx-xxxx		14-4-2004						evaluate radiographs before 23-6-2004
Patient 9	xx-xx-xxxx		21-4-2005		11-9-2005				
Patient 10	xx-xx-xxxx		28-10-1996						

CHAPTER 5

Central adrenal insufficiency is not a common feature in CHARGE syndrome: a cross-sectional study in two cohorts

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5.1 Abstract

Objective: To evaluate whether central adrenal insufficiency (CAI) is present in CHARGE syndrome, a complex malformation disorder that includes central endocrine dysfunction.

Study design: Two cross-sectional studies were performed in Dutch (September 2013-February 2015) and Australian (January 2012-January 2014) Expert Clinics for CHARGE syndrome. Twenty-seven Dutch and 19 Australian patients (16 months-18 years) with genetically confirmed CHARGE syndrome were included in the cohorts. The low dose adrenocorticotropin (ACTH) test (LDAT) was used to assess CAI in the Dutch cohort. A peak cortisol response below 18.1 µg/dL (500 nmol/L) was suspected for CAI and a glucagon stimulation test (GST) was performed for confirmation. Australian patients were screened by single measurements of ACTH and cortisol levels. If adrenal dysfunction was suspected, a standard dose ACTH test was performed.

Results: LDAT was performed in 23 patients (median age 8.4 (1.9-16.9) years). Seven patients showed an insufficient maximum cortisol level (10.3-17.6 µg/dL, 285-485 nmol/L), but CAI was confirmed by GST in only one patient (maximum cortisol level 15.0 µg/dL, 415 nmol/L). In the Australian cohort, 15 patients (median age 9.1 (1.3-17.8) years) were screened, and none had CAI.

Conclusions: This is the first study investigating adrenal function in a large cohort of children with CHARGE syndrome. CAI was not common in our cohorts, and routine testing of adrenal function in children with CHARGE syndrome is not indicated.

5.2 Introduction

CHARGE (Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital hypoplasia, and Ear abnormalities, including deafness) syndrome (#MIM 214800) is caused by a dominant mutation in or deletion of the Chromodomain Helicase DNA-binding protein 7 (*CHD7*) gene (#MIM 608892) [1]. The clinical features of the syndrome that were the first to be recognized are included in the acronym CHARGE. Subsequently, the phenotype of CHARGE syndrome expanded to include other features: absent or hypoplastic semicircular canals, cranial nerve dysfunction, cleft lip and/or palate, anosmia, feeding difficulties, and skeletal abnormalities [2-4]. Clinical diagnosis of typical and atypical CHARGE syndrome can be made using the clinical criteria proposed by Blake et al. [5] or by Verloes [2]. There is no clear genotype-phenotype correlation, but *CHD7* variants leading to a premature stop codon or the complete absence of the gene are, in general, associated with a more severe phenotype than variants with a non-truncating effect (i.e. missense variants) [6].

In addition to the features mentioned above, hypothalamic-pituitary hormonal disorders like hypogonadotropic hypogonadism [3, 7-9], central hypothyroidism [9, 10], and growth hormone deficiency have been described in CHARGE syndrome [9-13]. Until now, central adrenal insufficiency (CAI) has never been systematically studied in children with CHARGE syndrome. This is surprising, since sudden and unexplained death in these children, most likely due to respiratory aspiration and/or circulatory arrest, has been described [14]. However, it is unclear whether CAI may have contributed to the unexpected death in these children. This is important because, if CAI is frequently present in CHARGE syndrome, mortality may be prevented through the use of corticosteroids during stressful conditions. We aimed to examine the frequency of CAI in two separate cohorts of patients with genetically confirmed CHARGE syndrome.

5.3 Patients and Methods

The inclusion criteria for this cross-sectional study were an age below 18 years and a proven mutation in *CHD7*. The use of systemic steroids or other medications that interfere with adrenal function was an exclusion criterion. Clinical information on medication, infections, hospital admissions, and surgical procedures were extracted from patient files. Mutations in *CHD7* were classified as truncating (nonsense, frame shift, or deletion) or non-truncating (missense or splice site). The study was approved by the local institutional review boards of both medical centers and written informed consent was obtained from parents or legal representatives.

5.3.1 Dutch cohort

The study was performed between September 2013 and February 2015. An invitation to participate in the study was sent to 83 eligible patients seen at the Dutch Expert

Clinic for CHARGE syndrome at the University Medical Center Groningen (UMCG).

5.3.1.1 Adrenal function tests

The low dose adrenocorticotropin (ACTH) test (LDAT) was chosen as the primary diagnostic test to detect CAI. This test is validated and used in routine patient diagnostics in the UMCG. The LDAT started at 0830 h with a baseline blood sample for plasma ACTH and cortisol. After an interval of 15 minutes, tetracosactrin (0.5 µg/1.73 m^2 body surface area; Synacthen®, Sigma Tau BV, Utrecht, the Netherlands) was administered intravenously. Blood samples for plasma cortisol were taken at 0, 30, and 60 minutes after tetracosactrin administration. A peak cortisol response ≥ 18.1 µg/dL (500 nmol/L) was considered a normal test response that excludes CAI [15]. Patients with an insufficient cortisol response were retested on a separate occasion using a glucagon stimulation test (GST) to confirm the presence of CAI.

The GST started at 0830 h after an overnight fast. Baseline blood samples for plasma ACTH, cortisol, and glucose were taken. After intramuscular administration of glucagon (0.05-0.1 mg glucagon per kg body weight, maximum 1 mg; GlucaGen®, Novo Nordisk BV, Alphen aan den Rijn, the Netherlands), plasma cortisol and glucose were measured at 30 minute intervals for three hours. After the test, patients had lunch and their glucose level was monitored for two hours. A peak cortisol response ≥ 18.1 µg/dL (500 nmol/L) was considered a normal response to the test [16, 17]. It should be mentioned that the exact mechanism by which glucagon stimulates ACTH and subsequent cortisol release is not yet clear [18, 19].

5.3.1.2 Biochemical analyses of plasma ACTH and cortisol

Blood samples for ACTH were collected into EDTA tubes, placed on ice and, after centrifugation, frozen immediately until analysis. The analysis was performed by electrochemiluminescence immunoassay using Cobas® 6000 E601 (Roche Diagnostics, intra-assay coefficient of variation (CV) 0.6-3.6% and inter-assay CV 3.5-5.4%). Plasma cortisol was analyzed by electrochemiluminescence immunoassay using Modular E170 (Roche Diagnostics, inter-assay CV 2.3-4.0%).

For two patients, the LDAT was performed at another university medical center for logistical reasons. The analysis of plasma ACTH was performed on Immulite® 2000 (Siemens Diagnostics, intra-assay CV 6.7-9.5% and inter-assay CV 6.1-10.0%) by chemiluminescent immunoassay. Analysis of plasma cortisol was performed with the same analyzer as at the UMCG.

5.3.2 Australian cohort

From January 2012 to January 2014, 21 eligible patients who had been previously seen at the Children's Hospital at Westmead, Sydney, were invited to the multidisciplinary CHARGE clinic.

5.3.2.1 Adrenal function screening and test

Adrenal function was screened by single measurement of ACTH and cortisol in blood, as per standard practice at the Children's Hospital at Westmead. Adrenal dysfunction was suspected if the ACTH level was > 45.5 pg/mL (10 pmol/L) and/or the cortisol level was < 7.3 µg/dL (200 nmol/L, 0900 h-1000 h) or < 2.2 µg/dL (60 nmol/l, after 1000 h; 1/3rd of the value of the morning cutoff level). These cutoff levels have been used by the endocrinology laboratory for many years at the Children's Hospital at Westmead. If suspected, repeat ACTH and cortisol measurements were performed, and if either was abnormal on repeat testing, a standard dose ACTH test (SDAT) was recommended. The SDAT was performed at any time of the day with a single dose of 250 µg tetracosactrin (Synacthen®, Novartis, North Ryde, Australia) intramuscular or intravenously. Blood samples for ACTH and cortisol were taken at 0, 30, and 60 minutes after tetracosactrin administration. A serum cortisol rise > 10.1 µg/dL (280 nmol/L) with a peak response > 21.7 µg/dL (600 nmol/L) was considered to exclude adrenal insufficiency.

5.3.2.2 Biochemical analyses of plasma ACTH and cortisol

Blood samples for ACTH were collected and analyzed on ice. Both ACTH and cortisol blood samples were analyzed by immunoassay with Immulite® 1000 (Siemens Diagnostics). The intra-assay CVs were 5.6% and 7.1% for ACTH and cortisol, respectively. The inter-assay CV was 7.8% for both ACTH and cortisol.

5.3.3 Statistical analysis

The statistical software program IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp.) was used for statistical analysis. Descriptive statistics were used. To compare groups, the Student's T test for continuous variables and the Fisher's exact test for categorical variables were used. A *P*-value < 0.05 was considered statistically significant.

