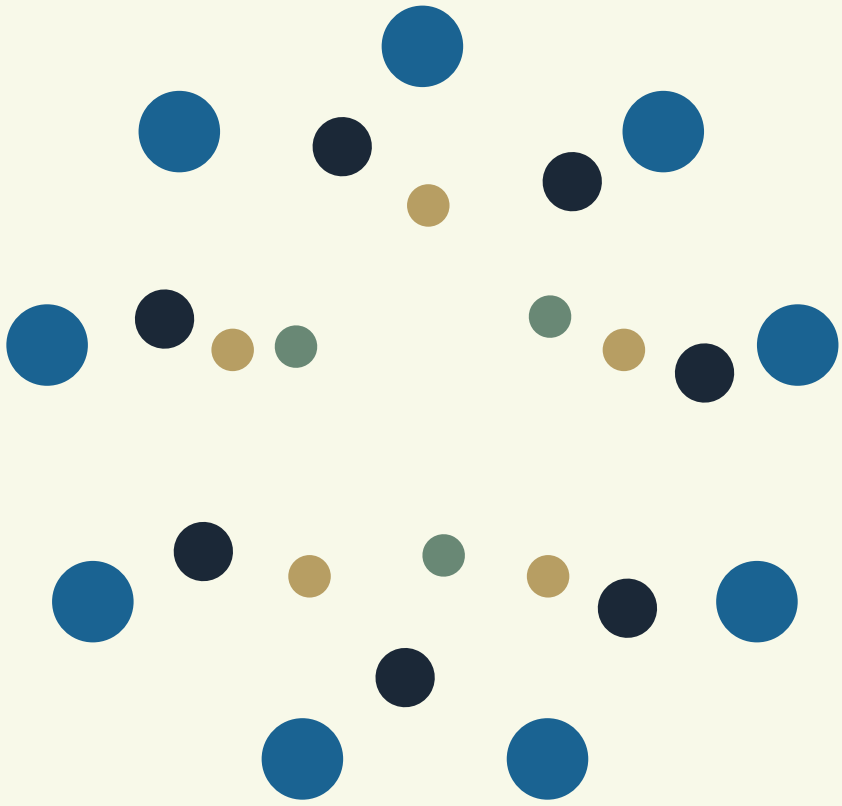
Humoral Immunity in Children with Down Syndrome

relevance to respiratory disease



Ruud Verstegen

Humoral Immunity in Children with Down Syndrome

relevance to respiratory disease

Ruud Verstegen

Molecular Medicine
Postgraduate School

The research for this thesis was in part performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

The studies described in this thesis were performed at the Department of Paediatrics and the Laboratory of Clinical Chemistry and Haematology, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands, the Department of Immunology, Erasmus MC, Rotterdam, The Netherlands and collaborating centers.

ISBN: 978-94-91811-05-0

Cover: Ruud Verstegen
Lay-out: Caroline Jongepier-Linker and Ruud Verstegen
Print: Ipskamp B.V., The Netherlands

Humoral Immunity in Children with Down Syndrome
relevance to respiratory disease

Humorale afweer bij kinderen met het syndroom van Down
relevantie voor luchtwegaandoeningen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 28 januari 2015 om 9.30 uur

door

Ruud Hendrikus Johannes Verstegen
geboren te Zeeland

Promotiecommissie

Promotoren	Prof.dr. J.J.M. van Dongen Prof.dr. E. de Vries
Overige leden	Prof.dr. H. Hooijkaas Prof.dr. A.J. van der Heijden Prof.dr. R. de Groot
Copromotor	Dr. M.C. van Zelm

Contents

Chapter 1

Introduction

Figure 1. The adoration of the Christ child (circa 1515).
A. Painting by Jan Joest van Kalkar (born circa 1450-1460, died in 1519 in Haarlem, The Netherlands) that shows multiple individuals with characteristics suggestive of Down syndrome. **B.** These characteristics include flattened midface, epicanthic folds, upslanted palpebral fissures, small and upturned nasal tip and downward curving mouth corners.
Printed under the Open Access for Scholarly Content (OASC) initiative of The Metropolitan Museum of Art, New York, United States of America.

PART A: DOWN SYNDROME

Clinical and genetic features of Down syndrome

In 1866, John Langdon Down was the first physician to describe intellectual disability and facial dysmorphia as the clinical features of what now is known as Down syndrome (OMIM 190685).[1] However, before then, the phenotypical characteristics have been shown in antiquities, especially figurines from South American and Mexican cultures (1500BC-300AD) and paintings from the European Renaissance (15-17th century; Figure 1).[2] In 1959, the presence of an extra chromosome 21 was identified as the cause of Down syndrome. Detection of trisomy 21 is since then used to confirm the diagnosis.[3] Genetic studies have shown that ~90% of cases with Down syndrome originate from meiotic nondisjunction of maternal origin.[4, 5] Paternal and mitotic origin of trisomy 21 as well as unbalanced translocation involving chromosome 21 are less common and affect approximately 5% each (Figure 2).[4, 5]

Individuals with Down syndrome display various health issues that manifest variably in different stages of life and can affect almost all organ systems. Children are often diagnosed shortly after birth due to hypotonia and facial dysmorphia that include upslanting palpebral fissures, epicanthic folds and macroglossia. In infants with Down syndrome, congenital malformations are found frequently and can affect all organ systems. Congenital heart disease and gastrointestinal malformations are most common and found in approximately 50% and 5-10% of all children, respectively.[6-8] Other problems during infancy include feeding difficulties and delayed motor and mental development.[9, 10] Ear, nose and throat (ENT) disease such as hearing loss and obstructive sleep apnoea are common and often related to anatomical abnormalities.[11-15] When growing up, orthopaedic problems of the feet, knees, hips and spine may arise as well as problems with their dental development.[16-20] Although the intellectual disability in individuals with Down syndrome is highly variable, it significantly impairs practical and social functioning in almost all individuals with Down syndrome.[21]

Due to the complex problems that may arise, diagnostic and management guidelines have been developed in many countries including The Netherlands and the United States of America.[22, 23] In The Netherlands, follow-up by a specialized paediatrician is recommended for all children with Down syndrome at least once a year. At specific ages or when needed, healthcare professionals such as ophthalmologist, ear, nose and throat-specialist, orthopaedic surgeon, rehabilitation specialist, psychiatrist, physical therapist and speech therapist are involved. For

adults, treatment by physicians specialized in people with intellectual disability is advised.

Risk factors for Down syndrome

Each year approximately 250 children with Down syndrome are born (1 in every 725 live born children; Figure 3A). Therefore it remains the most common genetic cause of intellectual disability.[24] This number has remained stable over the past decade, despite the decrease in total births in The Netherlands from 200,000 in 2000 to 170,000 in 2013. As a result, the prevalence of Down syndrome has slightly increased from 12 to 15 children per 10,000 live-born children (Figure 3B).[24, 25]

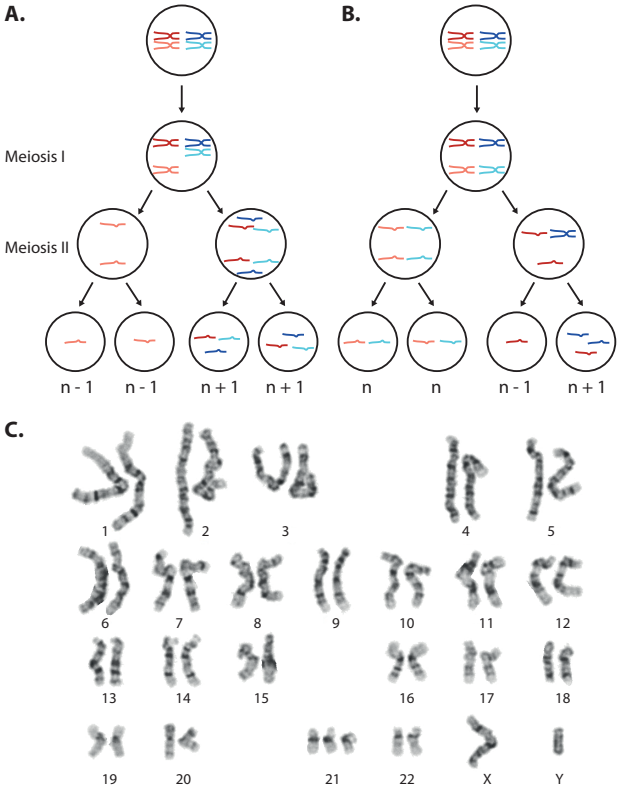


Figure 2. Genetics of Down syndrome
A-B. Nondisjunction during meiosis is the most important cause of Down syndrome (~90%) and can occur during the first (**A**) or second (**B**) meiotic division and results in the two-fold presence of chromosome 21. When the maternal and paternal germ cells fuse, a trisomy of chromosome 21 will occur. Blue and red chromosomes represent chromosome 21 and control chromosome, respectively. **C.** Karyotype of an individual with Down syndrome. Chromosomes are organized based on their size and aspect. A trisomy of chromosome 21 is the most common cause of Down syndrome, although translocation can occur as well. This figure was kindly provided by the Department of Human Genetics, Radboud University Medical Center, Nijmegen, the Netherlands.

Multiple medical as well as socio-economic variables have a significant impact on the prevalence of Down syndrome, which leads to a substantial variation within and between countries from 1 per 600-1,000 live born children.[26]

Already in the 1930s, it was established that maternal age is an important risk factor for the birth of a child with Down syndrome.[24, 26-28] For women aged 25 years old, there is a 1/1000 risk to give birth to a child with Down syndrome. However, this risk is increased to 1/19 for women over 45 years (Figure 3C).[29] This finding has been observed in The Netherlands as well: the average age of mothers giving birth to a child with Down syndrome was 34.0 years between 1997-2007, in contrast to the average maternal age of 30.8 years ($p<0.001$; Figure 3D).[24]

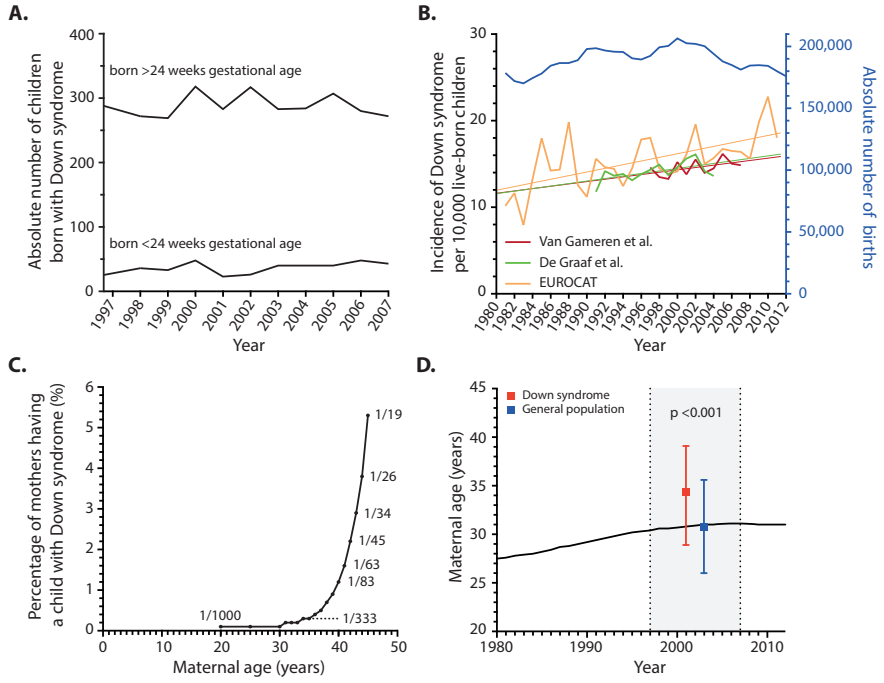


Figure 3. Demographics of Down syndrome
A. Births of number of children with Down syndrome obtained from Van Gameren-Oosterom et al.[24] Children born before 24 weeks of gestational age are suggestive for termination of pregnancy. **B.** The incidence of Down syndrome per 10,000 live born children in The Netherlands. The data with trend lines from Van Gameren-Oosterom et al.,[24] De Graaf et al.,[25] and the European Surveillance of Congenital Anomalies (EUROCAT) database of Northern Netherlands[166] are shown in red, green and orange, respectively. The total number of live births in The Netherlands was obtained from Statistics Netherlands (CBS) and shown in blue.[167] **C.** The risk of Down syndrome increases with increasing maternal age. In this figure, the percentages concern the gestational age of 20 weeks. Data are obtained from Snijders et al.[29] **D.** Maternal age in The Netherlands. Over the last 30 years, the mean maternal age as reported by Statistics Netherlands (CBS) increased to 31 years (black line).[167] Between 1997 and 2007 (dashed lines), the mean maternal age of mothers giving birth to a child with Down syndrome (red) was significantly increased compared to the mean maternal age in the population (blue).[24] More than 50% of children with Down syndrome are born to mothers that are < 35-years-old.

Other maternal risk factors include low socio-economic status, obesity and polymorphisms for folate metabolism.[30-32] It is noticeable that the risk for Down syndrome is lower in multiple than singleton pregnancies.[33] The influence of maternal race appears to be limited.[26] It is unknown how these risk factors increase meiotic nondisjunction.

Prenatal detection

Multiple screening methods have been developed to enable early diagnosis of Down syndrome. In The Netherlands, the combined screening test is used at 11-14 weeks gestational age.[34] In addition to maternal age, the gestational age is taken into account in this test because the risk of Down syndrome decreases as a result of more spontaneous abortions in early pregnancy.[29] Pregnancy-associated plasma protein A (PAPP-A) and beta human chorionic gonadotrophin (β-hCG) are proteins that are produced by the placenta. These values are measured in maternal plasma because lower PAPP-A and higher β-hCG levels are related to Down syndrome.[35] However, it is unclear why these values are altered. At last, the nuchal translucency is measured by ultrasound because higher measurements are found in Down syndrome, possibly caused by abnormal collagen deposition.[36, 37] The results of these variables are analysed using a complex algorithm to estimate the risk of the child having Down syndrome. In case an increased risk is identified, confirmation is possible through karyotyping upon chorionic villus sampling or amniocentesis.

Recently, a non-invasive prenatal test has been developed that allows detection of Down syndrome in the first trimester of pregnancy by analysis of foetal DNA in maternal blood.[38-40] At this moment, this test is only available to women who have an increased risk of having a child with Down syndrome. In absence of an early prenatal diagnosis, Down syndrome can be suspected later during pregnancy when congenital abnormalities are visible on ultrasound studies. However, the specificity of most congenital abnormalities for Down syndrome is low.[41]

In The Netherlands, up to 12.8% of pregnancies are terminated when a diagnosis of Down syndrome is made,[24] but these rates may vary between 0 and 75% in other countries (Figure 3A).[42, 43] Mothers aged >36 years who were reimbursed for the combined screening test by the health insurance showed both a higher uptake of this screening test as well as an increased likelihood to subsequently terminate the pregnancy.[44] From 2015 onwards reimbursement for the combined screening test will stop. How this will affect the screening uptake as well as the number of live born children with Down syndrome in The Netherlands is unclear.