5.4 Results

5.4.1 Dutch cohort

Twenty-seven of 83 (33%) eligible patients were included in this study. The LDAT could not be performed in three patients for technical reasons and one patient withdrew from the study. Table 1 shows the characteristics of the remaining 23 children. The group consisted of 14 boys and nine girls with a median age of 8.4 (range 1.9-16.9) years. Eighteen of the 23

Table 1. Patient characteristics of 23 children with CHARGE syndrome from the Dutch cohort

Patient	Age	Sex	Clinical CHARGE syndrome[1]	*CHD7* Mutation		ACTH LDAT	Maximum cortisol LDAT[2]	Maximum cortisol GST[2]
	years					pg/mL	µg/dL (nmol/L)	µg/dL (nmol/L)
D1	14.9	F	typical	5222G>C	missense	29	21.9 (605)	n/a
D2	8.3	M	typical	1480C>T	nonsense	25	**17.6 (485)**	18.5 (510)
D3	3.8	F	atypical	8077-1G>A	splice site	14	19.0 (525)	n/a
D4	13.8	M	typical	2442+5G>C	splice site	22	**13.8 (380)**	**15.0 (415)**
D5	16.9	M	atypical	2442+5G>C	splice site	20	**15.6 (430)**	18.5 (510)
D6	11.6	F	atypical	5405-17G>A	splice site	7.1	21.2 (585)	n/a
D7	3.2	M	typical	5405-17G>A	splice site	44	22.5 (620)	n/a
D8	2.3	F	typical	5428C>T	nonsense	22	23.4 (645)	n/a
D9	13.5	M	typical	8016G>A	nonsense	2.8	**17.2 (475)**	25.0 (690)
D10	5.9	F	typical	5944_5989dup	frame shift	15	**17.0 (470)**	25.8[3] (713)
D11	5.8	M	typical	7160C>A	nonsense	74	24.8 (685)	n/a
D12	6.6	M	atypical	5241-5244del	frame shift	16	23.6 (650)	n/a
D13	14.4	F	typical	5833C>T	nonsense	30	22.1 (610)	n/a
D14	15.5	M	atypical	7650_7651del	frame shift	23	**16.3 (450)**	22.1 (610)
D15	2.9	M	typical	964delTT	frame shift	15	22.7 (625)	n/a
D16	1.9	F	typical	4542dup	frame shift	23	27.7 (765)	n/a
D17	14.9	M	typical	5405-17G>A	splice site	21	21.6 (595)	n/a
D18	8.4	M	typical	3514_3515del	frame shift	22	19.0 (525)	n/a
D19	14.9	F	typical	5181C>G	nonsense	5.4	22.7 (625)	n/a
D20	8.1	M	typical	4731delA	frame shift	30	18.5 (510)	n/a
D21	4.1	M	typical	4783C>T	nonsense	12	**10.3 (285)**	21.7 (600)
D22	11.8	M	typical	5051-15T>A	splice site	95[4]	24.5[4] (677)	n/a
D23	10.7	F	typical	2572C>T	nonsense	17[4]	23.5[4] (649)	n/a

F, female; M, male; n/a, not applicable. [1]Based on the criteria by Blake et al [5] and Verloes [2] [2]Maximum cortisol levels below 500 nmol/L (18.1 mg/dL) are shown in bold. [3]Patient underwent SDAT instead of GST. [4]LDAT was performed at the Leiden University Medical Center.

(78%) patients fulfilled the clinical criteria for typical CHARGE syndrome as defined by Blake et al. [5] and/or Verloes [2]. Fifteen (65%) patients had a truncating mutation in the *CHD7*gene. Additional information from clinical records showed that all patients had a history of (frequent) infections, mainly otitis media and pneumonia [20]. Four patients (D10, D19, D21 and D23) used intranasal or inhaled steroids in a dose varying between 100 µg and 500 µg per day, which we considered to have minimal systemic effects and these four patients were not excluded from the study.

Seven of 23 (30%) children had an insufficient peak cortisol response with a median maximum cortisol level of 16.3 µg/dL (range 10.3-17.6 µg/dL, 450 (285-485) nmol/L) in the LDAT and were suspected to have CAI. There were no significant differences in age, gender, fulfillment of clinical criteria for typical CHARGE syndrome and type of *CHD7* mutation between patients without CAI (n=16) and those with suspected CAI (n=7).

Of the seven patients with suspected CAI, the GST was performed in six patients and the SDAT in one. The reason for performing the SDAT was to adhere to the policy of the local hospital where the primary care of patient D10 was set. CAI was confirmed in one of the seven patients by the GST with a maximum cortisol level of 15.0 µg/dL (415 nmol/L). This patient (D4) has been previously described as patient no. 9 and 2 in two separate papers in which the adrenal function had not been assessed [3, 21]. He was known to have a *CHD7* intronic variant close to the exon 6/intron 6 boundary that is predicted to disrupt splicing (IVS6+5G>C). The same variant was present in his older brother (D5) and it was proven to be due to paternal (germ line) mosaicism after their father was tested in 2015. The older brother was suspected of having CAI, but this was not confirmed by the GST. The father has only mild features including a square face, small earlobes, and a hockey-stick palmar crease. In the past history, patient D4 had had frequent infections and underwent several surgical procedures (orchidopexia and correction of cleft lip and palate) without complications. However, his mother recalled that he needed more time to recover from infections and surgeries compared with his older brother. No major abnormalities were seen on the cranial MRI images of the patient, although a small lesion of 6 mm in the sella was suspected to be a Rathke's cleft cyst (RCC). As a consequence of the results of the LDAT and GST, the patient was advised to take a stress dose of hydrocortisone during stressful conditions. During two separate infectious periods (sinusitis and otitis) which persisted for more than a week, the patient showed a fast recovery after a stress dose of hydrocortisone.

5.4.2 Australian cohort

A total of 19 eligible patients attended the clinic and blood was drawn for genetic, immunological, and endocrine testing prior to their visit [22]. One patient was deemed not to have CHARGE syndrome both by clinical assessment and normal *CHD7* testing. Screening of adrenal function was performed in 15 out of 18 remaining patients because the request slip was misplaced in three patients. The characteristics of the 15 patients are shown in Table 2. The cohort consisted of 4 boys and 11 girls with a median age of 9.1 (range 1.3-17.8) years. All patients fulfilled the Verloes diagnostic

criteria for typical CHARGE syndrome [2] and 12 (80%) patients had a truncating mutation in the *CHD7* gene. None of the 15 children tested had symptoms suggestive of adrenal insufficiency, such as prolonged infections or a history of surgical procedures with a complicated course. No patient was on corticosteroid medication at the time of sampling.

None of the tested children had reduced cortisol levels on initial screening, but four (A6, A8, A14, and A15) had elevated levels of ACTH (> 45.5 pg/mL, 10 pmol/L). On repeat testing, three patients (A8, A14, and A15) had a normal ACTH level (cortisol levels remained within normal range). One patient (A6) was advised to have a SDAT following the initial elevated ACTH, as she was having major cardiac surgery two weeks later. The SDAT showed a normal peak cortisol response of 31.4 µg/dL (866 nmol/L).

Table 2. Patient characteristics of 15 children with CHARGE syndrome from the Australian cohort

Patient	Age	Sex	Clinical CHARGE syndrome[1]	*CHD7* Mutation		Time of adrenal screening hours	ACTH[2]	Cortisol
	years						pg/mL (pmol/L)	µg/dL (nmol/L)
A1	1.3	M	typical	3082A>G	missense	1642	25.9 (5.7)	6.1 (169)
A2	11.4	F	typical	282delT	frame shift	1055	18.6 (4.1)	6.3 (174)
A3	10.1	F	typical	2362C>T	nonsense	1115	14.1 (3.1)	4.4 (121)
A4	13.0	F	typical	5181G>A	nonsense	1100	24.5 (5.4)	11.9 (328)
A5	15.7	F	typical	Exon 3 del	deletion	1130	15.0 (3.3)	12.4 (342)
A6	1.3	F	typical	7441C>T	nonsense	1106	**48.6 (10.7)**	9.4 (258)
A7	5.9	M	typical	4353G>T	splice site	1225	14.5 (3.2)	8.2 (225)
A8	9.1	M	typical	1989dupA	frame shift	1020	**115.0 (25.3)**	13.7 (378)
A9	7.0	F	typical	7727A>G	missense	1248	32.3 (7.1)	15.2 (419)
A10	17.8	F	typical	8028delinsAGGAA	frame shift	1215	13.6 (3.0)	5.0 (137)
A11	15.6	F	typical	5458C>T	nonsense	1205	6.8 (1.5)	9.5 (263)
A12	2.6	M	typical	604C>T	nonsense	0850	41.4 (9.1)	12.2 (337)
A13	8.7	F	typical	2394del	frame shift	1154	25.5 (5.6)	13.3 (367)
A14	12.6	F	typical	6705delA	frame shift	1445	**64.5 (14.2)**	4.8 (133)
A15	5.7	F	typical	Exons 19-38 del	deletion	0945	**92.3 (20.3)**	7.8 (214)

[1]Based on the criteria by Verloes [2]. [2]ACTH levels greater than 10 pmol/L (45.5 pg/mL) are shown in bold.

5.5 Discussion

Knowledge about the phenotype of CHARGE syndrome keeps expanding, including new information on endocrine features involved in the hypothalamic-pituitary axis [3, 7-13]. Adrenal insufficiency had not yet been explored despite reported cases of sudden mortality in CHARGE syndrome [14]. Since CAI, specifically partial CAI, is mostly asymptomatic in unstressed situations, active evaluation of the hypothalamic-pituitary-adrenal axis is needed to exclude CAI. If symptoms and signs of CAI are present, they are often non-specific and are similar to those seen in primary adrenal insufficiency [23]. However, some symptoms and signs are more indicative of primary or central adrenal insufficiency. For example, hyperpigmentation and salt craving are specific signs for primary adrenal insufficiency and CAI is frequently associated with other hypothalamic-pituitary disorders [23]. In stressful situations, primary adrenal insufficiency can be life-threatening due to the total lack of cortisol secretion. In this regard, CAI has a milder presentation during stressful events because cortisol secretion, although decreased, is still partially present. Nonetheless, sudden deaths have been reported in patients with CAI or who were suspected of having CAI [24].

In our Dutch cross-sectional study, we found CAI in one out of 23 well-studied patients. In this patient, the CAI can be classified as partial since the cortisol response is marginally reduced. Furthermore, his partial CAI might be secondary to a RCC, as has been reported in the non-CHARGE population. Cohan et al. [25] reported a cohort of 24 patients with symptomatic RCC of whom two patients presented with CAI. In addition, several single case reports have been published on the relation between RCC and CAI [26-28]. RCC is not a common feature in CHARGE syndrome and thus the combination of RCC and CAI in patient D4 may be unrelated to his CHARGE syndrome. In the Australian cohort, serum ACTH and cortisol were assessed as part of a general screening in 15 patients. Adrenal dysfunction was not seen, which supports the conclusion that adrenal insufficiency is not common in CHARGE syndrome.

This is the first study to systematically explore CAI in a large cohort of phenotypically well-defined children who had genetically confirmed CHARGE syndrome. Previously published data on CAI in CHARGE syndrome only come from case reports. A PubMed search using ("CHARGE syndrome" OR "CHARGE association" OR "CHD7") AND (adrenal OR ACTH OR cortisol) revealed eight publications, of which four were not on CHARGE syndrome and adrenal function. In one publication, information was lacking to confirm the diagnosis of both CHARGE syndrome and adrenal insufficiency [29]. Of the remaining three papers, Gregory et al. [10] described two male patients with genetically confirmed CHARGE syndrome whose adrenal function was assessed with SDAT. The test confirmed CAI in one patient. This patient fulfilled the clinical criteria for typical CHARGE syndrome and had other endocrine dysfunctions including growth hormone deficiency, hypogonadotropic hypogonadism, and central hypothyroidism. A small anterior pituitary and an absent pituitary stalk with an ectopic posterior pituitary were seen in cranial MRI. The patient had a splice site variant of the *CHD7* gene (IVS35+6T>C) that was also found in the unaffected mother, who had no obvious endocrinopathy. Another patient with diagnostically confirmed CAI was described by

James et al. [30]. This newborn boy with clinically, but not molecularly, confirmed CHARGE syndrome presented with persistent hyponatremia and hypoglycemia. Endocrine screening showed a low baseline cortisol level of 1.7 µg/dL (47 nmol/L, normal 7.2-19.9 µg/dL (200-550 nmol/L)) and an inappropriately normal ACTH of 15.5 pg/mL (3.4 pmol/L, normal 9.1-50 pg/mL (2.0-11.0 pmol/L)). CAI was diagnosed with a LDAT (1 µg) that showed an inadequate peak cortisol response of 13.0 µg/dL (360 nmol/L). Brain abnormalities were not described. The last paper described normal adrenal screening in a clinically, but not genetically, confirmed CHARGE patient [31]. In summary, including our own patient, CAI has been described in three patients with CHARGE syndrome, and two of these patients had structural abnormalities of the pituitary gland which could explain the presence of CAI.

The gold standard for testing adrenal function is the insulin tolerance test, but this test is not recommended for children because the induced hypoglycemia is potentially dangerous [15]. The metyrapone test is a good alternative test but may trigger an acute adrenal crisis [32] and requires an overnight stay at the intensive care unit. Therefore, alternative tests are often used to assess the adrenal function in children. These tests are the GST, the SDAT, and the LDAT. Notably, no consensus has been reached which test is the best alternative to the insulin tolerance test in children [33]. The GST is considered to be safe in children but is time-consuming with a duration of five hours and includes an intramuscular or subcutaneous injection [34, 35]. The SDAT and LDAT are both safe and convenient for children, although the SDAT is less sensitive and thus less able to detect subtle CAI when compared to the LDAT [15, 36, 37].

In addition to the variety of methods of testing central adrenal function, there are also various cutoff values to diagnose CAI for each test. One reason for this lack of uniformity is the non-standardization of cortisol assays [38]. In the insulin tolerance test in children, a rise in plasma cortisol of > 19.9 µg/dL (550 nmol/L) with a hypoglycemia of < 39.6 mg/dL (2.2 nmol/L) is considered a normal response [15]. The peak cortisol level to exclude CAI in the GST varies between 14.6 to 19.9 µg/dL (403-550 nmol/L) [15-17, 39]. We have chosen the cutoff level of 18.1 µg/dL (500 nmol/L) as this is used in clinical practice in our hospital. The SDAT has peak cortisol levels to exclude CAI varying between 16 to 19.9 µg/dL (440-550 nmol/L) [15, 36, 38]. The definition of a normal peak cortisol response in the LDAT ranges from 15.4-21.8 µg/dL (415-600 nmol/l) [33], but, in general, a peak cortisol response of < 18.1 µg/dL (500 nmol/L) is used to define CAI [15].

After considering the advantages and disadvantages of the different alternative tests, the LDAT was chosen as the primary test in the Dutch cohort. The test is safe and convenient, which were important aspects for a test used in a research setting. Moreover, broad experience in performing the LDAT was available in the Dutch clinic. The sensitivity and specificity of the LDAT, as used in our study, were 96% and 37%, respectively [40]. To compensate for the low specificity, a GST with an 87.5% sensitivity and 10.5% specificity [41] was performed as a second test to confirm CAI.

A limitation of the study is the low participation rate in the Dutch cohort (27 out of 83 eligible patients), which reflects the reluctance of parents to subject their children to the longer travel times to reach our regional hospital for invasive tests. However, an ascertainment bias towards more healthier children is not likely, because, in general, parents are more eager to participate if children are suspected to have problems. Thus, we do not think that the low participation rate influenced our results. Unfortunately, different methodologies were used between the Dutch and Australian cohort. Nonetheless, the results of the Australian cohort support the findings in the Dutch cohort.

In conclusion, CAI is not common in CHARGE syndrome and does not seem to be an explanation for the unexpected mortality seen in CHARGE patients [14]. Studies with larger cohorts, preferably via international collaborations, are needed to confirm our findings. Based on this cohort, we advise that screening CHARGE patients for adrenal insufficiency is not necessary. This recommendation is relevant for professionals who are involved in the care of these children.

5.6 Acknowledgement

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CHAPTER 6

Prominent scapulae mimicking an inherited myopathy expands the phenotype of CHD7-related disease

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6.1 Abstract

CHD7 variants are a well-established cause of CHARGE syndrome, a disabling multi-system malformation disorder that is often associated with deafness, visual impairment and intellectual disability. Less severe forms of *CHD7*-related disease are known to exist, but the full spectrum of phenotypes remains uncertain. We identified a *de novo* missense variant in *CHD7* in a family presenting with musculoskeletal abnormalities as the main manifestation of *CHD7*-related disease, representing a new phenotype. The proband presented with prominent scapulae, mild shoulder girdle weakness and only subtle dysmorphic features. Investigation revealed hypoplasia of the trapezius and sternocleidomastoid muscles and semicircular canal defects, but he did not fulfill diagnostic criteria for CHARGE syndrome. Although the shoulders are often sloping and anteverted in CHARGE syndrome, the underlying neuromuscular cause has never been investigated. This report expands the phenotypes associated with *CHD7* mutations to include a musculoskeletal presentation, with hypoplasia of the shoulder and neck muscles. *CHD7* should be considered in patients presenting in childhood with stable scapular winging, particularly if accompanied by dysmorphic features and balance difficulties.

6.2 Introduction

CHARGE syndrome is a disabling complex of congenital malformations associated with variants in *CHD7* in 60-90% of patients [1-3]. Various clinical diagnostic criteria have been proposed [4-6], but musculoskeletal abnormalities outside the face are uncommonly reported. Nonsense and frameshift variants are most common, but missense variants are also reported [7-9]. A small number of patients with mild phenotypes, who do not fulfill the diagnostic criteria, have been reported in association with missense variants [9]. Commonly these patients have had ear abnormalities, with or without one or two other minor criteria.

We studied a proband with scapular winging who had been extensively investigated for genetic myopathies. He was found to have a novel *de novo* missense variant in *CHD7* associated with hypoplasia of shoulder and neck muscles, and hypoplasia of the semicircular canals. Review of a large cohort of patients with *CHD7*-related disease revealed a further patient with atrophic shoulder muscles and cavovarus foot deformities. This report enlarges the spectrum of *CHD7*-related disease to include patients with musculoskeletal presentations.

6.3 Methods

Patient 1 was referred with proximal muscle weakness and scapular winging, suggestive of an inherited myopathy and was included in a whole-exome sequencing (WES) study. Patient 2 is the mildly affected son of Patient 1. The database of the University Medical Center Groningen (UMCG) CHARGE clinic was screened for patients with neuromuscular problems. Patient 3 was identified as presenting with a suspected myopathy.

WES was performed in Patient 1 and his unaffected parents using a well-established pipeline at the Broad Institute [10]. The *CHD7* variant was confirmed by Sanger sequencing in the proband and close family members. The coding regions of *CHD7* were sequenced in Patient 3. All variants were submitted to the Leiden Open Variant CHD7 database (www.LOVD.nl/CHD7; patient IDs 51495 and 51531). The variants and phenotypes will also be available in updated CHD7 database (www.chd7.org).

6.4 Results

WES in Patient 1 showed a heterozygous missense variant in *CHD7* c.3398C>T, p.(Thr1133Met); (NM_017780.3). Sanger sequencing confirmed that this variant arose *de novo* in Patient 1, and was also present in his mildly affected son (Patient 2). The p.(Thr1133Met) variant has not been previously reported in the CHD7 database (www.chd7.org, accessed July 2015) or the Exome Aggregation Consortium (ExAC) database (www.exac.broadinstitute.org, accessed July 2015). The variant is located in the helicase ATP-binding domain [11]. Four in silico prediction software packages

predicted the variant to be pathogenic (PolyPhen-2 1.0 [12]; SIFT deleterious (0.000); Provean deleterious (−5.610) [13]; MutationTaster disease causing (0.9990) [14]). On the basis of the prediction programs (score +2) and *de novo* occurrence (score +3), this missense variant probably affects function according to the scoring classification published by Bergman [15].

In Patient 3, *CHD7* sequencing revealed an intronic variant predicted to disrupt the exon 6 donor splice site (c.2442+5G>C; NM_017780.3; g.127819G>C; NG_007009.1). The same variant was present in his affected older brother [9] and their mildly affected father. The variant was not present in the ExAC database.