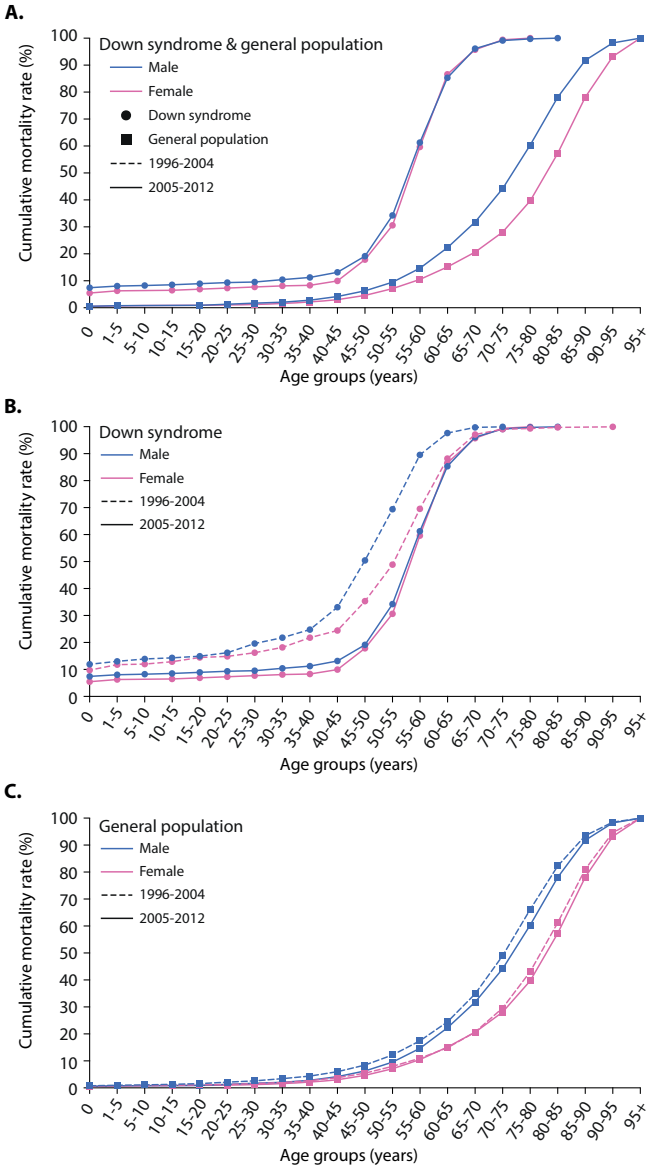


Figure 4. Life expectancy in individuals with Down syndrome.
The age distribution of mortality in individuals where Down syndrome has been noted as the primary cause of death is shown in blue for men and pink for women. Data are shown as the cumulative relative contribution of age groups to mortality. **A.** Life expectancy of individuals with Down syndrome is decreased compared to the general population (2005-2012). **B.** Life expectancy has further improved in 2005-2012 compared to 1996-2004 in individuals with Down syndrome, this improvement has not been observed as much in the general population (**C**). Down syndrome and general population are depicted in circles and squares, respectively. Men and women are shown in blue and pink, respectively. Dashed and solid lines represent 1996-2004 and 2005-2012, respectively. Data are obtained from Statistics Netherlands.[50]

Life expectancy

In the last fifty years, the life expectancy of individuals with Down syndrome has improved significantly. However, it remains limited as compared to the general population (Figure 4).[45, 46] One of the main reasons is the increased mortality in infants, and especially of those who are suffering from congenital heart disease.[7, 47] As a result of improved care for congenital heart disease in general, a decline in mortality was observed in children with Down syndrome as well.[45] In Sweden for example, infant mortality in Down syndrome decreased from 14.2% in 1973-1980 to 2.3% in 1995-1998.[47] Data from Statistics Netherlands confirm the decreasing trend. In 2003 the reported mortality in infants with Down syndrome was 4%.[7] In adults with Down syndrome a major improvement has been obtained in survival. Mean age of individuals with Down syndrome was 26 years in 1983, which has increased to approximately 60 years nowadays.[45, 48] It is unclear whether this is only caused by improved medical care. Strikingly, where in the general population women tend to get older than men, this difference was not found in Down syndrome (Figure 3B and C).[49, 50]

Thus, despite generally lower birth rates and the introduction of prenatal diagnostic options with possible subsequent termination of pregnancy, the absolute number of children born with Down syndrome in The Netherlands has been stable due to increased maternal age. Further improvement of the medical and social care for these children and adults is therefore important.

PART B: IMMUNOLOGICAL DISEASES IN DOWN SYNDROME

Malignancies of immune cells

Children with Down syndrome are more likely to develop acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML), which are characterized by clonal expansion in the bone marrow of a lymphocyte or myeloid precursor subset, respectively. ALL is the most common malignancy in childhood and affects approximately 3 in 100,000 children with a peak incidence of 7.6/100,000 children at the age of 2-3 years.[51-53] In children with Down syndrome the risk for ALL is ~24 times increased.[54, 55] In The Netherlands, every year 100-120 children are diagnosed with ALL of whom 4-5 children have Down syndrome (Figure 5).[56] Although the prognosis for ALL is very good (i.e. five-year-survival), individual prognosis may vary between 75% and >95% and is depending on risk stratification, based on treatment response and genetic characteristics of the leukaemia clone.[57] Children with Down syndrome show other cytogenetic leukaemia profiles (i.e. more *IKZF1* mutations) and higher treatment related toxicity that is related to poor prognosis in terms of relapse rate as well as survival in both children with and without Down syndrome.[58-61] Infant leukaemia, meaning leukaemia in young children up to 1 years of age with *mixed lineage leukaemia* (MLL) gene rearrangement,[62] is rarely found in children with Down syndrome.[58] This is confirmed by data from the Dutch Childhood Oncology Group (Figure 5).[56]

In the general population, AML is less common than ALL with an incidence rate of 0.5 in 100,000 children.[52, 53] Each year ~25 children are diagnosed in The Netherlands of whom 2-3 children have Down syndrome (Figure 5).[56] Therefore, children with Down syndrome are 20 times more at risk to develop AML compared to children without Down syndrome.[54, 55] AML is categorized in different subtypes according to the *World Health Organization (WHO) Classification of Tumours of the Hematopoietic and Lymphoid Tissues*.[63] In Down syndrome especially acute megakaryoblastic leukaemia is found. Because this malignancy is different in children with Down syndrome compared to children without Down syndrome, this type of AML is classified as "Myeloid leukaemia associated with Down syndrome".[63] The prognosis of AML is generally better compared to children without Down syndrome.[64] The relapse rate is 12% in Down syndrome versus 30-40% in non-Down syndrome and their overall survival rate 79% and 60-65%, respectively.[64, 65]

Myelodysplastic syndrome (MDS) is a rare heterogeneous group of disorders characterized by abnormal cell morphology and cell numbers of blood and/or bone marrow components.[66] The incidence of MDS is 3.2 per 1,000,000 children, and of those approximately 15-25% have Down syndrome.[67, 68] Because of the similarities to

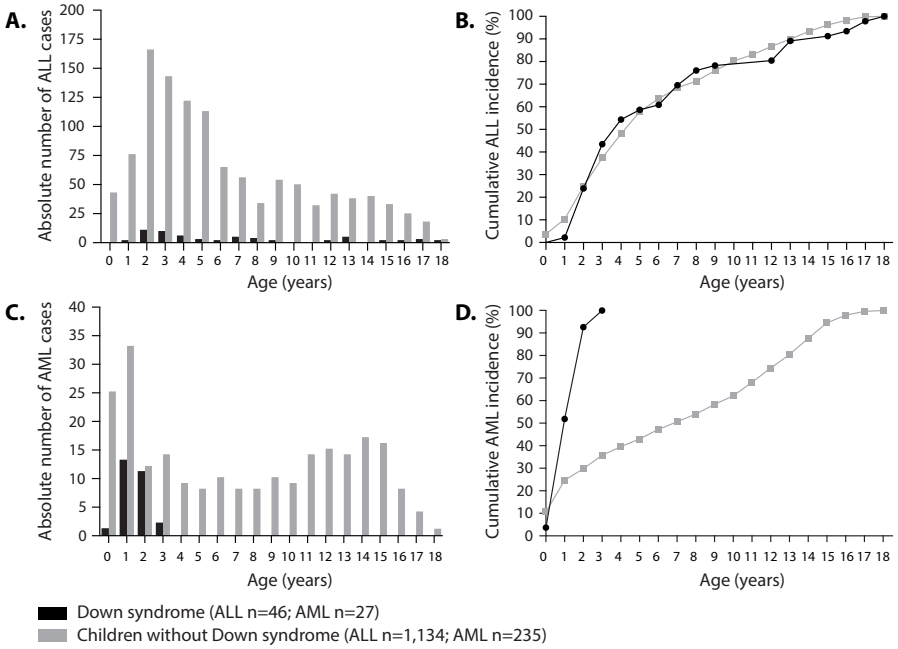


Figure 5. Leukaemia in Down syndrome
Absolute numbers of children with and without Down syndrome diagnosed with acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) in the Netherlands between 2002 and 2011 (**panel A and C**). The cumulative incidence rate illustrates the relative contribution of age on the incidence of ALL and AML (**panel B and D**). The data shown in this figure were kindly provided by the Dutch Childhood Oncology Group (DCOG) Trial and Data Centre.[56]

AML, MDS in Down syndrome is classified as "Myeloid leukaemia associated with Down syndrome" and treated accordingly.[63, 69]

In addition to the above-described malignancies, transient myeloproliferative disorder (TMD) is another megakaryoblastic proliferative disease with variable clinical signs that has been found uniquely in Down syndrome and affects foetuses as well as newborns up to the age of 3 months. The exact incidence is not known but has been estimated to be 4-10%.[70] TMD regresses in most children within the first months of life.[71] However, chemotherapy may be required in patients with high white blood cell count, thrombocytopenia and/or liver dysfunction.[70] Approximately 20% of children with a history of TMD that had regressed in peripheral blood, developed AML later in life.[71] Cytogenetic analysis of Down syndrome leukaemia has shown *GATA1* mutations in AML and TMD but not in ALL or MDS.[72, 73]

Thus, children with Down syndrome not only show a unique proliferative disease in the form of TMD, they are more at risk to develop ALL and AML in a Down syndrome specific pattern. However, individuals with Down syndrome are not at increased risk to develop lymphomas and solid tumours.[54, 55, 74-76]

Autoimmune diseases

Children as well as adults with Down syndrome are more at risk to develop autoimmune diseases, which mainly includes autoimmune hypothyroidism, celiac disease and type 1 diabetes mellitus. For Crohn's disease, ulcerative colitis and psoriasis however, the risk is comparable to the general population.[55]

Hypothyroidism is common in Down syndrome.[55, 77] Newborns show a 30-fold increased risk of congenital hypothyroidism.[78] Later in life, up to 50% of all older children as well as adults show a decline in thyroid function.[79-81] However, treatment is not always required. Besides structural differences of the thyroid gland,[82] Hashimoto's thyroiditis, which is accompanied by the production of autoantibodies (i.e. thyroid peroxidase (TPO) antibodies), is an important cause of hypothyroidism in Down syndrome.[80]

It has been shown that individuals with Down syndrome have a 6 to 10 fold increased risk to develop celiac disease for which routine laboratory screening has been advised.[83-86] However, if patients are negative for both human leukocyte antigen (HLA)-DQ2 and HLA-DQ8, the chance of developing celiac disease is very low and routine screening is not indicated.[84] Finally, there is a threefold increase in the likelihood for individuals with Down syndrome to develop type 1 diabetes mellitus.[87, 88]

Thus, autoimmunity is more common in children and adults with Down syndrome; this is especially true for thyroid disease, celiac disease and type 1 diabetes mellitus.

Respiratory tract infections

Children with Down syndrome suffer more bacterial and viral respiratory tract infections that cause significant morbidity and mortality. In young infants with Down syndrome, the risk for hospitalization due to respiratory syncytial virus (RSV) infection is increased as compared to their siblings.[89] Moreover, up to 80% of paediatric intensive care unit admissions of children with Down syndrome is caused by lower respiratory tract infections.[90] The risk for death due to respiratory tract infections is increased in Down syndrome and almost one third of all deaths are related to pneumonia, influenza infection and/or aspiration.[91, 92] Although respiratory tract infections in children with Down syndrome are often accompanied by wheezing, there is no increased risk to develop asthma.[55, 93-97]

Many causes for recurrent respiratory tract infections have been described in Down syndrome, but it is unknown how they contribute individually to the number and severity of these infections. First, there are multiple anatomical abnormalities of the upper and lower respiratory tract that include macroglossia,

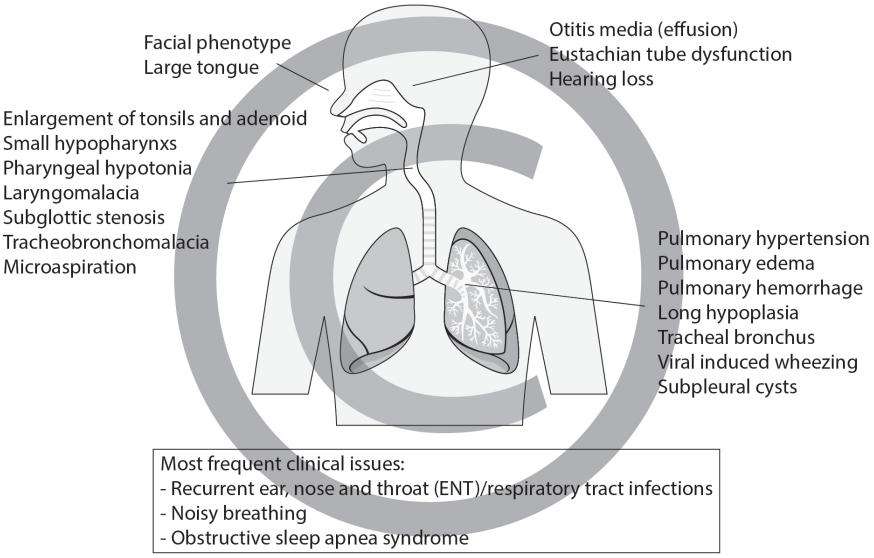


Figure 6. Diseases of the respiratory tract in Down syndrome.
Overview of common abnormalities in the respiratory tract in Down syndrome.[11, 12, 14, 96-98, 168-171]

midface hypoplasia, narrow nasopharynx and trachea, tracheal bronchus as well as laryngo- and tracheomalacia (Figure 6).[98] Second, physiological differences of the respiratory tract include decreased ciliary beat frequency and increased production of mucus.[98, 99] Third, children with Down syndrome suffer from hypotonia especially in the first years of life, which leads to insufficient mucus clearing by coughing and enhances the abnormalities of the respiratory tract.[96, 98] Fourth, congenital heart diseases have been proposed as an attributable risk factor in the development of respiratory tract infections.[100-102] And last, gastroesophageal reflux may cause chronic microaspiration leading to pneumonia.[103]

It is clear that respiratory tract infections are a multifactorial problem in children with Down syndrome that is associated with high morbidity as well as mortality.