6.4.1 Clinical features

Patient 1 had prominent scapulae, shoulder girdle weakness and pain from 7 years of age. He was assessed in his early 20 s with a suspected inherited myopathy. He had no history of gross motor delay or poor balance, but had mild learning difficulties at school. At 27 years he had subtle dysmorphic features, including small ears and hockey-stick palmar creases. His neck was short and broad, and his shoulders had an abnormal anterior position. His scapulae were laterally rotated, and with shoulder abduction they showed cranial elevation and dorsal winging, more pronounced on the right (Figures 1a and c). He had mild weakness (MRC grade 4/5) of shoulder abduction and neck flexion. He had no facial asymmetry or weakness. Sense of smell, genital development, ophthalmology review, audiology assessment, echocardiogram and respiratory examinations were normal. Scoliosis was not present. Creatine kinase (CK) level was normal. A deltoid muscle biopsy taken at age 23 years was non-specific. Southern blot analysis for facio-scapular-humeral dystrophy (FSHD) type 1 was negative. Nerve conduction studies (NCS) were normal. At 27 years of age, stimulation of the spinal accessory nerves elicited normal amplitude compound muscle action potentials (CMAP). Electromyography of both trapezii showed polyphasic motor units with reduced recruitment.

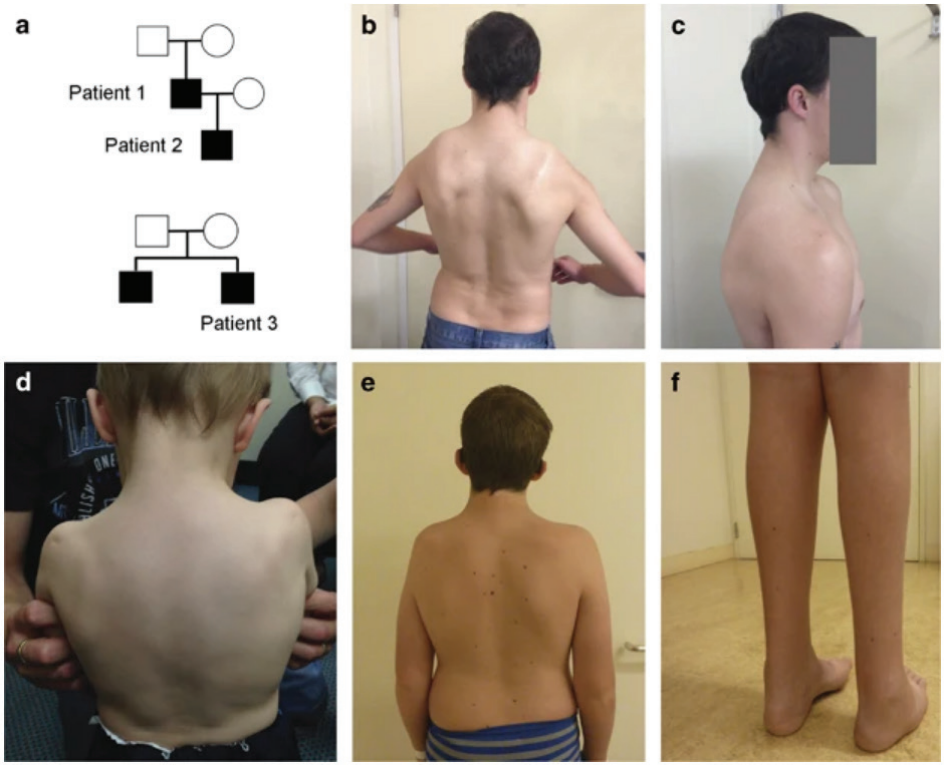

Figure 1. (a) Pedigrees illustrating the relationships of Patient 1 and 2, and Patient 3. (b) Posterior view of Patient 1 showing sloping shoulders, dorsal winging and lateral displacement of the scapulae during arm abduction. (c) Lateral view of Patient 1 showing anterior position of the shoulders, small ears and mild retrognathia. (d) Posterior view of Patient 2 showing laterally displaced scapulae and bilateral acromial dimples. (e) Patient 3 showing mild prominence of the right scapula. (f) The lower legs of Patient 3 showing calf atrophy and pes cavovarus.

An MRI brain scan, with fine cuts through the semicircular canals, showed aplasia of the superior semicircular canal bilaterally and dysplastic horizontal canals. The brain and cervical spinal cord were normal, although the XIth cranial nerve was not seen. Muscle MRI showed reduced trapezius muscle bulk with changes more marked on the right (Figure 2a). The sternocleidomastoid and paravertebral muscle bulk (C3-C4 level) were reduced bilaterally (Figure 2b). Serratus anterior and the lower limb musculature were normal.

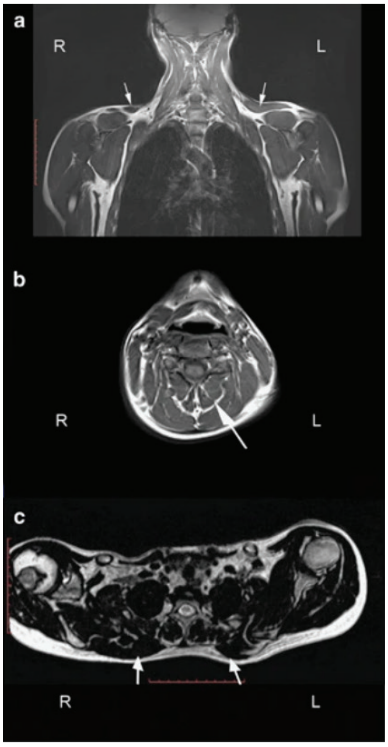

Figure 2. (a) Coronal T1 muscle MRI image from Patient 1. The arrows point to the trapezius muscle, which is hypoplastic bilaterally, with the right more severely affected. (b) Axial T1 section through the neck of Patient 1. The arrow indicates hypoplasia of the paraspinal muscles. (c) Axial T2 section from Patient 3. The arrows point to the trapezius muscles showing asymmetry with the left smaller than the right.

Patient 2, the 18 month-old son of Patient 1, had normal developmental milestones, no dysmorphic features and no muscle weakness. He had an abnormal shoulder shape with hypermobile, laterally positioned scapulae with bilateral dimples over the acromial processes. Genital development, echocardiogram, audiology and ophthalmology assessments were normal. He was not investigated with MRI imaging.

Patient 3, and his older brother, have been previously described (as family 1) in a paper on familial CHARGE [9]. Both fulfilled diagnostic criteria for a typical CHARGE syndrome. Their father was recently available for testing and review. He was mildly affected with a square face, small earlobes and a hockey-stick palmar crease. Patient 3 was referred for a neuromuscular evaluation at 8 years of age because of pes cavovarus and fatigue. He was re-evaluated at age 13 because of mildly progressive pes cavovarus and calf atrophy. He had an abnormal gait with inversion of the ankle and impaired dorsiflexion of the right foot. He had bilateral scapular winging and an atrophic appearance to the shoulder muscles, with neck webbing (Figures 1b and

c). Scoliosis was not present. CK was normal. NCS showed reduction of the right peroneal nerve CMAP amplitude. EMG was normal. MRI showed mild asymmetry of the trapezius muscles (Figure 2c) and preserved sternocleidomastoids. There was a slight reduction in the size of the right calf compared with the left, and mild atrophic fatty change in the right flexor halluces longus and tibialis anterior.

To investigate the frequency of musculoskeletal features affecting the shoulder region and limbs in *CHD7*-related disease, we searched the database of the UMCG CHARGE expert clinic (n=104). Clinical photographs of the shoulder region were available for 41 patients with CHARGE syndrome. Abnormal shoulder posture was present in 31 out of 41 (76%) patients, involving anteverted (n=18), sloping (n=29) and/or asymmetric (n=18) shoulder posture. In addition to patient 4, underlying neuromuscular pathology was suspected in 6 out of 104 patients. In these six patients, neuromuscular symptoms involved reduced exercise tolerance (n=2), proximal muscle weakness (n=2), pes adductus varus (n=1), shortened pectoral muscles (n=1), limited arm abduction (n=2) and atrophic shoulder muscles (n=1).

6.5 Discussion

Patient 1 was investigated for a suspected inherited myopathy over several years due to abnormal scapular position and movements. Limb girdle muscular dystrophy and FSHD were considered; however, a specific diagnosis could not be established. We were surprised to identify a *de novo* heterozygous missense variant in *CHD7* with WES.

Patient 1 did not fulfill diagnostic criteria for CHARGE syndrome but the presence of dysplastic semicircular canals, an uncommon finding that is strongly associated with *CHD7*-related disease, makes it highly likely that the p.(Thr1133Met) *de novo* variant affects function. Patient 2, his son, also had a mild phenotype with an abnormal shoulder shape. Missense variants in *CHD7* are relatively uncommon in the literature, and it is possible that mild or atypical CHARGE phenotypes, such as we report, have been under-recognized. Variable expressivity is common in autosomal dominant transmission of *CHD7* variants [9], and it is uncertain whether the p.(Thr1133Met) variant predisposes to a mild phenotype or could lead to typical CHARGE syndrome in some individuals.

Patient 1 had hypoplastic trapezius and sternocleidomastoid muscles on muscle MRI explaining his prominent scapulae, abnormal shoulder shape and shoulder girdle weakness. Although scapular winging was noted in Patient 3, this was less marked than in Patient 1 and the trapezius muscles were only mildly asymmetric in bulk, suggesting there is a spectrum of severity of neuromuscular involvement. Patient 3 also demonstrates that abnormal neuromuscular features can accompany typical CHARGE syndrome. In addition to the shoulder abnormalities, Patient 3 had pes cavovarus and mild calf muscle atrophy. These observations of muscle hypotrophy have not been previously described in association with *CHD7* mutations.

Sloping, asymmetric and anteverted shoulders are a common feature of CHARGE syndrome [5]. A retrospective evaluation of the UMCG database showed this to be present in 31 out of 41 patients. A systematic analysis of these findings has never been performed and the cause for this phenomenon has not been previously identified. More interestingly, we have demonstrated in Patient 1 that muscle hypoplasia can be the most prominent clinical abnormality associated with *CHD7* mutations.

The *CHD7* protein is a transcriptional regulator, which regulates nuclear gene expression and rRNA biogenesis [16]. It has an essential role in the formation of multipotent neural crest cells and their migration, and development into a range of head and neck structures [17]. Chick embryos have high *CHD7* protein expression in the optic, otic and nasal placodes, and branchial arches, which corresponds with the structures classically affected in CHARGE syndrome [18].

The sternocleidomastoid and trapezius muscles are both innervated by the XIth cranial nerve, which has its embryological origins in the neural crest. The EMG findings in Patient 1 were suggestive of chronic denervation and the spinal accessory nerves were not visualized on MRI imaging, raising the possibility that the primary defect is in the development of and innervation from the XIth cranial nerve. The abnormalities of the VIIth and other cranial nerves are common in CHARGE syndrome. In zebrafish models, *CHD7* is important in neuronal development and axonal projections. Zebrafish with reduced *CHD7* expression showed disrupted organization of cranial motor neurons [19]. However, the involvement of the paraspinal muscles, and the peroneal weakness seen in Patient 3 is not easily explained by this hypothesis.

An alternative explanation is that the abnormalities may result from abnormal patterning, migration and differentiation of muscle progenitor cells, a process dependent on normal cranial neural crest cell function. In chick embryo models, myogenesis was initiated in the absence of neural crest cells, but the migratory pathways and anterior-posterior registration of the paraxial mesoderm was severely impaired and overall myofiber organization was disrupted [20,21].

Making a diagnosis of *CHD7*-related disease has implications for health surveillance and genetic counseling, given the autosomal dominant inheritance and well-described risk of germline mosaicism [8]. *CHD7*-related disease should be considered in the differential diagnosis of children with early-onset prominent scapulae and a stable clinical course, particularly if associated with dysmorphic facial features and balance difficulties, and in our clinic has lead to the identification of a further unpublished patient. If suspected, investigation with a CT or MRI of the brain for semicircular canal malformations is recommended, which if present give strong support to this diagnosis. A next-generation sequencing approach to the genetic diagnosis of scapular winging is advantageous given the heterogeneity of this presentation and *CHD7* should be considered for inclusion in neuromuscular gene panels.

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CHAPTER 7

General discussion

7.1 What did my research contribute to our knowledge about CHARGE syndrome?

My research focused on three less well-studied clinical aspects of CHARGE syndrome: immunology, adrenal function and abnormalities of the shoulder musculature. I was able to show that recurrent infections are common in patients with CHARGE syndrome and that immunological abnormalities can contribute to that. I also showed that chest radiography is insufficient to study thymic aplasia and that more data on the thymus in CHARGE syndrome are needed. Furthermore, I have shown that (central) insufficiency of the adrenal function is not common in children with CHARGE syndrome. Finally, I identified that musculoskeletal deformities of the shoulder region are a common but overlooked aspect in patients with CHARGE syndrome.

In sum, my work further detailed the complex and variable phenotype of CHARGE syndrome. Since my main professional interests are the societal aspects of sickness and health, I wondered how this knowledge can benefit patients with CHARGE syndrome? How can I implement this knowledge in the practice of clinicians? Furthermore, although I have contributed to more knowledge on CHARGE syndrome, there are still gaps. What can researchers and policymakers do to fill those gaps, specifically for CHARGE syndrome, but also for rare syndromes in general? Therefore, I discuss our most important findings in a broader context for patients, clinicians, researchers and policymakers.

7.2 Implications for patients: acknowledgement and empowerment

An important aspect of my research is that it focused on issues that may be easily overlooked by clinicians yet still have great impact on the daily life of patients. By emphasizing these issues in my research, I acknowledge the daily problems patients face.

7.2.1 Immune system

Parents of patients with recurrent, common and (at times) prolonged infections will often hear that these events will become less frequent and/or severe in future years. This may lead to the perception that the issue is not important or cumbersome. Although most of these infections are indeed not life-threatening, they do affect the quality of life of patients, who miss out on school and social events. I have now shown that immunological defects are present in CHARGE syndrome (Chapter 3). Investigations of the immune system are indicated when infections are frequent and/or more serious than is usual in non-affected children of comparable age [1]. Recurrent need for antibiotics or benefit from prophylactic antibiotics might be other indicators of immunological defects. Medical attention to the “common infections of childhood” empowers patients and their parents, as well as collaborating medical

colleagues.

7.2.2 Central adrenal function

By contrast, my work has also shown that it is not necessary to screen children with CHARGE syndrome for their adrenal function (Chapter 5). This means a lot for children and their parents because they often encounter diverse medical issues, which require different examinations. It is therefore helpful to know when medical aspects do not need to be assessed, especially when it comes to invasive tests like the functional tests used to assess adrenal function. This finding also means that patients do not need specific peri-operative measures to counter adrenal deficiency, e.g. corticosteroids in stress doses. As Hefner and Fassi stated: "...knowing what is normal/negative can be just as important as knowing what is wrong" [2].

7.2.3 Musculoskeletal function

Musculoskeletal deformities of the shoulder and neck region are easily overlooked in CHARGE syndrome but can have a major effect on patients' daily life. Impeded mobility of the shoulders means that patients may have trouble bathing, dressing and engaging in physical activities like sports and play. As I showed in Chapter 6, the deformity may be the only visible presentation of CHARGE syndrome. Patients should therefore not feel restricted in mentioning musculoskeletal problems to their clinicians even though it seems less relevant compared to other more urgent medical problems.

7.2.4 Other issues that need attention

Due to improvements in medical care, children with CHARGE syndrome are reaching adulthood and thus requiring age-appropriate care. The transition from childhood to adulthood is an important process that does not receive enough attention. One of the underexamined issues around this transition is sexuality. Delayed sexual development due to hypogonadotropic hypogonadism is a known aspect of CHARGE syndrome, but the attention paid to this is mostly focused on medical issues, with little attention paid to psychological and behavioural issues. A recent study showed reduced sexual satisfaction in individuals with isolated hypogonadotropic hypogonadism [3]. Other studies have focused mostly on individuals with developmental disability in general [4,5], but individuals with CHARGE syndrome often have additional disabilities (e.g. deaf blindness) and medical issues (e.g. hypogonadotropic hypogonadism) that can complicate a healthy sexual life.

7.2.5 Empowerment

Patients and their parents could advocate for more attention to be paid to the issues that they encounter in their daily lives that are neglected by clinicians, researchers, or policymakers. Patient organizations can play an important role in collecting the common issues experienced by their members and putting them on the agendas of the other stakeholders. An important stakeholder here is the Centre of Expertise

for CHARGE syndrome [6], which plays a pivotal role in the coordination of care and research. In addition to advocacy, active patient participation in research should be achieved, for example by forming a patient panel as part of a research team.

7.3 Implications for clinicians: awareness and guideline development

Time is precious for clinicians. Guidelines are therefore helpful to enable clinicians to quickly gain insight into rare syndromes and to know what is needed to provide optimal care for the patient. This is especially important for rare syndromes with a complex clinical presentation. Guidelines can raise awareness of less prevalent issues and lead to more insight into the prevalence of these less frequently occurring issues by noting them in the patient's records.

7.3.1 Immune system

Distinct immunological features are not specifically prominent in CHARGE patients, at least outwardly. Only rarely do these children present with life-threatening diseases indicating severe immunodeficiency. However, if missed, immunological deviations can lead to prolonged or recurrent infections and thus to increased morbidity. There are other recently published studies that conclude with the advice to perform immunological screening [7] or specific immunological diagnostics if indicated by the clinical history [7,8]. Therefore, immunological assessment should be incorporated into the (existing) guidelines to assure that it is not overlooked. Fortunately, Trider et al [9] have done so, including a description of the thymus during open heart surgery in their extensive checklist of the management of patients with CHARGE syndrome.

When an immunodeficiency is diagnosed, patients and their parents should be advised to follow general guidelines on prevention of transmission of infectious diseases. If cellular deficiency is combined with an absent thymus, there is currently no effective treatment other than supportive care, like strict infection prevention measures or immunoglobulin replacement. Clinical trials for treatment are also still being performed, such as the recent study on cultured thymus tissue [10].

When insufficient antibody responses are found, booster vaccinations should be considered. Prophylactic antibiotics or substitution therapy with gamma globulins may be warranted in individual patients.

7.3.2 Central adrenal function

According to the Trider et al [9] checklist and the recommendations collected from literature by de Geus et al [11], attention should be paid to several endocrinological problems in CHARGE syndrome, including growth hormone deficiency, hypogonadotropic hypogonadism and thyroid dysfunction. I showed through an international study that routine testing of adrenal function is not indicated and thus should not be included in a guideline for CHARGE syndrome.

7.3.3 Musculoskeletal function

Musculoskeletal issues were also addressed by Trider et al [9] in their checklist. They advise monitoring for scoliosis and/or kyphosis and to evaluate mobility, especially when hypotonia is present. I suggest adding special attention to the shoulder region because of the impact it has on daily independent living (see section 7.2). Some problems can be helped by physiotherapy or, if necessary, operative procedures [12].

7.3.4 Guideline development and implementation

It is important to keep exploring the phenotypic spectrum of CHARGE syndrome to find signs and symptoms that could be overlooked and thereby lead to unnecessary loss of quality of life. These aspects should be included in (existing) guidelines to raise clinician awareness of these issues and to optimize care. But is there a consensus guideline for CHARGE syndrome?

The information on clinical management in CHARGE syndrome is too dispersed in form (online or offline), in platform (scientific journals, scientific institutes, professional and patient organisations) and in region or country. Therefore, I suggest developing one international online guideline for CHARGE syndrome to improve efficiency and quality of care. The chapter on *CHD7* Disorder in GeneReviews [13] is quite comprehensive and provides a good basis for further optimalization. An important issue, however, is that implementation is not widespread. The guideline seems only to be known by genetic clinicians and not by other clinicians, e.g. paediatricians and general practitioners. So, how should the optimization of this guideline be done?

I suggest executing a proper guideline development and implementation, a complex process consisting of different steps [14,15]. One important step in guideline development and implementation is to involve relevant stakeholders, including patients and health professionals [14,15]. The European Reference Network ITHACA (ERN-ITHACA) [16] could be a good candidate to oversee the processes because of its link with European medical experts and patient organisations. This network, established in 2017, consists of all European centres of expertise on rare (multiple) malformation syndromes. One of the aims of ERN-ITHACA is to develop high-quality, evidence-based guidelines and to disseminate, utilise and evaluate these. A workgroup has been established, but this is in its start-up phase [17]. An advantage of ERN-ITHACA is that the dissemination of the guideline could be delegated to the different members (centres of expertise). In the Netherlands, the Dutch National multidisciplinary CHARGE outpatients' clinic [18], a member of ERN-ITHACA, could further implement the European guideline into national or local practices.