Thus, throughout their lives, individuals with Down syndrome are more likely to suffer from recurrent respiratory tract infections, haematological malignancies and autoimmune diseases. The combination of these diseases is suggestive for impaired immunity and has led to many immunological studies. Until now, attention has been mainly focussed on the development and function of T cells, but the clinical phenotype of Down syndrome is also reminiscent of impaired humoral immunity. For this reason, we have studied the B-cell compartment in children with Down syndrome.

PART C: B-CELL DEVELOPMENT AND ANTIBODY RESPONSES

The immune response

The human body is exposed each day to many different pathogens such as bacteria and viruses. The skin and mucosal surfaces of the respiratory tract and gut serve as the first physical and chemical barrier to prevent invasion by these microorganisms. Once invasion does occur, the immune system will first try to eliminate the intruder by innate immune responses. Its proteins (e.g. complement) and immune cells (e.g. neutrophils, macrophages, natural killer cells) have the ability to produce a fast but non-specific response against pathogens. Finally, in the following 3-6 days, B and T cells will provide an antigen specific response. These lymphoid cells recognize pathogens with their antigen specific receptors, generate long-term immunological memory, and are able to improve their response upon repetitive exposition.

Antigen independent development of B and T cells

All cellular components of the immune system originate from the bone marrow where they develop in an antigenic independent manner from haematopoietic stem cells under the tight regulation of growth factors. Haematopoietic stem cells can either differentiate into common myeloid or lymphoid progenitors.[104, 105] Common myeloid progenitors give rise to erythrocytes, platelets and many cellular components of the innate immune system that include monocytes, macrophages and neutrophils. In contrast, B, T and NK cells develop from common lymphoid progenitors (Figure 7). B and NK cells fully develop in the bone marrow and enter the bloodstream as transitional B cells and functional NK cells, respectively. T cells however, will partially develop in the bone marrow and then migrate to the thymus to undergo further development into naive T cells.

The B- and T-cell antigen receptors

The B-cell antigen receptor (BCR) consists of two identical heavy and light chains. The heavy chain consists of one variable domain and 3 or 4 constant domains, whereas the light chain consists of one variable and one constant domain (Figure 8A). The variable regions of the heavy and light chain form a pocket, or antigen-binding site, which allows antigen recognition. As a result, each BCR contains two antigen-binding sites. The T-cell receptor (TCR) consists of two chains that each consist of one variable and one constant domain. The variable regions form one antigen-binding site. Most TCRs consist of an α and β chain (~85%), but a minority has a configuration with a γ and δ chain (~5%; Figure 8B).[106]

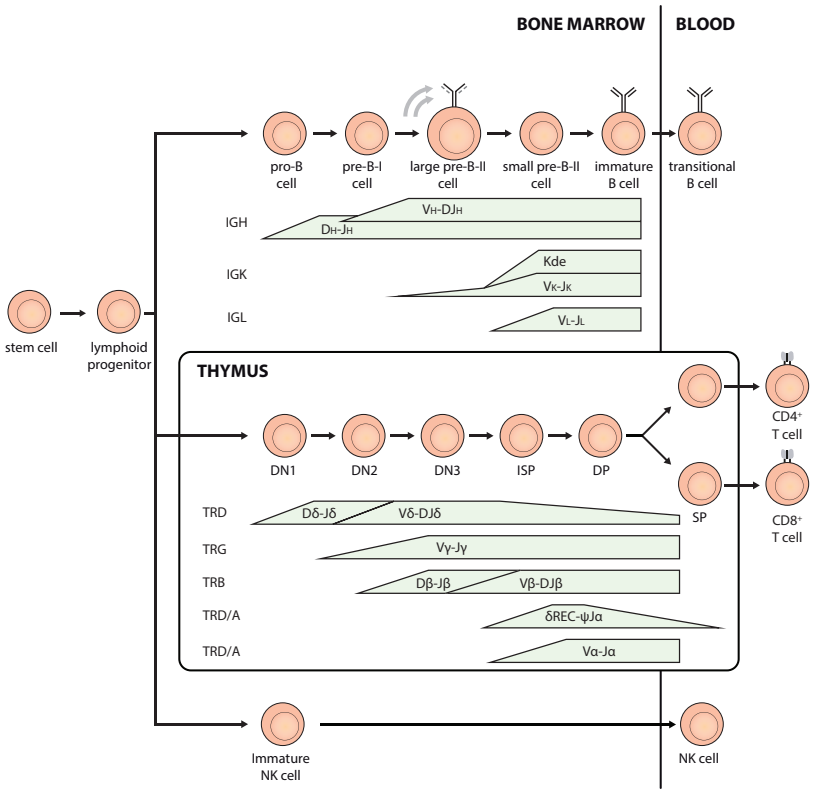


Figure 7. Development of the lymphoid compartment in bone marrow and thymus.
Whereas naive B cells and NK cells fully develop in the bone marrow, T cells migrate to the thymus for further development. V(D)J recombination in applicable loci are shown under the corresponding developmental stage. DN, double negative; ISP, immature single positive; DP, double positive; SP, single positive. Figure adapted from Van Zelm et al.[112] and Dik et al.[172]

A combination of one of the variable (V), diversity (D) and joining (J) genes on the *IGH* locus and a combination of V and J genes on the *IGK* or *IGL* locus codes the variable region of the Ig heavy and light chain, respectively (Figure 8C). In T cells, V, D and J genes on the *TRB* and *TRD* loci are recombined to code for the β and δ chain, respectively. The α and γ chain are coded by *TRA* and *TRG*, respectively. These loci contain V and J genes that are recombined to code for the variable region.

There are 9 constant genes that encode the constant region of the heavy chain and determine the function of the secretory immunoglobulin that is produced (i.e. IgM, IgG and IgA). For example, IgA and IgM have their main effector function at mucosal surfaces and in the bloodstream, respectively. The Ig light chain has a similar composition to the heavy chain. However, the constant region of the light chain has no influence on the functionality of the receptor and can be of the Kappa or Lambda isotype.

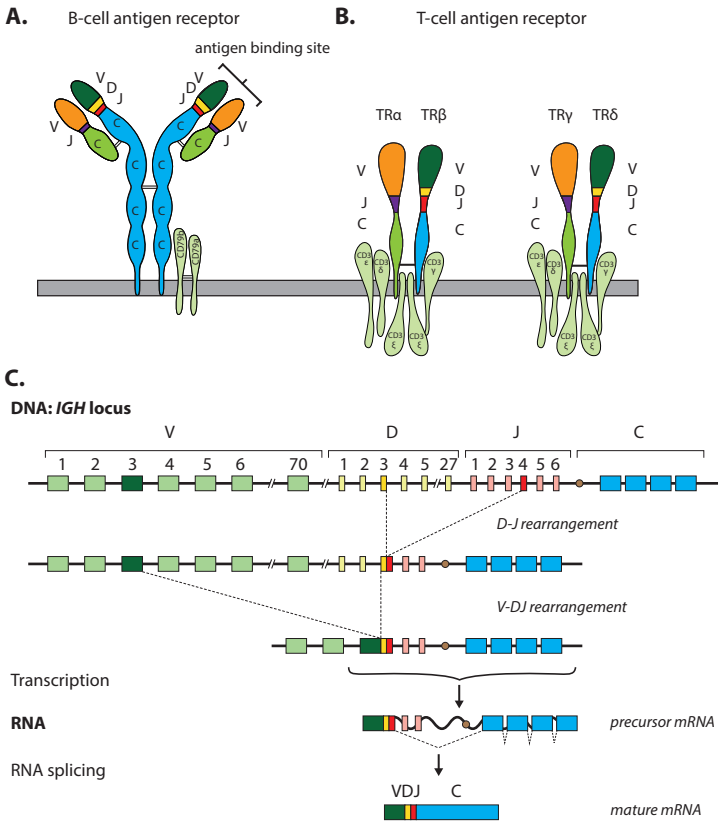


Figure 8. Antigen recognition receptors and V(D)J recombination
A. B-cell antigen receptor of IgM isotype is expressed with CD79A/B. The membrane bound molecule consists of two heavy and two light chains. **B.** T-cell antigen receptors consist of either an α and β chain or a γ and δ chain and are expressed by the CD3 complex. **C.** V(D)J recombination of the IGH locus combines a V, D and J gene that code for the variable region. D to J recombination is followed by V to DJ recombination.

V(D)J recombination and excision circle formation

Each B and T cell contains a unique receptor that allows recognition of a specific antigen. Therefore, a high diversity of receptors (>10^{15}) is provided to ensure wide enough coverage of pathogens.[107] V(D)J recombination is responsible for generation of the receptor repertoire by combining different V, D and J genes (Figure 8C).[108, 109] This process is highly regulated and occurs in a specific consecutive order. In B cells, first DH to JH rearrangement occurs in both *IGH* alleles (pre-pro-B cell stage), which is followed by VH to DJH rearrangement in one of the alleles (pro-B cell stage).[110, 111] Subsequently, development will continue with Vκ to Jκ rearrangement in one *IGK* allele that will be tested for functionality. New rearrangements of Vκ and Jκ genes will be created until a functional junction is formed. If functionality cannot

be obtained, further intron RSS to kappa-deleting element (Kde) rearrangement takes place, which will remove the Cκ of this allele from the genome and forms a Kappa-deleting excision circle (KREC; Figure 9). This rearrangement occurs in ~70% of all B cells.[112] Then, rearrangement of the second *IGK* allele takes place. If these rearrangements are also non-functional, rearrangement of the *IGL* allele will occur in an identical manner. Once a functional BCR is formed that does not show high affinity for auto-antigens, the B cell will further mature and enter the bloodstream as a transitional B cell.

During T-cell development, first rearrangements occur in the *TRD* locus. Very frequently, no functional TCR will develop and excision of the *TRD* locus will occur. Analogue to the development of the KREC in the development of the BCR, a T-cell excision circle (TREC) is formed with a δRec-ψJα signal joint (Figure 9). As a result, almost all T cells that enter the bloodstream have a TREC.[113, 114]

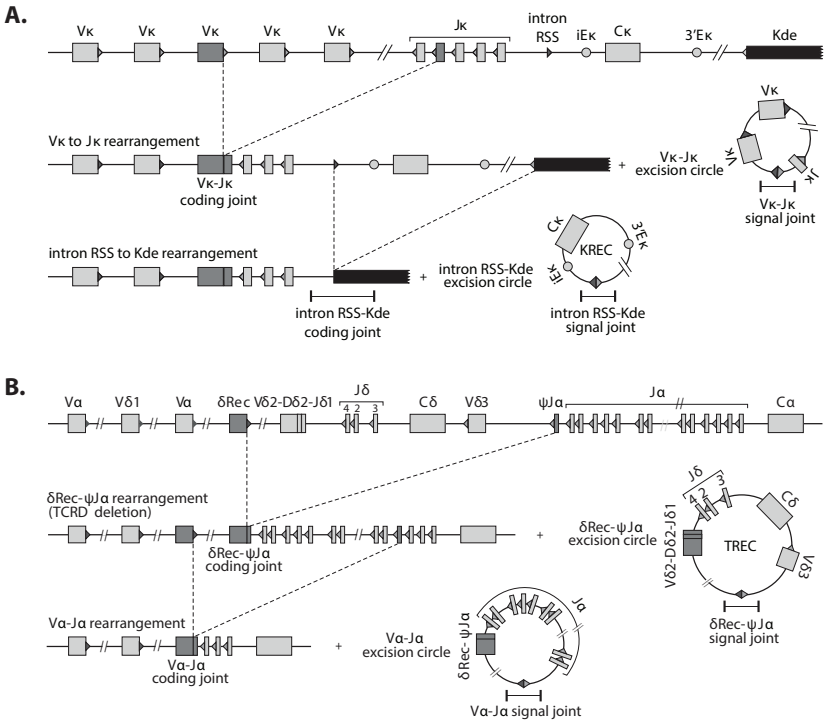


Figure 9. Formation of KRECs and TRECs.
The formation of excision circles occurs when DNA is excised from the genome during V(D)J recombination. **A.** During intron RSS to Kappa deleting element (Kde) rearrangement in B cells a Kappa-deleting recombination excision circle (KREC) is formed. **B.** V(D)J recombination in TCRD and TCRG are often not functional. To enable TRA rearrangement, δRec to ψJα rearrangement will occur first to delete the TCRD locus from the genome. During this rearrangement, a T-cell receptor excision circle (TREC) is formed. This figure is adapted from Van Zelm et al.[113]

B-cell survival

As naive B cells recirculate, their survival and homeostasis is tightly regulated by external signals such as B-cell activating factor (BAFF).[115] BAFF is an important signal that interacts with the BAFF-receptor (BAFFR). Studies in mice and mutations in the human BAFFR have demonstrated that deficient BAFF-BAFFR signalling results in decreased naive mature B cells.[116-118] Besides survival, limited homeostasis takes place in the naive compartment that consists of ±2 cell divisions.[119] Macrophage migration inhibitory factor (MIF) induces proliferation by CD74-CD44 interaction and is produced by dendritic cells in bone marrow.[120]

B-cell activation

B cells exit the bone marrow as transitional B cells, which are functionally immature and do not yet respond to antigen stimulation until they mature into naive mature B cells. In order to provide an adequate immune response, B cells need to be activated by at least two signals (Figure 10). Antigen binding to the BCR-CD79-complex serves as the primary activation signal. However, the BCR signalling depends on the CD19-complex that consists of CD19, CD21, CD81 and CD225.[121-123]

Secondary signals can come from follicular helper T cells (TFH) that reside in germinal centers. B cells will process the antigen recognized and present it through an MHC class II molecule. Upon recognition by the TFH, multiple receptors are upregulated that bind to their ligands on the B cell. The most important signal comes from CD40L, but other co-stimulatory signals come from receptors such as ICOS (inducible T-cell costimulator), CTLA-4 (cytotoxic T lymphocyte-associated 4) and PD-1 (programmed cell death 1) as well as soluble factors such as IL21. Upon activation, intracellular signalling is followed by nuclear translocation of NF-κB to

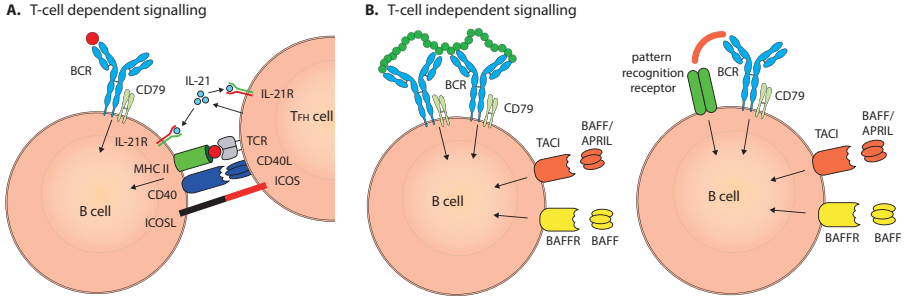


Figure 10. B-cell activation by T-cell dependent and independent stimuli.
A. B-cell activation is induced by antigen recognition by the B-cell receptor (BCR) and T-cell dependent co-stimulatory signalling through CD40-CD40L and ICOS-ICOSL interaction as well as IL-21 signalling.
B. T-cell independent responses can occur by BCR cross-linking or BCR stimulation in conjunction with stimulation of pattern recognition molecules (e.g. Toll-like receptors, TLR).