In addition to the guideline, a standard of care should be developed, including of the transition of care from childhood to adulthood. The purpose of a standard of care is to describe the whole process of care around a patient with a specific disease. In this trajectory, the role, timing and actions of each health professional are described [19]. I suggest involving a broader group of health professionals that includes both specialists (clinical geneticists and paediatricians) and generalists (general practitioners,

intellectual disability physicians and preventive child health care professionals) in development. Care by generalists should be organized nearby the patient as much as possible, but specialized care should be available and accessible when needed.

ERN-ITHACA is an important step towards uniformity of care for rare diseases in Europe. The next step is to broaden the network internationally, as much of the expertise and knowledge also lies outside of Europe, especially in the USA. In the next sections, I will further discuss collaboration and how policymakers can support this.

7.4 Implications for researchers: collaboration and innovation

Research on rare diseases such as CHARGE syndrome requires a multidisciplinary and (inter)national approach. I collaborated with clinical geneticists, subspecialists of paediatrics (immunology, endocrinology and neurology), immunologists, radiologists and statisticians. I also collaborated with colleagues in Australia [20,21]. These collaborations improved the quality of the studies in terms of sample size, knowledge and effective use of resources. However, as mentioned in the section for patients, there are still issues in CHARGE syndrome that need more attention. In this section, I provide recommendations for researchers on how to address these issues via research on CHARGE syndrome and on rare diseases in general.

7.4.1 Collaborate with other researchers

Collaborations with other researchers or research groups at an (inter)national level will not only result in larger sample sizes, it will also improve cost-effectiveness by limiting repetition of the same kind of study. To stimulate this initiative, I suggest starting an online platform where researchers who are interested in CHARGE syndrome and/or the gene *CHD7* can post research projects, discuss them and collaborate with other research groups around the world. The platform could be embedded in ERN-ITHACA via their research workgroup, which seems to be in a start-up phase. In the meantime, the platform could be incorporated by the CHARGE Syndrome Foundation [22]. This American foundation has established a professional research section with funding from donations and a scientific advisory board of accomplished researchers.

I also suggest combining different kind of studies on the same subject. For example, Yu et al [23] combined mouse models with clinical observations to study cerebellar deformities in CHARGE syndrome. Additionally, surveillance or natural history databases are an important source to collect information. I recommend setting up an international surveillance database by combining existing (online) databases like the CHARGE Syndrome Clinical Database Project [24] and the CHD7 mutation database [25].

Finally, I suggest working together with researchers or research groups who study other (rare) syndromes that overlap genetically and/or clinically with CHARGE syndrome. The aim here is to explore newly discovered features in other overlapping

syndromes and thereby also create awareness in clinicians (see section 7.3). The studies on immunology and adrenal function in CHARGE syndrome were ignited by its strong overlap with 22q11.2 deletion syndrome [26] and the endocrinological overlap with Prader-Willi syndrome [27,28], respectively. Another aim is to find possible links to further elucidate the pathogenesis of syndromes. For example, Ufartes et al [29] provided new insights into the pathogenesis of CHARGE syndrome by studying *SEMA3E*, a gene involved in Kallmann syndrome, which has clinical overlap with CHARGE syndrome [30,31]. In a recent study, the same research group also described a pathogenetic link between CHARGE syndrome and the overlapping Kabuki syndrome [32].

7.4.2 Collaborate with patients, parents and clinicians

Patient involvement in scientific medical research can improve research in multiple ways. It improves societal impact by better understanding, addressing and answering patient needs. It stimulates the study participation rate and chances of researchers receiving funding as patient involvement has become an important evaluation criterion. One example of patient involvement is the current PhD project on growth and puberty in CHARGE syndrome, which was initiated by parents' need for a dedicated growth chart for children with CHARGE syndrome like the one that exists for children with Down syndrome [33]. Children with CHARGE syndrome have a specific growth pattern [34], so a syndrome-specific growth chart is necessary to screen for abnormal growth and development. Another great example of patient involvement is the Chromosome 6 project [35] in which parents are involved in the study design, funding and inclusion of participants.

It is also important to involve relevant clinicians in research. A multidisciplinary approach is a requirement for a Centre of Expertise, but it is mostly restricted to (para)medical disciplines within an academic hospital. I suggest broadening the scope outside the centre and including health professionals in preventive child health care, general practitioners and intellectual disability physicians, as well as paramedical professionals like physiotherapists, occupational therapists and psychologists. Involvement of an integral network of caregivers stimulates societal scientific research and can supply input for the standard of care (see paragraph 7.3.3). Results from these kinds of studies can also be relevant for policymakers (see section 7.5).

7.4.3 Collaborate with people outside the academic sector

My last recommendation for researchers is to think outside the (academic) box. Why not collaborate with public and private sector partners outside academia? I could imagine collaborations with educational institutions, public health services, municipalities, companies producing pharmaceutics/medical devices/IT/ toys and games, and many more. This kind of collaboration will improve creativity and the translation of scientific results into practical applications. It will also improve visibility and public-awareness of CHARGE syndrome or other rare diseases/syndromes. In one example of this kind of collaboration, researchers from Kentalis, the Dutch organisation for deafblind and communication disability, have developed a game

about coping with emotions [36].

7.5 Implications for policymakers: coordination and facilitation

In my research I encountered how difficult it was to find financial support to conduct research on a rare disease, specifically on clinical aspects that seem not to be important but could affect a patient's quality of life. The unfamiliarity of a rare disease makes it hard to get the attention of the public and the policymakers, who won't prioritize the subject. Fortunately, the Council of the European Union (EU) signed the Council Recommendation of the 8th of June 2009 on an action in the field of rare diseases [37]. This means that all EU member states, including the Netherlands, are obliged to establish and implement a plan or strategy for rare diseases with the aim of improving the (infrastructure of) healthcare by the end of 2013 at the latest. In October 2013, a National Plan Rare Diseases was presented as part of the Dutch strategy. The plan consists of suggestions on six subjects to improve the situation of people with a rare disease in the Netherlands:

1. Awareness by clinicians
2. Information and communication
3. Organisation of the healthcare system and (availability of) treatment
4. Research
5. Patient empowerment
6. Coordination and continuity of the plan

Most subjects have already been discussed in the previous sections aiming at patients, clinicians and researchers. In this section, I will discuss how policymakers can contribute to improving the situation of people with a rare disease, in particular CHARGE syndrome, by coordination and facilitation at each level: patient, healthcare and research.

7.5.1 Improving the position of people with rare diseases

The Dutch government recognizes and structurally subsidizes only three nationwide patient organizations, which includes the umbrella organization for people with physical and/or intellectual disability and chronic diseases: Ieder(in) [38]. This financial support is meant for the operational activities of the organization. Separate short-term funding is available for advocational activities. But advocational activities or initiatives, like patient participation in the development of guidelines, are just as important or even more so. The Dutch government should therefore consider structurally funding these activities. Furthermore, it is important to incorporate the voice of people with rare diseases in policy making, e.g. when making new regulations on financial support or when changing the infrastructure of the healthcare system.

7.5.2 Improving the healthcare system for people with rare diseases

The patient should be the leading actor in the healthcare system. This is especially true

for people with rare diseases, who encounter multiple healthcare professionals and institutions, each with its own perspective and policy. As laid out in paragraph 7.3.3, a broader integrated network of professionals is needed to develop a standard of care around a patient. Policymakers could facilitate this by supplying an infrastructure that stimulates collaborations, e.g. in terms of manpower and IT infrastructure. Additionally, health insurance agencies could facilitate this initiative by an adequate cost coverage system, which supports a shared care system from home to hospital and back.

7.5.3 Improving research on rare diseases

It is crucial to collaborate internationally, especially in the field of rare diseases. There are currently European (E-Rare) and worldwide (International Rare Diseases Research Consortium) initiatives to stimulate research on an international level. It is important to continue financing these initiatives, especially for research on the natural course of disease, which requires long-term follow-up studies needing continuous funding.

Additionally, I suggest more funding for translational research in which researchers collaborate with medical and educational professionals and policymakers. In this way, questions from the field are answered by the research, which is performed preferably in the field, and the study results are incorporated in evidence-based policy. One example of this is the Academic Collaborative Centres Public Health [39], a long-term partnership between one or more community health services (municipal public health departments) and a university.

Furthermore, extra funding should be reserved for research initiatives that are daring and out-of-the-box (see paragraph 7.4.3). Creativity and pioneering research should be stimulated to enable innovations and keep pace with societal developments.

7.6 Concluding remarks

Integration is the key word for both providing the best care for patients with CHARGE syndrome and for generating new knowledge about CHARGE syndrome. The syndromic nature of CHARGE means that it requires integrated care that covers the many different elements of the condition. The rarity of CHARGE syndrome means that it is best approached as an international project so that the information generated in one country can be integrated and translated elsewhere. To achieve integration and transcending existing structures and systems, we need boldness from every stakeholder: patients, parents, healthcare professionals, researchers and policymakers.

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CHAPTER 8

Addendum

8.1 Summary

This thesis begins with a general introduction on CHARGE syndrome in **Chapter 1**. It starts with a brief history of CHARGE syndrome that shows how it has developed into a more complex syndrome with an expanding clinical presentation that has outgrown the features highlighted in its initial acronym: **C**oloboma, **H**eart defects, **A**tresia of the choanae, **R**etardation of growth and/or development, **G**enital abnormalities, and **E**ar abnormalities. Milder clinical features have been adding up since the discovery of the causative gene, *CHD7*, and with the rising use of genome-wide genetic techniques. Therefore, the clinical diagnostic criteria and surveillance guidelines need continuous updates. However, three clinical aspects seem insufficiently covered in the guidelines even though they can have a major impact on the patient's well-being: immunological, adrenal, and scapular function. By studying these three functions, we aimed to further improve the recognition and clinical management of underexposed features in patients with CHARGE syndrome.