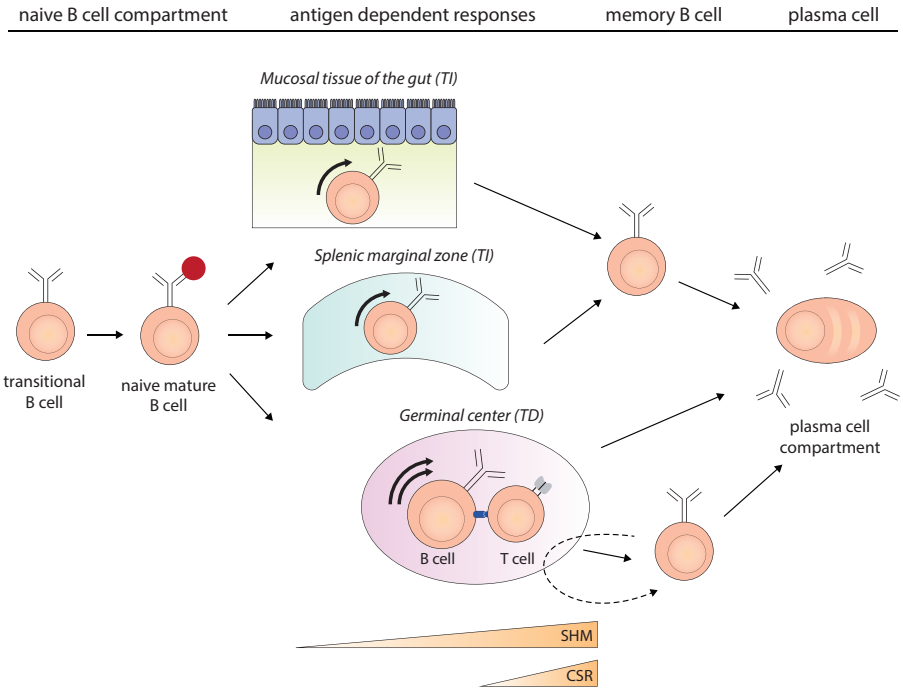


Figure 11. Antigen driven responses of B cells.
Upon activation of B cells, T-cell dependent responses will occur in the germinal centres. T-cell independent responses take place in the mucosal layer of the intestines and marginal zone of the spleen. TI, T-cell independent; TD, T-cell dependent, SHM, somatic hypermutation; CSR, class switch recombination.

enhance the transcription of pro-inflammatory genes. In case T-cell help is present, B cells start a germinal center reaction where they undergo extensive proliferation, affinity maturation and Ig class switch recombination of the BCR (Figure 11). These processes improve the binding affinity to the antigen and change the effector function of the antibody. Simultaneously, B cells will develop into memory B cells as well as plasma cells.

Extrafollicular reactions take place in absence of T-cell help in the spleen or gut-associated lymphoid tissues (GALT; Figure 10). In these instances, the additional signals that are required for B-cell activation can come from BCR crosslinking or pattern recognition receptor signalling (i.e. Toll-like receptor, TLR; Figure 11). In addition, BAFF-BAFFR and BAFF/APRIL-TACI interactions stimulate the antigen driven response.[124-126]

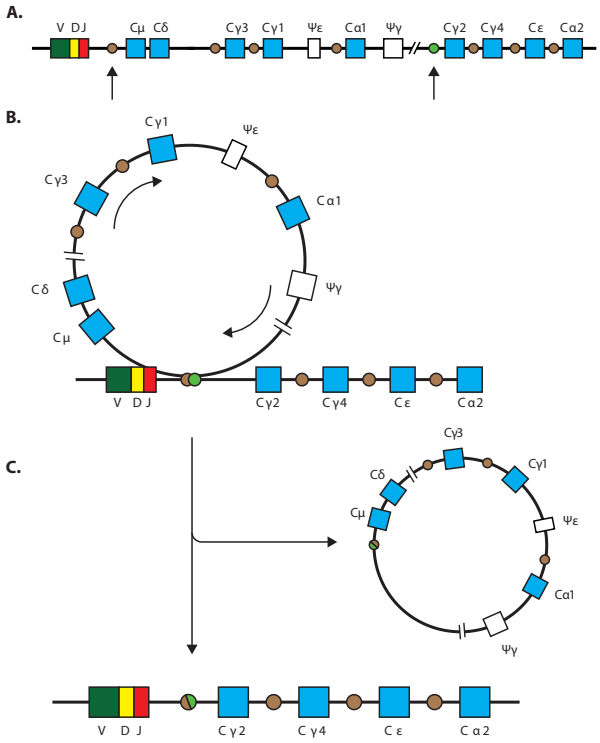


Figure 12. Class switch recombination (CSR).
A. The IGH locus contains 9 immunoglobulin constant genes that code for the immunoglobulin heavy chain (Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2). Arrows indicate the IgM and IgG2 switch regions, respectively. **B.** Switch regions in the proximity to constant genes on the IGH locus contain a high density of activation-induced cytidine deaminase (AID) hotspots, which become accessible under the influence of specific cytokines. **C.** Double stranded breaks are initiated and the intervening DNA is excised, resulting in an excision circle and CSR to IgG2. The involved switch regions merge, resulting in hybrid switch regions.

Affinity maturation and class switch recombination

Affinity maturation consists of induction of somatic hypermutations (SHM) in the variable Ig gene by activation-induced cytidine deaminase (AID) and subsequent selection for SHMs that improve binding affinity of the BCR.[127] These clones will proliferate further and undergo additional SHM.

During an immune response, the Ig heavy chain isotype changes from Igμ to one of the more distal isotypes that is responsible for changing the function of the excreted immunoglobulins (Figure 12).[127] On the *IGH* locus multiple constant genes are present that encode for the constant region of the heavy chain. Each constant gene has an Ig switch region upstream that contains multiple AID hotspot motifs. Due to the high density of AID hotspots, a double stranded DNA break can be induced at multiple sites. And if these breaks occur at the Cμ gene and a downstream

constant gene, the intervening DNA will be excised and rearrangement will occur. In contrast to V(D)J recombination, CSR is not a random process but is regulated by cytokine signalling that affects the accessibility of AID to particular IGHC switch regions. For example, IL21 induces CSR towards IgG3, IgG1 and IgA1,[128, 129] whereas IL4 and IL13 promote downstream IgG4 and IgE class switching.[130, 131]

D. CLINICAL AND DIAGNOSTIC ASPECTS OF IMPAIRED B-CELL DEVELOPMENT AND FUNCTION

Primary immunodeficiencies

A primary immunodeficiency (PID) is a disease that is characterized by insufficient function of one or multiple components of the immune system. As a consequence PID patients suffer from an increased infection rate. In addition, other non-infectious features can be present at diagnosis or develop throughout life. PIDs can be classified based upon the affected components and specific genetic mutations.[132] The European Society for Immunodeficiencies (ESID) has developed an online registry for patients with PIDs (www.esid.org). Over 19,000 patients have been registered between 2004 and 2014 of whom 56% have a predominantly antibody deficiency (Figure 13). The spectrum of B-cell disorders is diverse and varies from complete agammaglobulinemia to the inability to produce specific antibodies.

In patients with clinical problems due to an antibody deficiency, treatment consists of antibacterial prophylaxis and/or immunoglobulin substitution. However, non-infectious complications may warrant specific treatment as well.

Antibody deficiencies

X-linked agammaglobulinemia (XLA) is characterized by a complete absence of peripheral B cells caused by a mutation in the X-linked Bruton's tyrosine kinase (BTK) gene that results in a developmental block at the pre-B to immature B-cell

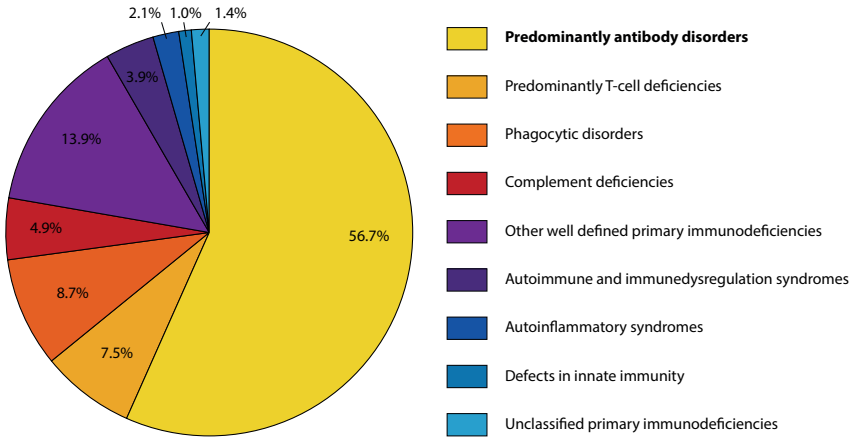


Figure 13. Distribution of primary immunodeficiencies (PIDs) in Europe.
Overview of 19,355 patients registered in the online registry of the European Society for Immunodeficiencies (ESID),[173] updated to June 25th 2014. The majority of patients have an antibody disorder.

stage.[133-135] Since patients with XLA lack B cells, they are not able to develop plasma cells and thus cannot produce immunoglobulins. This causes a high susceptibility to – mainly respiratory tract – infections. Being X-linked, XLA is most prevalent. Still, other autosomal recessive causes of agammaglobulinemia as a result of absent B cells have been identified and include mutations in genes encoding components of the pre-BCR and downstream signalling proteins such as CD79A/B, λ5, IGHM and BLNK.[136-141]

Patients with a defect in B-cell activation present with hypo- or agammaglobulinemia but have normal or low B cells. As mentioned, B-cell activation requires two signals. The primary signal is initiated by antigen recognition through the BCR. Because this signal depends on the CD19 complex, mutations in CD19, CD21 and CD81 result in strongly reduced serum IgG despite normal B-cell numbers.[121-123]

Defects in co-stimulatory signalling have been described extensively. T-cell dependent co-stimulation consists of multiple cell bound molecules as well as soluble factors. Mutations in CD40, CD40L and AID result in Ig class switch defects that are characterized by absent IgG, impaired affinity maturation and absence of germinal centers.[142-145] Patients with ICOS mutations also have antibody deficiency and impaired germinal center formation.[146] However, CSR is not strictly depending on ICOS and patients can generate IgG in small amounts.[146] Therefore, clinical features become apparent much later in life as compared to the other Ig class switch defects.

Common Variable Immunodeficiency Disorders (CVID) is a heterogeneous group of diseases that is characterized by a decreased level of IgG and IgA with or without low IgM.[147] In addition, these patients have poor antibody responses to vaccines and/or absent isohemagglutinins or decreased switched memory B cells. All other defined causes of hypogammaglobulinemia must be excluded.[147] The clinical phenotype of patients with CVID is highly variable. Most patients will present with infectious diseases and/or complications (i.e. bronchiectasis) but autoimmune phenomena and malignancies are present at higher frequencies as well.[148]

In the last decade, several mutations have been identified in patients that were diagnosed as CVID. These patients illustrate how different cellular processes result to a CVID phenotype (i.e. ICOS and CD21 deficiency).[123, 146] At this moment >90% of patients do not have a known mutation but it can be expected that new mutations will be found that will further unravel the pathophysiology of CVID.

Many PIDs result in decreased total serum IgG but deficiencies of specific IgG can be diagnosed as well. Patients with isolated IgG subclass deficiency have decreased serum levels of IgG2, IgG3 and/or IgG4. Because IgG1 comprises approximately two-third of the total IgG, a deficiency in this subclass will always lead to hypogammaglobulinemia and is not included in this category. Most patients are asymptomatic, but when symptoms occur these consist of recurrent infections and are especially found in IgG2 deficiency.[149-151] Some patients have decreased responses to polysaccharide antigens.[152, 153] There are no known genetic mutations that result in IgG subclass deficiency.

In specific antibody deficiency (SPAD), patients have insufficient responses to polysaccharide antigens but normal responses to protein antigens.[154] Moreover, they show normal serum Ig levels. The pathogenesis of SPAD is not understood.

Selective IgA deficiency is the most common antibody deficiency with a prevalence of 1 in 600 persons.[155] The pathogenic mechanisms and underlying molecular processes have not been elucidated. Although most individuals are asymptomatic, there are higher incidences of respiratory tract infections, allergy and autoimmune diseases reported in selective IgA deficiency.[156] Moreover, some selective IgA deficient patients may develop CVID later in life.[157, 158]

Diagnostics of B-cell disorders

Patients suspected for an antibody deficiency are evaluated in a stepwise manner.[159] The order of studies depends on the result of preceding studies. In general, Ig serum levels will be assessed first to determine whether patients lack one or multiple immunoglobulin isotypes. Depending on the results, the functionality of antigen responses can be studied by vaccination studies. In these studies, patients are immunized by vaccines that contain protein or polysaccharide antigens and specific antibodies are determined after 4-6 weeks. Moreover, the blood B-cell compartment can be studied by flow cytometry.[160, 161] Sometimes, a bone marrow biopsy will be taken as well. These studies often allow patients to be classified within a disease group. Moreover, the results may allow focus to specific functional assays or genetic studies in order to come to a precise diagnosis.

Neonatal screening for B- and T-cell defects

In many Western countries, neonatal screening programs are implemented to detect a wide spectrum of serious inherited diseases that offer improved outcomes if an early diagnosis is made.[162] These tests are performed on dried blood spots that are collected 3-5 days after birth on a Guthrie-card.

In the last decade, analysis of TRECs and KRECs in dried blood spots has been developed to detect children with PID.[163, 164] Severe Combined Immune Deficiency (SCID) is a heterogeneous disease that is related to very high mortality in the first year of life due to opportunistic infections when not diagnosed and treated in time. Almost all patients have low numbers of T cells, which can be accompanied by decreased numbers of B and NK cells as well. Therefore, a screening assay has been developed to determine the number of TRECs present in the dried blood spots as a means to estimate the number of T cells present.

More recently, screening of KRECs in neonates has been applied to detect XLA.[165] This disease is associated with serious increased respiratory tract infections that can result in long-term lung damage. Since these complications can be prevented by immunoglobulin substitution it is desirable to make a diagnosis as soon as possible after birth.