8.1.1 Immunological function

Chapter 2 provides an overview of the literature on immunologic abnormalities in CHARGE syndrome and a comparison between CHARGE syndrome and other multiple congenital anomaly (MCA) syndromes on their immunological phenotypes. We performed a systematic search of publications on immunological aspects of CHARGE syndrome, with all the literature found being either case reports or retrospective studies. The clinical presentation of the patients ranged from relatively mild infections of the (upper) respiratory tract to more severe systemic infections in a few patients. The information available about immune cells showed decreased T-cell numbers (T-cell lymphopenia) in 80% of the patients, which is associated with a reduced T-cell function and low gammaglobulins, yet normal B-cell and NK-cell numbers. Thymic aplasia was reported in almost half of the patients and might be the underlying cause of the T-cell lymphopenia.

CHARGE syndrome overlaps clinically with other MCA syndromes. The most remarkable phenotypic overlap is with 22q11.2 deletion syndrome, and this seems to also be true for the prevalence of T-cell lymphopenia. Immunological functions in other overlapping MCA syndromes are less well described. T-cell dysfunction is only mentioned sporadically in Alagille syndrome, Pfeiffer syndrome, and Kabuki syndrome, but the clinical overlap of these syndromes with CHARGE syndrome indicates that their underlying genetic defects may result in shared embryonic pathways that lead to T-cell abnormalities.

Publications on immunology in CHARGE syndrome are scarce and difficult to compare due to differences in how the results are reported. Reporting bias may also play a role in these differences as most publications are case reports.

To better understand the frequency and nature of immune dysfunction in CHARGE syndrome, we performed a cross-sectional study (**Chapter 3**). Extensive clinical and immunological data of 24 patients with CHARGE syndrome were collected using

questionnaires and laboratory tests. All CHARGE patients had a history of infections, mainly otitis media and pneumonia, leading to frequent use of antibiotics and to hospital admissions. T-cell lymphopenia was found in 50% of the patients with diminished T-receptor excision circle amounts, which can be used as a reflection of thymic T-cell output. Although the numbers of T-cells were decreased, we did not find deficiencies in T-cell function. Despite normal peripheral B-cell differentiation and immunoglobulin production in all patients, 83% of patients had insufficient antibody titres to one or more early childhood vaccinations. Based on these results, I recommend performing specialised immunologic assays, which consist B- and T-cell numbers and vaccination responses, in patients with persistent infections who need prophylactic antibiotics. It may be worthwhile to give these patients booster vaccinations and recheck the antibody responses.

The thymus plays an important role in the development and function of T-cells, but the prevalence of thymic anomalies in CHARGE syndrome remains unknown. In **Chapter 4**, we obtained an estimated prevalence of thymic aplasia in children with CHARGE syndrome via retrospective examination of chest radiographs for a thymic shadow. We also screened cardiac surgery reports for a description of the thymus. Thymic shadow was present in 6 of the 37 patients. However, the observation that the thymus was present during surgery even when it was not visible on chest radiographs in six other patients underscores the inadequacy of current radiographic methods for this diagnostic purpose. Although we could not further elucidate the prevalence of thymic aplasia in patients with CHARGE syndrome, we confirmed that chest radiography has minimal diagnostic value in determining thymic aplasia. Prospective studies with other methods that include age-related controls are therefore needed.

8.1.2 Adrenal function

Central adrenal insufficiency (CAI) has never been systematically studied in children with CHARGE syndrome. This is surprising because, if CAI is frequently present, mortality may be prevented through the use of corticosteroids during stressful conditions such as surgeries and infections. In **Chapter 5**, I examined the prevalence of CAI in two separate cohorts of patients with CHARGE syndrome (a Dutch and an Australian cohort) using a cross-sectional study design. CAI was assessed using routine functional tests, and only one of the 38 patients from both cohorts had confirmed CAI. CAI thus seems not to be common in CHARGE syndrome. Routine screening for adrenal function and specific peri-operative measures to counter adrenal deficiency are therefore not needed in children with CHARGE syndrome. This is a crucial finding because it reduces uncertainty for physicians and concern for patients and parents as well as removing the need for extra testing and other measures.

8.1.3 Scapular function

Sloping, asymmetric and anteverted shoulders are common features of CHARGE syndrome but receive little attention in practice. In **Chapter 6**, we describe three patients who presented with hypoplasia of the shoulder and neck muscles, which further expands the phenotype associated with *CHD7* mutations. In one patient,

muscle hypoplasia was the most prominent clinical abnormality. *CHD7*-related disease should therefore be considered in the differential diagnosis of children with early-onset prominent scapulae and a stable clinical course, particularly if associated with dysmorphic facial features and balance difficulties.

8.1.4 Implications in a broader context

I have put the knowledge gained from my work studying underexposed features in CHARGE syndrome in a broader and societal context in **Chapter 7**, where I discuss the implications of my work for all stakeholders: patients, clinicians, researchers, and policymakers.

- For patients, this work acknowledges the daily problems they face by emphasising issues that do not receive enough attention from clinicians, researchers, and policymakers. To keep the patient's interests in mind, it is important to empower the patient's voice in the practices of the other stakeholders.
- For clinicians, the knowledge and recommendations generated by my work should be included in a guideline, in order to create awareness. This should be a uniform and easily accessible guideline, which requires proper guideline development and implementation with involvement of other stakeholders.
- For researchers, I have emphasised that it is important to collaborate with different stakeholders to achieve research that has societal impact. Furthermore, I have affirmed that collaborations could improve the quality and cost-effectiveness of research while also stimulating innovative ideas.
- For policymakers, I have stressed that coordination and facilitation of other stakeholders is important to enable them to do their part in improving care for individuals with CHARGE syndrome but also for those with other rare diseases. Coordination could be delegated to centres of expertise and international reference networks because they combine patient care and research.

Integration of other stakeholders is needed at every level, and this requires boldness from all stakeholders.

8.2 Nederlandse samenvatting

CHARGE syndroom is een zeldzame en complexe genetische aandoening. Kinderen met het syndroom kunnen een combinatie van verschillende aangeboren afwijkingen, ontwikkelings- en gedragsproblemen hebben. In **hoofstuk 1** wordt een algemene inleiding over CHARGE syndroom gegeven en wordt de kennis over het syndroom in historisch perspectief geplaatst. Aanvankelijk werden vooral de klinische kenmerken beschreven die opgenomen zijn in het acroniem: **C**oloboma, **H**artafwijkingen, **A**tresie van de choanae, **R**etardatie van groei en/of ontwikkeling, **G**enitale afwijkingen en **E**venwichtsorgaan- en oorafwijkingen. Na de ontdekking van het oorzakelijke gen *CHD7* en met het toenemende gebruik van genoombrede genetische technieken werden steeds meer mensen gediagnosticeerd met CHARGE syndroom. Hierdoor werd duidelijk dat er meer, ook mildere, klinische kenmerken bij het syndroom voorkomen. De bestaande richtlijnen bleken niet meer passend bij het huidige spectrum van klinische kenmerken. Het is daarom belangrijk om, voortdurend, de richtlijnen bij te werken zodat behandelaren de beste zorg kunnen leveren aan patiënten met CHARGE syndroom. We merkten dat drie klinische aspecten tot nu toe onvoldoende aan bod waren gekomen in de richtlijnen terwijl deze aspecten grote impact kunnen hebben op het welzijn van de patiënt. Het gaat om de functies van de afweer (immuunsysteem), de bijnier en de schoudergordel. Door deze drie functies te bestuderen, streven we ernaar dat onderbelichte kenmerken bij patiënten met CHARGE syndroom beter herkend en behandeld worden, o.a. door de uitkomsten van het onderzoek op te laten nemen in nieuwe richtlijnen.

8.2.1 Afweerfunctie

Allereerst wordt in **hoofstuk 2** een overzicht gegeven over wat er tot nu toe bekend is over afwijkingen in de afweer bij CHARGE syndroom. We hebben ook de afweer bij andere syndromen met meervoudig aangeboren afwijkingen vergeleken met die van CHARGE syndroom. Dit hebben we gedaan door op een systematische manier te zoeken naar medisch wetenschappelijke publicaties over dit onderwerp. Het bleek dat alle geïdentificeerde publicaties over afweer bij CHARGE syndroom case reports of retrospectieve studies waren. Dit kan mogelijk een vertekend beeld geven door publicatiebias (bijzonderheden worden makkelijker gepubliceerd). Daarnaast waren de publicaties schaars en moeilijk onderling te vergelijken vanwege verschillen in de manier waarop de resultaten worden gerapporteerd.

Uit de publicaties over CHARGE syndroom bleek dat de klinische presentatie van de beschreven patiënten varieerde van het (vaak) hebben van relatief milde infecties van de (bovenste) luchtwegen tot ernstigere systemische infecties bij enkele patiënten. De beschikbare informatie over immuuncellen toonde verminderde T-cel aantallen (T-cel lymfopenie) bij 80% van de patiënten, hetgeen geassocieerd kan zijn met een verminderde T-celfunctie en lage gammaglobulinen. Toch waren de B-cel en NK-cel aantallen normaal. Afwezigheid van de thymus (thymusaplasie) werd gemeld bij bijna de helft van de patiënten en kan de onderliggende oorzaak zijn van de T-cel lymfopenie.

CHARGE syndroom heeft klinische kenmerken die overeenkomen met die van andere syndromen met meervoudig aangeboren afwijkingen. De meeste klinische overlap bestaat met het 22q11.2 deletiesyndroom, ook wat betreft de T-cel lymfopenie. Afweerfuncties in andere overlappende syndromen zijn minder goed beschreven. T-cel dysfunctie wordt slechts sporadisch genoemd bij Alagille syndroom, Pfeiffer syndroom en Kabuki syndroom, maar de duidelijke klinische overlap van deze syndromen met CHARGE syndroom suggereert dat ook hun onderliggende genetische defecten kunnen leiden tot T-cel afwijkingen via eenzelfde embryologische route.