AIMS AND OUTLINE OF THIS THESIS

Recurrent respiratory tract infections are a major health issue in children with Down syndrome. Still, there is a lack of information regarding the causes as well as consequences of these infections. Therefore, we designed a study to increase knowledge on the epidemiology of respiratory symptoms in children up to 18 years of age (**Chapter 2**). In **Chapter 3**, the correlation between recurrent respiratory tract infections and development, behaviour and health related quality of life is described.

In the 1970s the hypothesis has been formulated that Down syndrome resembles an immunodeficiency syndrome based on the increased number of respiratory tract infections, autoimmune diseases and haematological malignancies. In **Chapter 4** an overview is given of the immunological studies that have been performed in Down syndrome. Since there has been limited attention for B cells and humoral immunity in Down syndrome, we have focussed on this topic. **Chapter 5** presents the composition of the peripheral B-cell compartment in relation to age, respiratory tract infections and immunoglobulin values of children with Down syndrome. The B-cell response in blood and tonsils is studied in detail and described in **Chapter 6**. **Chapter 7** focuses on apoptosis of blood lymphocytes as an underlying mechanism of reduced B-cell numbers in Down syndrome. In **Chapter 8** neonatal B- and T-cell numbers are studied using novel newborn screening tests for XLA and SCID based on KRECs and TRECs in a cohort of children with Down syndrome.

Finally, the discussion in **Chapter 9** focuses on the impact of respiratory infections on children with Down syndrome and the pathophysiology of the impaired B-cell development, as well as its possible consequences on the diagnostics and treatment of respiratory tract infections in children with Down syndrome.

REFERENCES

1. Down JL. Observations on an ethnic classification of idiots. 1866. Ment Retard 1995; 33:54-6.
2. Starbuck JM. On the Antiquity of Trisomy 21: Moving Towards a Quantitative Diagnosis of Down Syndrome in Historic Material Culture. Journal of Contemporary Anthropology 2011; 2:17-44.
3. Lejeune J, Gautier M, Turpin R. [Study of somatic chromosomes from 9 mongoloid children]. C R Hebd Seances Acad Sci 1959; 248:1721-2.
4. Petersen MB, Mikkelsen M. Nondisjunction in trisomy 21: origin and mechanisms. Cytogenet Cell Genet 2000; 91:199-203.
5. Hassold T, Sherman S. Down syndrome: genetic recombination and the origin of the extra chromosome 21. Clin Genet 2000; 57:95-100.
6. Weijerman ME, van Furth AM, van der Mooren MD, van Weissenbruch MM, Rammeloo L, Broers CJ, et al. Prevalence of congenital heart defects and persistent pulmonary hypertension of the neonate with Down syndrome. Eur J Pediatr 2010; 169:1195-9.
7. Weijerman ME, van Furth AM, Vonk Noordegraaf A, van Wouwe JP, Broers CJ, Gemke RJ. Prevalence, neonatal characteristics, and first-year mortality of Down syndrome: a national study. J Pediatr 2008; 152:15-9.
8. Freeman SB, Torfs CP, Romitti PA, Royle MH, Druschel C, Hobbs CA, et al. Congenital gastrointestinal defects in Down syndrome: a report from the Atlanta and National Down Syndrome Projects. Clin Genet 2009; 75:180-4.
9. Spender Q, Stein A, Dennis J, Reilly S, Percy E, Cave D. An exploration of feeding difficulties in children with Down syndrome. Dev Med Child Neurol 1996; 38:681-94.
10. Cunningham C. Down Syndrome: An Introduction for Parents and Carers: Souvenir Press Limited; 2006.
11. Shott SR. Down syndrome: common otolaryngologic manifestations. Am J Med Genet C Semin Med Genet 2006; 142C:131-40.
12. Rodman R, Pine HS. The otolaryngologist's approach to the patient with Down syndrome. Otolaryngol Clin North Am 2012; 45:599-629, vii-viii.
13. Thomas K, Bourke J, Girdler S, Bebbington A, Jacoby P, Leonard H. Variation over time in medical conditions and health service utilization of children with Down syndrome. J Pediatr 2011; 158:194-200 e1.
14. Shott SR, Joseph A, Heithaus D. Hearing loss in children with Down syndrome. Int J Pediatr Otorhinolaryngol 2001; 61:199-205.
15. Rosen D. Management of obstructive sleep apnea associated with Down syndrome and other craniofacial dysmorphologies. Curr Opin Pulm Med 2011; 17:431-6.
16. Concolino D, Pasquzzi A, Capalbo G, Sinopoli S, Strisciuglio P. Early detection of podiatric anomalies in children with Down syndrome. Acta Paediatr 2006; 95:17-20.

17. Milbrandt TA, Johnston CE, 2nd. Down syndrome and scoliosis: a review of a 50-year experience at one institution. Spine (Phila Pa 1976) 2005; 30:2051-5.
18. Merrick J, Ezra E, Josef B, Hendel D, Steinberg DM, Wientroub S. Musculoskeletal problems in Down Syndrome European Paediatric Orthopaedic Society Survey: the Israeli sample. J Pediatr Orthop B 2000; 9:185-92.
19. Oliveira AC, Paiva SM, Campos MR, Czeresnia D. Factors associated with malocclusions in children and adolescents with Down syndrome. Am J Orthod Dentofacial Orthop 2008; 133:489 e1-8.
20. Sakellari D, Arapostathis KN, Konstantinidis A. Periodontal conditions and subgingival microflora in Down syndrome patients. A case-control study. J Clin Periodontol 2005; 32:684-90.
21. Van Gameren-Oosterom HB, Fekkes M, Reijneveld SA, Oudesluys-Murphy AM, Verkerk PH, Van Wouwe JP, et al. Practical and social skills of 16-19-year-olds with Down syndrome: independence still far away. Res Dev Disabil 2013; 34:4599-607.
22. Borstlap R, van Gameren-Oosterom HBM, Lincke C, Weijerman ME, van Wieringen H, van Wouwe JP. An update of the multidisciplinary guideline for the medical treatment of children with Down syndrome [Een update van de multidisciplinaire richtlijn voor de medische begeleiding van kinderen met Downsyndroom]. 2011.
23. Bull MJ, Committee on G. Health supervision for children with Down syndrome. Pediatrics 2011; 128:393-406.
24. Van Gameren-Oosterom HB, Buitendijk SE, Bilardo CM, van der Pal-de Bruin KM, Van Wouwe JP, Mohangoo AD. Unchanged prevalence of Down syndrome in the Netherlands: results from an 11-year nationwide birth cohort. Prenat Diagn 2012; 32:1035-40.
25. De Graaf G, Haveman M, Hochstenbach R, Engelen J, Gerssen-Schoorl K, Poddighe P, et al. Changes in yearly birth prevalence rates of children with Down syndrome in the period 1986-2007 in The Netherlands. J Intellect Disabil Res 2011; 55:462-73.
26. Carothers AD, Hecht CA, Hook EB. International variation in reported livebirth prevalence rates of Down syndrome, adjusted for maternal age. J Med Genet 1999; 36:386-93.
27. Penrose LS. The relative effects of paternal and maternal age in mongolism. Journal of Genetics 1933; 27:219-24.
28. Hassold T, Chiu D. Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. Hum Genet 1985; 70:11-7.
29. Snijders RJ, Sundberg K, Holzgreve W, Henry G, Nicolaides KH. Maternal age- and gestation-specific risk for trisomy 21. Ultrasound Obstet Gynecol 1999; 13:167-70.
30. Hildebrand E, Kallen B, Josefsson A, Gottvall T, Blomberg M. Maternal obesity and risk of Down syndrome in the offspring. Prenat Diagn 2013.
31. Hunter JE, Allen EG, Shin M, Bean LJ, Correa A, Druschel C, et al. The association of low socioeconomic status and the risk of having a child with Down syndrome: a report from the National Down Syndrome Project. Genet Med 2013; 15:698-705.

32. Yang M, Gong T, Lin X, Qi L, Guo Y, Cao Z, et al. Maternal gene polymorphisms involved in folate metabolism and the risk of having a Down syndrome offspring: a meta-analysis. Mutagenesis 2013; 28:661-71.
33. Boyle B, Morris J, McConkey R, Garne E, Loane M, Addor M, et al. Prevalence and risk of Down syndrome in monozygotic and dizygotic multiple pregnancies in Europe: implications for prenatal screening. BJOG 2014.
34. Wortelboer EJ, Koster MP, Stoutenbeek P, Elvers LH, Loeber JG, Visser GH, et al. First-trimester Down syndrome screening performance in the Dutch population; how to achieve further improvement? Prenat Diagn 2009; 29:588-92.
35. Brambati B, Tului L, Bonacchi I, Shrimanker K, Suzuki Y, Grudzinskas JG. Serum PAPP-A and free beta-hCG are first-trimester screening markers for Down syndrome. Prenat Diagn 1994; 14:1043-7.
36. Pandya PP, Brizot ML, Kuhn P, Snijders RJ, Nicolaides KH. First-trimester fetal nuchal translucency thickness and risk for trisomies. Obstet Gynecol 1994; 84:420-3.
37. Von Kaisenberg CS, Krenn V, Ludwig M, Nicolaides KH, Brand-Saberi B. Morphological classification of nuchal skin in human fetuses with trisomy 21, 18, and 13 at 12-18 weeks and in a trisomy 16 mouse. Anat Embryol (Berl) 1998; 197:105-24.
38. Song Y, Liu C, Qi H, Zhang Y, Bian X, Liu J. Noninvasive prenatal testing of fetal aneuploidies by massively parallel sequencing in a prospective Chinese population. Prenat Diagn 2013; 33:700-6.
39. Dhallan R, Guo X, Emche S, Damewood M, Bayliss P, Cronin M, et al. A non-invasive test for prenatal diagnosis based on fetal DNA present in maternal blood: a preliminary study. Lancet 2007; 369:474-81.
40. Norton ME, Brar H, Weiss J, Karimi A, Laurent LC, Caughey AB, et al. Non-Invasive Chromosomal Evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012; 207:137 e1-8.
41. Smith-Bindman R, Chu P, Goldberg JD. Second trimester prenatal ultrasound for the detection of pregnancies at increased risk of Down syndrome. Prenat Diagn 2007; 27:535-44.
42. Loane M, Morris JK, Addor MC, Arriola L, Budd J, Doray B, et al. Twenty-year trends in the prevalence of Down syndrome and other trisomies in Europe: impact of maternal age and prenatal screening. Eur J Hum Genet 2013; 21:27-33.
43. Cocchi G, Gualdi S, Bower C, Halliday J, Jonsson B, Myrelid A, et al. International trends of Down syndrome 1993-2004: Births in relation to maternal age and terminations of pregnancies. Birth Defects Res A Clin Mol Teratol 2010; 88:474-9.
44. Engels MA, Bhola SL, Twisk JW, Blankenstein MA, van Vugt JM. Evaluation of the introduction of the national Down syndrome screening program in the Netherlands: age-related uptake of prenatal screening and invasive diagnostic testing. Eur J Obstet Gynecol Reprod Biol 2014; 174:59-63.
45. De Graaf G, Vis JC, Haveman M, van Hove G, de Graaf EAB, Tijssen JGP, et al. Assessment of Prevalence of Persons with Down Syndrome: A Theory-based Demographic Model. Journal of Applied Research in Intellectual Disabilities 2011; 24:247-62.

46. Presson AP, Partyka G, Jensen KM, Devine OJ, Rasmussen SA, McCabe LL, et al. Current estimate of Down Syndrome population prevalence in the United States. J Pediatr 2013; 163:1163-8.
47. Frid C, Drott P, Otterblad Olausson P, Sundelin C, Anneren G. Maternal and neonatal factors and mortality in children with Down syndrome born in 1973-1980 and 1995-1998. Acta Paediatr 2004; 93:106-12.
48. Glasson EJ, Sullivan SG, Hussain R, Petterson BA, Montgomery PD, Bittles AH. The changing survival profile of people with Down's syndrome: implications for genetic counselling. Clin Genet 2002; 62:390-3.
49. Englund A, Jonsson B, Zander CS, Gustafsson J, Anneren G. Changes in mortality and causes of death in the Swedish Down syndrome population. Am J Med Genet A 2013; 161A:642-9.
50. Mortality of individuals with Down syndrome. Statistics Netherlands.
51. McNeil DE, Cote TR, Clegg L, Mauer A. SEER update of incidence and trends in pediatric malignancies: acute lymphoblastic leukemia. Med Pediatr Oncol 2002; 39:554-7; discussion 2-3.
52. Gurney JG, Severson RK, Davis S, Robison LL. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer 1995; 75:2186-95.
53. Ries LAG, Smith MA, Gurney JG, Linet M, Tamra T, Young JL, et al. Cancer Incidence and Survival among Children and Adolescents: United States SEER Program 1975-1995, National Cancer Institute, SEER Program. Bethesda, MD: National Cancer Institute, SEER Program., 1999.
54. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet 2000; 355:165-9.
55. Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down's syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-7.
56. Dutch Childhood Oncology Group (DCOG) Trial and Data Centre (2014). Incidences of acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) in children with and without Down syndrome in The Netherlands between 2002 and 2011.
57. Kamps WA, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. Leukemia 2010; 24:309-19.
58. Buitenkamp TD, Izraeli S, Zimmermann M, Forestier E, Heerema NA, van den Heuvel-Eibrink MM, et al. Acute lymphoblastic leukemia in children with Down syndrome: a retrospective analysis from the Ponte di Legno study group. Blood 2014; 123:70-7.
59. Buitenkamp TD, Pieters R, Gallimore NE, van der Veer A, Meijerink JP, Beverloo HB, et al. Outcome in children with Down's syndrome and acute lymphoblastic leukemia: role of IKZF1 deletions and CRLF2 aberrations. Leukemia 2012; 26:2204-11.
60. Kuiper RP, Waanders E, van der Velden VH, van Reijmersdal SV, Venkatachalam R, Scheijen B, et al. IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. Leukemia 2010; 24:1258-64.

61. O'Connor D, Bate J, Wade R, Clack R, Dhir S, Hough R, et al. Infection-related mortality in children with acute lymphoblastic leukemia: a retrospective analysis of infectious deaths on UKALL 2003. Blood 2014.
62. Jansen MW, Corral L, van der Velden VH, Panzer-Grumayer R, Schrappe M, Schrauder A, et al. Immunobiological diversity in infant acute lymphoblastic leukemia is related to the occurrence and type of MLL gene rearrangement. Leukemia 2007; 21:633-41.
63. Vardiman JW. The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. Chem Biol Interact 2010; 184:16-20.
64. Blink M, Zimmermann M, von Neuhoff C, Reinhardt D, de Haas V, Hasle H, et al. Normal karyotype is a poor prognostic factor in myeloid leukemia of Down syndrome: a retrospective, international study. Haematologica 2014; 99:299-307.
65. Kaspers GJ, Creutzig U. Pediatric acute myeloid leukemia: international progress and future directions. Leukemia 2005; 19:2025-9.
66. Vardiman JW. Hematopathological concepts and controversies in the diagnosis and classification of myelodysplastic syndromes. Hematology Am Soc Hematol Educ Program 2006:199-204.
67. Hasle H, Wadsworth LD, Massing BG, McBride M, Schultz KR. A population-based study of childhood myelodysplastic syndrome in British Columbia, Canada. Br J Haematol 1999; 106:1027-32.
68. Stary J, Baumann I, Creutzig U, Harbott J, Michalova K, Niemeyer C. Getting the numbers straight in pediatric MDS: distribution of subtypes after exclusion of down syndrome. Pediatr Blood Cancer 2008; 50:435-6.
69. Lange BJ, Kobrinsky N, Barnard DR, Arthur DC, Buckley JD, Howells WB, et al. Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group Studies 2861 and 2891. Blood 1998; 91:608-15.
70. Gamis AS, Alonzo TA, Gerbing RB, Hilden JM, Sorrell AD, Sharma M, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. Blood 2011; 118:6752-9; quiz 996.
71. Klusmann JH, Creutzig U, Zimmermann M, Dworzak M, Jorch N, Langebrake C, et al. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. Blood 2008; 111:2991-8.
72. Magalhaes IQ, Splendore A, Emerenciano M, Figueiredo A, Ferrari I, Pombo-de-Oliveira MS. GATA1 mutations in acute leukemia in children with Down syndrome. Cancer Genet Cytogenet 2006; 166:112-6.
73. Greene ME, Mundschau G, Wechsler J, McDevitt M, Gamis A, Karp J, et al. Mutations in GATA1 in both transient myeloproliferative disorder and acute megakaryoblastic leukemia of Down syndrome. Blood Cells Mol Dis 2003; 31:351-6.
74. Hasle H. Pattern of malignant disorders in individuals with Down's syndrome. Lancet Oncol 2001; 2:429-36.

75. Sullivan SG, Hussain R, Glasson EJ, Bittles AH. The profile and incidence of cancer in Down syndrome. J Intellect Disabil Res 2007; 51:228-31.
76. Hill DA, Gridley G, Cnattingius S, Mellemkjaer L, Linet M, Adami HO, et al. Mortality and cancer incidence among individuals with Down syndrome. Arch Intern Med 2003; 163:705-11.
77. Graber E, Chacko E, Regelmann MO, Costin G, Rapaport R. Down syndrome and thyroid function. Endocrinol Metab Clin North Am 2012; 41:735-45.
78. Roberts HE, Moore CA, Fernhoff PM, Brown AL, Khoury MJ. Population study of congenital hypothyroidism and associated birth defects, Atlanta, 1979-1992. Am J Med Genet 1997; 71:29-32.
79. Tuysuz B, Beker DB. Thyroid dysfunction in children with Down's syndrome. Acta Paediatr 2001; 90:1389-93.
80. Rubello D, Pozzan GB, Casara D, Girelli ME, Boccato S, Rigon F, et al. Natural course of subclinical hypothyroidism in Down's syndrome: prospective study results and therapeutic considerations. J Endocrinol Invest 1995; 18:35-40.
81. Karlsson B, Gustafsson J, Hedov G, Ivarsson SA, Anneren G. Thyroid dysfunction in Down's syndrome: relation to age and thyroid autoimmunity. Arch Dis Child 1998; 79:242-5.
82. Cebeci AN, Guven A, Yildiz M. Profile of hypothyroidism in Down's syndrome. J Clin Res Pediatr Endocrinol 2013; 5:116-20.
83. Marild K, Stephansson O, Grahnquist L, Cnattingius S, Soderman G, Ludvigsson JF. Down syndrome is associated with elevated risk of celiac disease: a nationwide case-control study. J Pediatr 2013; 163:237-42.
84. Wouters J, Weijerman ME, van Furth AM, Schreurs MW, Crusius JB, von Blomberg BM, et al. Prospective human leukocyte antigen, endomysium immunoglobulin A antibodies, and transglutaminase antibodies testing for celiac disease in children with Down syndrome. J Pediatr 2009; 154:239-42.
85. Cogulu O, Ozkinay F, Gunduz C, Cankaya T, Aydogdu S, Ozgenc F, et al. Celiac disease in children with Down syndrome: importance of follow-up and serologic screening. Pediatr Int 2003; 45:395-9.
86. Sattar N, Lazare F, Kacer M, Aguayo-Figueroa L, Desikan V, Garcia M, et al. Celiac disease in children, adolescents, and young adults with autoimmune thyroid disease. J Pediatr 2011; 158:272-5 e1.
87. Van Goor JC, Massa GG, Hirasing R. Increased incidence and prevalence of diabetes mellitus in Down's syndrome. Arch Dis Child 1997; 77:186.
88. Bergholdt R, Eising S, Nerup J, Pociot F. Increased prevalence of Down's syndrome in individuals with type 1 diabetes in Denmark: A nationwide population-based study. Diabetologia 2006; 49:1179-82.
89. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, van den Ende K, et al. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. Pediatrics 2007; 120:e1076-81.
90. Hilton JM, Fitzgerald DA, Cooper DM. Respiratory morbidity of hospitalized children with Trisomy 21. J Paediatr Child Health 1999; 35:383-6.

91. Day SM, Strauss DJ, Shavelle RM, Reynolds RJ. Mortality and causes of death in persons with Down syndrome in California. Dev Med Child Neurol 2005; 47:171-6.
92. Yang Q, Rasmussen SA, Friedman JM. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 2002; 359:1019-25.
93. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, Kimpen JL, et al. High incidence of recurrent wheeze in children with down syndrome with and without previous respiratory syncytial virus lower respiratory tract infection. Pediatr Infect Dis J 2010; 29:39-42.
94. Forni GL, Rasore-Quartino A, Acutis MS, Strigini P. Incidence of bronchial asthma in Down syndrome. J Pediatr 1990; 116:487-8.
95. Schieve LA, Boulet SL, Boyle C, Rasmussen SA, Schendel D. Health of children 3 to 17 years of age with Down syndrome in the 1997-2005 national health interview survey. Pediatrics 2009; 123:e253-60.
96. McDowell KM, Craven DI. Pulmonary complications of Down syndrome during childhood. J Pediatr 2011; 158:319-25.
97. Weijerman ME, Brand PL, van Furth MA, Broers CJ, Gemke RJ. Recurrent wheeze in children with Down syndrome: is it asthma? Acta Paediatr 2011; 100:e194-7.
98. Watts R, Vyas H. An overview of respiratory problems in children with Down's syndrome. Arch Dis Child 2013.
99. Piatti G, Allegra L, Ambrosetti U, De Santi MM. Nasal ciliary function and ultrastructure in Down syndrome. Laryngoscope 2001; 111:1227-30.
100. Chaney RH, Eyman RK, Miller CR. The relationship of congenital heart disease and respiratory infection mortality in patients with Down's syndrome. J Ment Defic Res 1985; 29 (Pt 1):23-7.
101. Faria PF, Nicolau JA, Melek MZ, Oliveira ND, Bermudez BE, Nisihara RM. Association between congenital heart defects and severe infections in children with Down syndrome. Rev Port Cardiol 2014; 33:15-8.
102. Medrano Lopez C, Garcia-Guereta Silva L, Lirio Casero J, Garcia Perez J, Grupo Civic, Grupo de Trabajo de Infecciones de la Sociedad Española de Cardiología Pediátrica y Cardiopatías Congénitas. [Respiratory infections, Down's syndrome and congenital heart disease: the CIVIC 21 study]. An Pediatr (Barc) 2009; 71:38-46.
103. Harding SM, Allen JE, Blumin JH, Warner EA, Pellegrini CA, Chan WW. Respiratory manifestations of gastroesophageal reflux disease. Ann N Y Acad Sci 2013; 1300:43-52.
104. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 2000; 404:193-7.
105. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 1997; 91:661-72.
106. Andreu-Ballester JC, Garcia-Ballesteros C, Benet-Campos C, Amigo V, Almela-Quilis A, Mayans J, et al. Values for alphabeta and gammadelta T-lymphocytes and CD4+, CD8+, and CD56+ subsets in healthy adult subjects: assessment by age and gender. Cytometry B Clin Cytom 2012; 82:238-44.

107. Schroeder HW, Jr. Similarity and divergence in the development and expression of the mouse and human antibody repertoires. Dev Comp Immunol 2006; 30:119-35.
108. Hozumi N, Tonegawa S. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc Natl Acad Sci U S A 1976; 73:3628-32.
109. Tonegawa S. Somatic generation of antibody diversity. Nature 1983; 302:575-81.
110. Osmond DG, Rolink A, Melchers F. Murine B lymphopoiesis: towards a unified model. Immunol Today 1998; 19:65-8.
111. Osmond DG. Population dynamics of bone marrow B lymphocytes. Immunol Rev 1986; 93:103-24.
112. Van Zelm MC, van der Burg M, de Ridder D, Barendregt BH, de Haas EF, Reinders MJ, et al. Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression. J Immunol 2005; 175:5912-22.
113. Van Zelm MC, van der Burg M, Langerak AW, van Dongen JJ. PID comes full circle: applications of V(D)J recombination excision circles in research, diagnostics and newborn screening of primary immunodeficiency disorders. Front Immunol 2011; 2:12.
114. Verschuren MC, Wolvers-Tettero IL, Breit TM, Noordzij J, van Wering ER, van Dongen JJ. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. J Immunol 1997; 158:1208-16.
115. Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol 2003; 21:231-64.
116. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science 2001; 293:2111-4.
117. Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci U S A 2009; 106:13945-50.
118. Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, et al. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 2001; 293:2108-11.
119. Van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med 2007; 204:645-55.
120. Gore Y, Starlets D, Maharshak N, Becker-Herman S, Kaneyuki U, Leng L, et al. Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex. J Biol Chem 2008; 283:2784-92.
121. Van Zelm MC, Reisli I, van der Burg M, Castano D, van Noesel CJ, van Tol MJ, et al. An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med 2006; 354:1901-12.
122. Van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest 2010; 120:1265-74.

123. Thiel J, Kimmig L, Salzer U, Grudzien M, Lebrecht D, Hagena T, et al. Genetic CD21 deficiency is associated with hypogammaglobulinemia. J Allergy Clin Immunol 2012; 129:801-10 e6.
124. Wu Y, Bressette D, Carrell JA, Kaufman T, Feng P, Taylor K, et al. Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLyS. J Biol Chem 2000; 275:35478-85.
125. Marsters SA, Yan M, Pitti RM, Haas PE, Dixit VM, Ashkenazi A. Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI. Curr Biol 2000; 10:785-8.
126. Thompson JS, Schneider P, Kalled SL, Wang L, Lefevre EA, Cachero TG, et al. BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. J Exp Med 2000; 192:129-35.
127. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 2000; 102:553-63.
128. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. J Immunol 2008; 181:1767-79.
129. Pene J, Gauchat JF, Lecart S, Drouet E, Guglielmi P, Boulay V, et al. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. J Immunol 2004; 172:5154-7.
130. Lundgren M, Persson U, Larsson P, Magnusson C, Smith CI, Hammarstrom L, et al. Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. Eur J Immunol 1989; 19:1311-5.
131. Pene J, Rousset F, Briere F, Chretien I, Paliard X, Banchereau J, et al. IgE production by normal human B cells induced by alloreactive T cell clones is mediated by IL-4 and suppressed by IFN-gamma. J Immunol 1988; 141:1218-24.
132. Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol 2014; 5:162.
133. Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell 1993; 72:279-90.
134. Vetrie D, Vorechovsky I, Sideras P, Holland J, Davies A, Flinter F, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature 1993; 361:226-33.
135. Bruton OC. Agammaglobulinemia. Pediatrics 1952; 9:722-8.
136. Minegishi Y, Coustan-Smith E, Wang YH, Cooper MD, Campana D, Conley ME. Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. J Exp Med 1998; 187:71-7.
137. Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, et al. An essential role for BLNK in human B cell development. Science 1999; 286:1954-7.

138. Ferrari S, Lougaris V, Caraffi S, Zuntini R, Yang J, Soresina A, et al. Mutations of the Igbeta gene cause agammaglobulinemia in man. J Exp Med 2007; 204:2047-51.
139. Dobbs AK, Yang T, Farmer D, Kager L, Parolini O, Conley ME. Cutting edge: a hypomorphic mutation in Igbeta (CD79b) in a patient with immunodeficiency and a leaky defect in B cell development. J Immunol 2007; 179:2055-9.
140. Minegishi Y, Coustan-Smith E, Rapalus L, Ersoy F, Campana D, Conley ME. Mutations in Igalpha (CD79a) result in a complete block in B-cell development. J Clin Invest 1999; 104:1115-21.
141. Yel L, Minegishi Y, Coustan-Smith E, Buckley RH, Trubel H, Pachman LM, et al. Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. N Engl J Med 1996; 335:1486-93.
142. Peters AL, Plenge RM, Graham RR, Altshuler DM, Moser KL, Gaffney PM, et al. A novel polymorphism of the human CD40 receptor with enhanced function. Blood 2008; 112:1863-71.
143. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, et al. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). Cell 2000; 102:565-75.
144. Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science 1993; 259:990-3.
145. Van Zelm MC, Bartol SJ, Driessen GJ, Mascart F, Reisli I, Franco JL, et al. Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation. J Allergy Clin Immunol 2014; 134:135-44 e7.
146. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol 2003; 4:261-8.
147. ESID Registry - Working definitions for clinical diagnosis of PID (2014). Available from http://esid.org/Working-Parties/Registry/Diagnosis-criteria.
148. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood 2008; 112:277-86.
149. Meyts I, Bossuyt X, Proesmans M, De B. Isolated IgG3 deficiency in children: to treat or not to treat? Case presentation and review of the literature. Pediatr Allergy Immunol 2006; 17:544-50.
150. Moss RB, Carmack MA, Esrig S. Deficiency of IgG4 in children: association of isolated IgG4 deficiency with recurrent respiratory tract infection. J Pediatr 1992; 120:16-21.
151. Bartmann P, Kleihauer E. Undetectable IgG4 in immunoprecipitation: association with repeated infections in children? Eur J Pediatr 1988; 148:211-4.
152. Sanders LA, Rijkers GT, Tenbergen-Meekes AM, Voorhorst-Ogink MM, Zegers BJ. Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr Res 1995; 37:812-9.
153. Siber GR, Schur PH, Aisenberg AC, Weitzman SA, Schiffman G. Correlation between serum IgG-2 concentrations and the antibody response to bacterial polysaccharide antigens. N Engl J Med 1980; 303:178-82.