De gevonden resultaten in hoofdstuk 2 gaf reden om de de frequentie en aard van afweerstoornissen bij CHARGE syndroom verder te onderzoeken. Hiervoor hebben we een cross-sectionele studie uitgevoerd bij 24 patiënten met CHARGE syndroom (**hoofdstuk 3**). Bij de patiënten hebben we gegevens over doorgemaakte infecties verzameld via een vragenlijst en hun afweer in kaart gebracht via laboratoriumtesten (volledig bloedbeeld en specifieke immunologische testen). We hebben broers of zussen gevraagd om bloed af te geven zodat referentiewaarden konden worden gegenereerd voor een aantal laboratoriumtesten. Alle CHARGE-patiënten hadden een voorgeschiedenis van infecties, voornamelijk middenoorontstekingen en longontstekingen, hetgeen leidde tot frequent gebruik van antibiotica en herhaalde ziekenhuisopnames. T-cel lymfopenie werd gevonden bij 50% van de patiënten. Dit komt waarschijnlijk door een verminderde aanmaak in de thymus omdat het aantal *T-receptor excision circles*, dat een maat is voor de T-cel output uit de thymus, gemiddeld genomen lager was in de groep met CHARGE syndroom dan in de referentie groep. Hoewel het aantal T-cellen verminderd was, vonden we geen afwijkingen in de T-cel functie. De perifere B-cel differentiatie en immunoglobulineproductie waren normaal bij alle patiënten. Toch had 83% van de patiënten te lage antilichaamtiters tegen één of meer vaccinaties die op jonge leeftijd waren gegeven. Op basis van deze resultaten, is het aan te bevelen om B- en T-celaantallen en vaccinatieresponsen te onderzoeken bij patiënten met aanhoudende infecties. Het kan tevens de moeite waard zijn om deze patiënten boostervaccinaties te geven en de antilichaamtiters opnieuw te controleren na 4 tot 6 weken.

Zoals in hoofstuk 3 aangegeven, speelt de thymus een belangrijke rol in de ontwikkeling en functie van T-cellen, maar de prevalentie van thymusafwijkingen bij CHARGE syndroom blijft onbekend. In **hoofdstuk 4** schatten we de prevalentie van thymusaplasie bij kinderen met CHARGE syndroom middels een retrospectief onderzoek. We hebben thoraxfoto's van 37 patiënten verzameld en deze werden door twee kinderradiologen beoordeeld op de aanwezigheid van een thymusschaduw. We hebben ook de hartoperatieverslagen doorgenomen op zoek naar een beschrijving van de thymus. Thymusschaduw was te zien bij 6 van de 37 patiënten. Echter, bij zes andere patiënten bleek uit de operatieverslagen dat de thymus aanwezig was, terwijl deze niet zichtbaar was op de pre-operatieve thoraxfoto's. Dit geeft aan dat de diagnostische waarde van thoraxfoto's om thymusafwijkingen te detecteren onvoldoende is. We stellen daarom voor vervolgonderzoek te doen bij kinderen met CHARGE syndroom waarbij de thymus in de tijd, bij voorkeur vanaf de geboorte,

wordt vervolgd met andere beeldvormende methoden, zoals echografie, CT-scan of MRI-scan, en bij leeftijd gematchte controles.

8.2.2 Bijnierfunctie

Een tweede onderbelicht kenmerk is de bijnierfunctie. Centrale bijnierschorsinsufficiëntie (CBI) door te weinig hormonale aansturing vanuit de hersenen is nooit systematisch onderzocht bij kinderen met CHARGE syndroom. Dit is verrassend omdat andere centraal aangestuurde hormonale aandoeningen wel zijn beschreven, zoals groeistoornissen en stoornissen in de puberteitsontwikkeling. Indien CBI regelmatig voor komt bij CHARGE syndroom dan is screening hierop zinol. Want dan kan toediening van corticosteroïden tijdens stressvolle situaties, zoals een operatie of een infectie, ernstige ontregeling van de bijnierfunctie (bijniercrisis) voorkomen. In **hoofdsuk 5** onderzochten we daarom hoe vaak CBI voorkomt in een Nederlands cohort van 23 kinderen en een Australisch cohort van 15 kinderen via een cross-sectionele onderzoeksopzet. In Nederland werd CBI bepaald met functionele testen. In Australia werden de kinderen eerst gescreend met een bloedtest en als het afwijkend is, werd er een functionele test gedaan. Van de totale groep van 38 kinderen, werd bij slechts één kind CBI bevestigd. CBI lijkt dus niet vaak voor te komen bij CHARGE syndroom. Screening op bijnierfunctie en het nemen van specifieke peri-operatieve maatregelen zijn daarom niet nodig bij kinderen met CHARGE syndroom. Dit is een cruciale bevinding voor zowel behandelaren als patiënten en hun ouders omdat hiermee de onzekerheid en bezorgdheid over een CBI wordt weggenomen. Daarnaast is er geen noodzaak tot extra testen en andere voorzorgsmaatregelen bij een patiëntengroep die al veel medische handelingen moet ondergaan.

8.2.3 Schoudergordelfunctie

Ook naar de schoudergordelfunctie is bij CHARGE syndroom nog weinig onderzoek gedaan. Maar in de praktijk lijkt een niet goed functionerende schoudergordel die veel impact heeft op de dagelijkse verrichtingen, zoals wassen, aankleden en spelen, vaak voor te komen bij deze patiënten. In **hoofdstuk 6** beschrijven we drie patiënten met een mutatie (verandering) in het CHD7 gen, die zich presenteerden met onderontwikkelde (hypoplasie) schouder- en nekspieren. Bij één patiënt was de spierhypoplasie zelfs de meest prominente klinische afwijking en werd pas op latere leeftijd de *CHD7*-mutatie gevonden. Op basis van deze bevindingen adviseren we om het *CHD7* gen te onderzoeken bij kinderen met vroeg ontstane prominente schouderbladen en een verder stabiel klinisch beloop, vooral als dit gepaard gaat met dysmorfe gelaatstrekken en evenwichtsproblemen.

8.2.4 Implicaties van mijn onderzoek in een bredere context

De kennis die ik heb opgedaan met mijn onderzoek naar onderbelichte kenmerken bij CHARGE syndroom, heb ik in een bredere en maatschappelijke context geplaatst in **hoofdstuk 7**. Hierin bespreek ik de implicaties van mijn onderzoek voor vier partijen: patiënten, behandelaren, onderzoekers en beleidsmakers.

- Voor patiënten betekent mijn onderzoek dat er erkenning is voor de dagelijkse problemen waarmee ze worden geconfronteerd. Door onderbelichte kenmerken te onderzoeken, komen deze beter onder de aandacht van behandelaren, onderzoekers en beleidsmakers. Om de belangen van de patiënt voor ogen te houden, is het belangrijk om de positie van de patiënt te versterken binnen de praktijk van de andere drie partijen.
- Voor behandelaren moeten de kennis en aanbevelingen uit mijn onderzoeken worden opgenomen in een richtlijn om zo meer bewustzijn te creëren. Dit moet een uniforme en makkelijk toegankelijke richtlijn zijn en dat vraagt om een goede richtlijnontwikkeling en -implementatie waar de andere drie partijen bij betrokken zijn.
- Voor onderzoekers is het belangrijk om samen te werken met de andere drie partijen. Dit zal leiden tot onderzoek met maatschappelijke impact en tot innovatieve ideeën. Verder heb ik laten zien dat samenwerken de kwaliteit van het onderzoek kan verbeteren en kosteneffectief kan zijn.
- Voor beleidsmakers is het belangrijk om de andere drie partijen te coördineren en te faciliteren bij het verbeteren van de zorg voor patiënten met CHARGE syndroom, maar ook voor mensen met andere zeldzame ziekten/aandoeningen. De coördinatie zou kunnen worden gedelegeerd aan expertisecentra en aan internationale referentienetwerken omdat zij ervaring hebben met het combineren van patiëntenzorg en onderzoek.

Integratie van verschillende partijen is nodig op elk niveau en dit vereist lef van alle betrokkenen.

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De kinderen en jongeren met CHARGE syndroom

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8.4 Publication list

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* These authors contributed equally to this work

8.5 Curriculum vitae

Monica Wong werd geboren in Hongkong op 7 november 1983. Zij emigreerde met haar ouders naar Groningen op de leeftijd van 6 maanden. Na het behalen van haar diploma aan het Praedinius Gymnasium te Groningen, werd zij aanvankelijk uitgeloot voor de studie geneeskunde. Echter na drie weken werd zij herplaatst voor de studie aan de Rijksuniversiteit Groningen.

Na de het behalen van haar artsendiploma in mei 2009, heeft Monica gewerkt als basisarts bij de afdelingen Obstetrie & Gynaecologie (UMCG en Erasmus MC) en de Intensive Care (UMCG). In januari 2013 is zij begonnen met haar baan als arts-onderzoeker bij de afdelingen Genetica en Kindergeneeskunde van het UMCG. Zij werd hierin begeleid door Prof. dr. Conny van Ravenswaaij, dr. Liesbeth Schölvinck en dr. Gianni Bocca.

Tijdens het onderzoek is Monica verder gaan oriënteren op haar loopbaan. Door een tip van haar copromotor Liesbeth ging zij zich verdiepen in de Infectieziektebestrijding bij de GGD. Na twee inspirerende meeloopweken bij GGD Fryslân, werd het enthousiasme voor het vak gewekt. In januari 2018 kwam Monica in opleiding voor het vak arts Maatschappij + Gezondheid. Zij werd opgeleid bij GGD Fryslân door Paul Tan en Everhard Hofstra.

In januari 2022 vervolgde Monica haar opleiding verder bij GGD Amsterdam onder begeleiding van dr. Tjalling Leenstra en dr. Gini van Rijckevorsel. In december 2022 rondt zij haar opleiding af en gaat zij haar onderzoeksvaardigheden verder inzetten voor de publieke gezondheidszorg.