154. Saxon A, Kobayashi RH, Stevens RH, Singer AD, Stiehm ER, Siegel SC. In vitro analysis of humoral immunity in antibody deficiency with normal immunoglobulins. Clin Immunol Immunopathol 1980; 17:235-44.
155. Pereira LF, Sapina AM, Arroyo J, Vinuelas J, Bardaji RM, Prieto L. Prevalence of selective IgA deficiency in Spain: more than we thought. Blood 1997; 90:893.
156. Jorgensen GH, Gardulf A, Sigurdsson MI, Sigurdardottir ST, Thorsteinsdottir I, Gudmundsson S, et al. Clinical symptoms in adults with selective IgA deficiency: a case-control study. J Clin Immunol 2013; 33:742-7.
157. Aghamohammadi A, Mohammadi J, Parvaneh N, Rezaei N, Moin M, Espanol T, et al. Progression of selective IgA deficiency to common variable immunodeficiency. Int Arch Allergy Immunol 2008; 147:87-92.
158. Espanol T, Catala M, Hernandez M, Caragol I, Bertran JM. Development of a common variable immunodeficiency in IgA-deficient patients. Clin Immunol Immunopathol 1996; 80:333-5.
159. De Vries E. Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol 2012; 167:108-19.
160. Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood 2011; 118:6814-23.
161. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood 2008; 111:77-85.
162. Wilson JMG, Jungner G. Principles and practice of screening for disease. Public Health Papers: World Health Organization (WHO), 1968.
163. Borte S, von Dobeln U, Fasth A, Wang N, Janzi M, Winiarski J, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood 2012; 119:2552-5.
164. Verbsky JW, Baker MW, Grossman WJ, Hintermeyer M, Dasu T, Bonacci B, et al. Newborn screening for severe combined immunodeficiency; the Wisconsin experience (2008-2011). J Clin Immunol 2012; 32:82-8.
165. Nakagawa N, Imai K, Kanegane H, Sato H, Yamada M, Kondoh K, et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. J Allergy Clin Immunol 2011; 128:223-5 e2.
166. European Surveillance for Congenital Anomalies (EUROCAT). Available from http://www.eurocat-network.eu ; Accession date May 28th 2014
167. Birth in the Netherlands. Statistics Netherlands. Available from http://statline.cbs.nl/StatWeb/publication/?VW=T&DM=SLNL&PA=37422ned&D1=0,42&D2=30-62&HD=140414-1359&HDR=T&STB=G1 ; Accession date May 28th 2014
168. Marcus CL, Keens TG, Bautista DB, von Pechmann WS, Ward SL. Obstructive sleep apnea in children with Down syndrome. Pediatrics 1991; 88:132-9.

169. Shott SR, Amin R, Chini B, Heubi C, Hotze S, Akers R. Obstructive sleep apnea: Should all children with Down syndrome be tested? Arch Otolaryngol Head Neck Surg 2006; 132:432-6.
170. Shott SR, Donnelly LF. Cine magnetic resonance imaging: evaluation of persistent airway obstruction after tonsil and adenoidectomy in children with Down syndrome. Laryngoscope 2004; 114:1724-9.
171. Cooney TP, Thurlbeck WM. Pulmonary hypoplasia in Down's syndrome. N Engl J Med 1982; 307:1170-3.
172. Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EF, Baert MR, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med 2005; 201:1715-23.
173. ESID Online Registry. Available from http://esid.org/Working-Parties/Registry/ESID-Database-Statistics ; updated to June 25th 2014.

Chapter 2

Epidemiology of respiratory symptoms in children with Down syndrome: a nationwide prospective web-based parent-reported study

R.H.J. Verstegen, R.W.N.M. van Hout, E. de Vries

BMC Pediatr. 2014;14:103.

ABSTRACT

Background

Children with Down syndrome suffer from recurrent respiratory tract and ear-nose-throat complaints that influence daily life. Little is known about the frequency of these complaints, as well as their relation to co-morbidity and ageing.

Methods

A prospective web-based parent-reported observational study was designed for parents having a child with Down syndrome (age 0 to 18 years). Upon registration, parents receive an email containing a link to a weekly questionnaire regarding respiratory symptoms during two consecutive years. Additionally, at the beginning, after one year and at the end of the study they receive an extended questionnaire concerning baseline data, daily activities and medical history. The data will be compared to the ongoing "child-is-ill" study, which collects weekly data in an identical fashion in children that are considered to be "normal as to being ill" by their parents.

Discussion

This study will provide important data on the epidemiology of respiratory symptoms in children with Down syndrome, which will be useful for further studies on treatment options. Also, this study will gain insight in healthcare usage and work absence due to the child's illnesses.

BACKGROUND

Down syndrome is the most common genetic cause of developmental delay in humans, affecting approximately 1 in 700 liveborn infants in The Netherlands.[1, 2] Numerous health issues are related to Down syndrome. In newborns, hypotonia, facial characteristics and congenital heart disease (CHD) are variably present. Later in life, Down syndrome is associated with an increased incidence of hematological malignancies and auto-immune diseases, such as celiac disease, hypothyroidism and type 1 diabetes mellitus.[3]

Respiratory complications and ear, nose and throat (ENT) diseases are a major contributor to morbidity and mortality in patients with Down syndrome. Up to 80% of all hospitalizations and admissions to a pediatric intensive care unit of children with Down syndrome is caused by lower respiratory tract infections.[4, 5] The risk of respiratory syncytial virus infection and viral induced wheezing, but not asthma, is increased in infants with Down syndrome.[6-11] Moreover, up to 29% of deaths in Down syndrome are related to pneumonia, influenza and aspiration, with increased standardized mortality odds ratio of 3.00-4.15 in children with Down syndrome aged <20 years.[12, 13] In addition, we recently showed that parent-reported recurrent respiratory tract infections in 8-year-old children with Down syndrome are associated with more impaired mental and motor development, lower health related quality of life and more behavioral problems compared to children whose parents report no increased respiratory tract infections in their child.[14]

In a retrospective Finnish chart study, up to 40% of children and young adults (aged <30 years) with Down syndrome have had at least one pneumonia, partly caused by aspiration.[15] Schieve et al. report that 27.6% of children with Down syndrome compared to 17.5% of children without Down syndrome or other cause of mental retardation had symptoms of head/chest cold in the 2 weeks prior to the conduction of the survey.[9] Another study showed that 17.6% of school-aged children with Down syndrome have a continual runny nose and that 12% have had more than 3 upper respiratory tract infections in the preceding year.[16] ENT diseases are more frequently found in children with Down syndrome and include ear infections, hearing impairment and obstructive sleep apnea syndrome,[17-21] which can subsequently lead to behavioral problems and more developmental delay.[22, 23]

Treatment, and especially prevention of recurrent respiratory tract infections is challenging. Anatomical and physiological changes in the respiratory and ENT tract in children with Down syndrome are inherent to the syndrome and leave little room for improvement, except adenotomy and/or tonsillectomy. The use of prophylactic antibiotics to decrease the frequency of respiratory infections has

never been studied in children with Down syndrome (nor in healthy children). Also, although palivizumab is sometimes prescribed in children with Down syndrome to prevent severe RSV disease, there have been no studies to evaluate the effect of this treatment. At last, other – more active – diagnostics and treatment by ENT specialists may be beneficial since adenotomy and/or tonsillectomy do not help all children.[24] In order to evaluate treatment options there is a need for more data on the exact incidence of respiratory symptoms and their evolvement in time to define patient groups who may benefit from these therapies. In this paper we describe the construction of a web-based observational study of respiratory tract infections in children with Down syndrome.

AIM OF THE STUDY

The primary goal of this study is to describe the frequency of respiratory symptoms in relation to age and comorbidity, with special focus on development of symptoms in time. The secondary goal is to show the medical and social consequences of respiratory symptoms such as doctor visits, absence from school and care leave of parents. Thirdly, we will relate the presence of co-morbidities in children with Down syndrome and their development in time with the obtained data.

STUDY DESIGN

We designed a prospective web-based parent-reported observational study for which inclusion started in March 2012. Approval of the study protocol was obtained from the Medical Ethical Review Board "METOPP", Tilburg, The Netherlands.

Inclusion of participants

Parents or legal guardians of a child with Down syndrome in The Netherlands aged 0-18 years are eligible for inclusion in this study. By estimation, the total age cohort consists of approximately 4500 children. Specialized pediatric outpatient clinics for Down syndrome were contacted and received posters for their waiting room. Also, the Dutch Down Syndrome Foundation and related organizations made the study more widely known by publishing a call on their websites. Finally, social media were used to increase awareness of this study. Parents who are interested can obtain more information about and register for this study though the study

website. According to Statistics Netherlands, almost all families with children in The Netherlands have access to the Internet and would therefore be eligible to participate.[25] However, parents should be able to understand Dutch in order to be capable to register for this study.

Data collection

All data for this study will be collected through web-based questionnaires. Participants receive email invitations, which are sent by an automatic data managing system (Research Manager, Nova Business Software, The Netherlands). At baseline, the parents are asked to complete a questionnaire, which includes questions on the composition of the household of the child, daily activities of their child (i.e. child care attendance or visiting primary or secondary school) and medical history of the child and family (Table 1). Thereafter, parents continue to receive a weekly questionnaire to ask whether their child has had symptoms in the past week. If so, additional questions, regarding the symptoms and consequences of the symptoms are asked (Table 2). After one year and at the end of the study (at two years) parents are asked to complete the baseline questionnaire again to determine any changes. Reminders will be sent for the baseline and two follow-up questionnaires twice.

Reference data is collected through the ongoing "child-is-ill" study, which collects weekly data in an identical fashion in children that are considered to be "normal as to being ill" by their parents. For this study over 750 children, aged 2-18 year, are registered.

Statistics

Because this is an observational study, we did not perform a power analysis. However, we estimate that 2,5% of parents of a child with Down syndrome participates in this study. Given the potential sample size of approximately 4,500 children with Down syndrome aged <18 years in The Netherlands, we estimate to include approximately 110 children. This group size will allow us to study a larger cohort of children with Down syndrome longitudinally. This cohort is a small fraction of the total Down syndrome population, and the way parents were selected is not random, having the consequence that bias may show up. We will carefully address potential bias, in particular by comparing all our outcomes with research data available on children with Down syndrome. A second restriction is that the size of the cohort may limit the possibilities of subgroup analyses. A final restriction is the uncertainty about the amount of missing data, as increasing numbers of missing data reduce power. It is conceivable that specific differences between children with Down syndrome and controls cannot be detected.

Table 1. Annual questionnaire regarding background, daily activities and medical history of participating child with Down syndrome.

General questions						
Child with Down syndrome	Date of birth			Gender		
Father/mother	Date of birth					
History of allergy, asthma and/or eczema?	Yes			No		
Siblings	Number of older siblings Number of younger siblings					
History of allergy, asthma and/or eczema?	Yes			No		
Does anyone smoke (almost) daily within the house?	Yes			No		
Daily activities						
Divide the 14 half-days present in each week between the following activities	Home Child day care Playgroup (age 2-4y) Primary school (age 6-12y) Secondary school Work placement Other			Grandparents/family/host family Special needs day care Pre-school kindergarten (age 4-5y) Special primary school Special secondary school Working		
If attending regular education, what grade is your child in?						
Medical history						
Compared to other children with the same age, the frequency of being ill is:	Lower		Equal	Higher		
Does your child have a history of any of the following illnesses, complaints or medication usage?						
Congenital heart disease	Yes			No		
If yes, please specify	VSD	ASD	AVSD	Tetralogy of Fallot	Other	Unknown
If yes, was surgery performed?	Yes			No		
Hypothyroid disease	Yes			No		
If yes, diagnosed at what age?						
Diabetes mellitus	Yes			No		
If yes, diagnosed at what age?						
Congenital malformations of the gastrointestinal tract	Yes			No		
If yes, please specify:	Oesophageal atresia		Duodenal atresia	Imperforate anus	Other	Unknown
Celiac disease	Yes			No		
If yes, diagnosed at what age?						

Table 1. Continued

Impaired hearing	Yes				No	
If yes, diagnosed at what age?						
Chronic snoring,	Yes				No	
If yes, present since what age?						
Breathing with open mouth	Yes				No	
If yes, present since what age?						
Frequently suffering from serious colds	Yes		Yes, but did in the past		No	
If complaints used to be present, until what age?						
Wheezing	Yes		Yes, but did in the past		No	
If complaints used to be present, until what age?						
Eye disorders	Yes				No	
If yes, please specify:	Cataract	Glaucoma	Amblyopia	Wears glasses	Strabismus	Other
Leukaemia	Yes				No	
If yes, diagnosed at what age?						
Antibiotic use for respiratory tract/ENT* infections in the past year	0-5 times		6-10 times		more than 10 times	
Hospital admission for RSV infection <2 years	Yes				No	
ENT-surgery	Yes				No	
If yes, please specify:	Tympanic tubes		Adenoidectomy		Tonsillectomy	
Daily antibiotic prophylaxis	Yes		Yes, but did in the past		No	
Inhaled corticoid for coughing, mucus and/or wheezing	Yes		Yes, but did in the past		No	

* ENT Ear-nose-throat

First, baseline characteristics of the study population will be presented in a descriptive manner using percentages. Second, mixed model analysis will be performed at the end of the study. This model allows analyzing data from repeated measurements as well as proper handling of missing data. Also, effect of age as well as time (including seasonal effects) can be taken into account. Early termination of study participation by request of the parents or by >4 weeks missing data prior to the endpoint will be marked as lost to follow-up, and dealt with accordingly. Analysis will be performed using IBM SPSS Statistics.

Table 2. Weekly questionnaire regarding medical symptoms in the past week.

Did your child have had any symptoms in the past week?	No		Yes
If yes,*			
Did you visit a doctor with your child?	No Yes, paediatrician Yes, other doctor		Yes, general practitioner Yes, ENT-specialist#
Did your child receive antibiotic treatment?			Yes
Which symptoms were present?	Earache Stuffy nose Hoarse voice	Running ear Runny nose Coughing/mucus	Sore throat Headache Shortness of breath
Was the temperature higher than 38.5°C (fever)?	No	Yes	Did not take a temperature
Did your child stay at home from school?	No	Yes	Not applicable
Did your child stay at home from work placement?	No	Yes	Not applicable
Did your child stay at home from work?	No	Yes	Not applicable
Did you or your partner stay at home from work?	No	Yes	Not applicable

* The additional questions are only shown after the first question is answered "yes".
# ENT Ear-nose-throat

Handling and storage of data

Data will be handled and stored according to legal and privacy guidelines. Parents will enter data concerning their child in a central web-based database. Security is guaranteed with login names, login codes and encrypted data transfer. The data of all subjects will be coded and this coding will not be retraceable to the individual patient. First, parents are not asked to enter the name of their child or exact address. Second, the email address of the parents will be blinded, stored encrypted and not accessible to the researchers of this study. Access to the database will be granted to the principal investigator and local investigator. Data will be stored for 15 years.

DISCUSSION

Although respiratory tract and ENT diseases are frequently encountered in the care for children with Down syndrome, little is known about their epidemiology. We have designed a prospective web-based study, which allows us to describe the incidence of respiratory symptoms, as well as their relation to comorbidity, age and season. This information can help to evaluate the effect of future treatment options.

To our knowledge this is the first prospective study in patients with Down syndrome on epidemiological data of respiratory symptoms. Earlier studies were often based on retrospective chart reviews, which excludes complaints for which no medical attention was sought by the parents.[5, 7, 15] Also, there have been many studies based on a single questionnaire where parents were asked if complaints were present in the past year(s), which leads to recall bias.[9, 11, 16, 21] Other authors have therefore used both chart review and retrospective questionnaires to minimize the effect of recall bias.[4, 6] Some studies are based on hospital admission/discharge records or mortality records only, and all outpatient symptoms are missed.[8, 12, 13] We expect our study to give more insight in respiratory symptoms in children with Down syndrome, which may help Down syndrome specialists to improve health care for this group.

REFERENCES

1. Weijerman ME, van Furth AM, Vonk Noordegraaf A, van Wouwe JP, Broers CJ, Gemke RJ. Prevalence, neonatal characteristics, and first-year mortality of Down syndrome: a national study. J Pediatr 2008; 152:15-9.
2. Mohangoo AD, van der Pal-Bruin KM, Buitendijk SE. TNO report on congenital defects in the Netherlands 1997-2008. Leiden: Netherlands Organization for Applied Scientific Research, TNO Quality of Life, 2010.
3. Roizen NJ, Patterson D. Down's syndrome. Lancet 2003; 361:1281-9.
4. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, van den Ende K, et al. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. Pediatrics 2007; 120:e1076-81.
5. Hilton JM, Fitzgerald DA, Cooper DM. Respiratory morbidity of hospitalized children with Trisomy 21. J Paediatr Child Health 1999; 35:383-6.
6. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, Kimpen JL, et al. High incidence of recurrent wheeze in children with down syndrome with and without previous respiratory syncytial virus lower respiratory tract infection. Pediatr Infect Dis J 2010; 29:39-42.
7. Forni GL, Rasore-Quartino A, Acutis MS, Strigini P. Incidence of bronchial asthma in Down syndrome. J Pediatr 1990; 116:487-8.
8. Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down's syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-7.
9. Schieve LA, Boulet SL, Boyle C, Rasmussen SA, Schendel D. Health of children 3 to 17 years of age with Down syndrome in the 1997-2005 national health interview survey. Pediatrics 2009; 123:e253-60.
10. McDowell KM, Craven DI. Pulmonary complications of Down syndrome during childhood. J Pediatr 2011; 158:319-25.

11. Weijerman ME, Brand PL, van Furth MA, Broers CJ, Gemke RJ. Recurrent wheeze in children with Down syndrome: is it asthma? Acta Paediatr 2011; 100:e194-7.
12. Day SM, Strauss DJ, Shavelle RM, Reynolds RJ. Mortality and causes of death in persons with Down syndrome in California. Dev Med Child Neurol 2005; 47:171-6.
13. Yang Q, Rasmussen SA, Friedman JM. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 2002; 359:1019-25.
14. Verstegen RH, van Gameren-Oosterom HB, Fekkes M, Dusseldorp E, de Vries E, van Wouwe JP. Significant impact of recurrent respiratory tract infections in children with Down syndrome. Child Care Health Dev 2012.
15. Maatta T, Maatta J, Tervo-Maatta T, Taanila A, Kaski M, Iivanainen M. Healthcare and guidelines: a population-based survey of recorded medical problems and health surveillance for people with Down syndrome. J Intellect Dev Disabil 2011; 36:118-26.
16. Selikowitz M. Health problems and health checks in school-aged children with Down syndrome. J Paediatr Child Health 1992; 28:383-6.
17. Shott SR. Down syndrome: common otolaryngologic manifestations. Am J Med Genet C Semin Med Genet 2006; 142C:131-40.
18. Shott SR, Amin R, Chini B, Heubi C, Hotze S, Akers R. Obstructive sleep apnea: Should all children with Down syndrome be tested? Arch Otolaryngol Head Neck Surg 2006; 132:432-6.
19. Shott SR, Joseph A, Heithaus D. Hearing loss in children with Down syndrome. Int J Pediatr Otorhinolaryngol 2001; 61:199-205.
20. Rodman R, Pine HS. The otolaryngologist's approach to the patient with Down syndrome. Otolaryngol Clin North Am 2012; 45:599-629, vii-viii.
21. Thomas K, Bourke J, Girdler S, Bebbington A, Jacoby P, Leonard H. Variation over time in medical conditions and health service utilization of children with Down syndrome. J Pediatr 2011; 158:194-200 e1.
22. Beebe DW. Neurobehavioral morbidity associated with disordered breathing during sleep in children: a comprehensive review. Sleep 2006; 29:1115-34.
23. Mitchell RB, Kelly J. Behavioral changes in children with mild sleep-disordered breathing or obstructive sleep apnea after adenotonsillectomy. Laryngoscope 2007; 117:1685-8.
24. Shott SR, Donnelly LF. Cine magnetic resonance imaging: evaluation of persistent airway obstruction after tonsil and adenoidectomy in children with Down syndrome. Laryngoscope 2004; 114:1724-9.
25. Statistics Netherlands: ICT use by households in The Netherlands (2013). Available from http://statline.cbs.nl/StatWeb/publication/?VW=T&DM=SLNL&PA=71102ned&D1=0-3,30-34&D2=0-10&D3=0,l&HD=131208-1743&HDR=G2,T&STB=G1.

Chapter 3

Significant impact of recurrent respiratory tract infections in children with Down syndrome

R.H.J. Verstegen, H.B.M. van Gameren-Oosterom, M. Fekkes, E. Dusseldorp, E. de Vries, J.P. van Wouwe

Child Care Health Dev. 2013;39(6):801-9.

ABSTRACT

Objective

Parents and health professionals believe that recurrent respiratory tract infections (RRTI) have a large impact on children with Down syndrome. We studied the relation between parent reported RRTI on development, behavior, and health related quality of life (HRQoL) in 8-year-old children with Down syndrome.

Method

During a 3-year period, 325 children with Down syndrome were recruited for inclusion in this observational study. Parents were asked to fill in the Child Behavior CheckList and TNO-AZL Children's Quality of Life Parent Form. A psychological assistant administrated the McCarthy Scales of Children's Abilities. The children were divided into a group with presence of RRTI ($RRTI^+$) and a group without RRTI ($RRTI^-$), on the basis of parental report. Linear regression analyses were performed to assess the effect of RRTI, while correcting for the influence of confounders.

Results

Compared to $RRTI^-$ children (n=176), $RRTI^+$ children (n=149, 46%) showed decreased mental and motor development (mean developmental age 3.67 vs. 4.08 years), more behavioral problems and lower scores on most HRQoL scales ($p<0.05$). Moreover, school enrollment is less favorable in $RRTI^+$ children.

Conclusion

In 8-year-olds with Down syndrome, the children with parent reported RRTI show more delayed development, more behavioural problems, and lower HRQoL compared to the children without RRTI. Although this association does not prove a causal relationship, further studies should focus on this, because RRTI are potentially preventable.

Key messages

Children with Down syndrome are known to be at increased risk of recurrent respiratory tract infections. In 8 year old children with Down syndrome, parental report of recurrent respiratory infections was associated with more delayed development, increased risk of behavioral problems and lower HRQoL.

INTRODUCTION

Down syndrome is one of the most common genetic causes of intellectual disability in children. In The Netherlands, the prevalence is approximately 1 in 714 live born infants.[1, 2] Facial dysmorphic features, hypotonia, and congenital heart defects (CHD) are variably present in newborns with Down syndrome. Also, Down syndrome is associated with celiac disease, thyroid disease, diabetes mellitus, and hematological malignancies.

Respiratory complications are common in children with Down syndrome. The risk of anatomic abnormalities, respiratory syncytial virus infection and viral induced wheezing is increased.[3-5] Recurrent lung and/or airway infections (recurrent respiratory tract infections; RRTI) are frequently encountered in children with Down syndrome.[5] Parents often report delayed development due to these RRTI. Up to now, this has been studied only once in toddlers: motor development was delayed 0.88 months in 2-year-old children with Down syndrome suffering from recurrent lung or airway disease, however mental development was not affected.[6]

In this study, we measure the association between RRTI, based on parental report, and development, behavior, and health related quality of life (HRQoL) in 8-year-old children with Down syndrome.

METHODS

Study population

All members of the Dutch Down syndrome Foundation having a child with Down syndrome turning 8 years of age between January 2000 and January 2003 were invited to participate in this study. If parents agreed to participation, they returned their written informed consent to the researchers. Parents were then contacted to plan a home visit for psychological testing, and a set of questionnaires was sent to them.

Social and medical background

A questionnaire asked for information on social background and demographic variables concerning family situation, breastfeeding (>1 month after birth), and attendance to childcare and school. The level of parental education was used as indicator for socioeconomic status. The medical history of the child was evaluated by routine questions. We asked parents "Does your child suffer from chronic airway infections (i.e., often severe common cold or bronchitis)" and "Was your child

diagnosed with asthma?“ to evaluate respiratory complaints. Response categories for both questions were “yes” or “no”. Based on the response to the first question, children were divided into children with RRTI ($RRTI^+$) and children without RRTI ($RRTI^-$). In the same way, the presence of frequently encountered diseases in Down syndrome, such as CHD, gastrointestinal disease, thyroid dysfunction, diabetes mellitus, impaired hearing and/or eye disease was noted.

Developmental skills

The Dutch version of the McCarthy Scales of Children's Abilities (MSCA) for children aged 2.5 to 8.5 years was used to measure general developmental skills[7]. An experienced and trained psychological assistant performed this test as soon as possible after the eighth birthday of the child. The MSCA contains 18 subtests, which are grouped into verbal, perceptual, quantitative, memory, and motor skills. In addition, a general cognitive scale and the developmental age of the child can be determined as well with the MSCA.

Emotional and behavioral functioning

The presence of emotional and behavioral problems was determined by the Dutch version of the Child Behavior Checklist (CBCL).[8] This test was created for children aged 4 to 12 years and contains a total of 118 items on the following 9 scales: withdrawn, somatic complaints, anxious/depressed, social problems, thought problems, attention problems, delinquent behavior, aggressive behavior and sexual problems. The scales of withdrawn, somatic complaints and anxious/depressed are combined for assessing internalizing problems as a separate score. The score for externalizing problems is comprised by the scales of delinquent and aggressive behavior.

We chose the CBCL because we wanted to compare the presence of behavioral problems in children with Down syndrome to healthy Dutch children, for which reference data is available. Besides, the manual and data on validity of the Developmental Behavior Checklist – specifically designed for children with developmental problems – were not yet available in The Netherlands at the time of writing of the study-proposal.

Health related quality of life

The TNO-AZL Children's Quality of Life Parent Form (TACQOL-PF) questionnaire for children aged 5 to 15 years was used to determine the HRQoL.[9] The 57 items are grouped in physical complaints, gross motor skills, autonomy, cognitive functioning, social functioning, positive emotions, and negative emotions. With

this questionnaire parents indicated health status problems in their child and also reported negative emotions expressed by the child to these problems.

Statistical analysis

To determine differences between the group of children with parent-reported RRTI and the group without RRTI ($RRTI^+$ and $RRTI^-$, respectively), we performed chi-square tests for all variables mentioned in "Social and medical background". Age was compared between both groups by an independent t-test. Linear regression analyses were performed to assess the association between RRTI and each of the three outcome variables separately: MSCA, CBCL, and TACQOL-PF. Gender, level of parental education, presence of siblings, childcare attendance, being breastfed and morbidity (CHD, diagnosis of asthma, gastrointestinal disease, eye disease, impaired hearing, and thyroid dysfunction) were used as confounders. The effect sizes were computed as Cohen's f^2, which is the effect size index for multiple regression (see for formula Cohen, 1988, p. 410). If f^2 equals 0.01 for a variable, it means that this variable uniquely accounts for 1% of the variance in the outcome variable (expressed as a proportion of the unexplained variance). When comparing the effect sizes for different outcome variables, f^2 is more appropriate than R^2-change, because the latter depends on the total variance accounted for. For interpretation of relevant effect sizes we used the following reference values: small effect ($0.01 \leq f^2 < 0.10$), moderate effect ($0.10 \leq f^2 < 0.33$), and large effect ($f^2 \geq 0.33$).[10] In addition, to determine whether the effects for gender, CHD, and impaired hearing on the outcome variables were equal for both groups of RRTI, the influence of interaction terms were assessed by hierarchical regression analyses. For this purpose, cross-products were computed between RRTI (plus or minus) and, respectively, gender, CHD, and impaired hearing. These cross-products were added as an extra step to the regression equation (which included all main effects). Although asthma was significantly more reported in $RRTI^+$, this subgroup was too small for further analysis.

Power analysis showed at least 137 patients per group were needed to detect a 3-month difference in developmental delay (power 80%, alpha 0.05). All analyses were performed by SPSS for Windows 17.0; statistical significance was defined as a two-sided $p<0.05$.

RESULTS

Patient characteristics and confounding factors

The data on patient characteristics and potential confounding factors are presented in Table 1. In total, 531 children with Down syndrome aged 8 years

Table 1. Patient characteristics and additional morbidity of 8-year-old Down syndrome population in relation to parent-reported presence of recurrent respiratory tract infections (RRTI).

	RRTI+		RRTI-		Total		Chi square test
	n	(%)	*n*	(%)	*n*	(%)	*p-value*
Male	85	(57)	84	(48)	169	(51)	NS
Female	64	(43)	92	(52)	156	(49)	
Age at inclusion [a] (mean, range, and SD in years)	8.14 (7.8-8.8) ± 0.14		8.15 (7.8-9.1) ± 0.16		8.14 (7.8-9.1) ± 0.15		
Ever attended regular education	99	(30)	142	(44)	241	(74)	0.003
Regular education attendance at inclusion	65	(20)	92	(28)	156	(48)	NS
Preschool *(normally age 4-5 years)*	21	(32)[b]	11	(12)	31	(20)	0.018
First grade *(normally age 6 years)*	33	(51)[b]	62	(67)	95	(61)	0.010
Second grade *(normally age 7 years)*	11	(17)[b]	19	(21)	30	(19)	NS
Level of parental education							
Primary or secondary education	24	(7)	31	(10)	55	(17)	NS
Higher secondary education	55	(17)	63	(19)	118	(36)	NS
University education	70	(22)	82	(25)	152	(47)	NS
Being breastfed (>1 month)	57	(18)	60	(18)	117	(36)	NS
Siblings	140	(43)	170	(52)	310	(95)	NS
Childcare (age <4 years)	142	(44)	160	(49)	302	(93)	NS
Additional morbidity [c]							
Congenital heart disease	73	(49)	64	(36)	137	(42)	0.022
Diagnosis of asthma	28	(19)	6	(3)	34	(10)	<0.001
Gastrointestinal disease	26	(17)	19	(11)	45	(14)	NS
Eye disease	77	(52)	81	(46)	158	(49)	NS
Impaired hearing	66	(44)	32	(18)	98	(30)	<0.001
Thyroid dysfunction	19	(13)	20	(11)	39	(12)	NS
Diabetes mellitus	1	(<1)	2	(1)	3	(1)	NS
Other morbidity, not specified	43	(29)	38	(22)	81	(25)	NS

RRTI+: children with respiratory tract infections. RRTI-: children without respiratory tract infections. NS: not significant. [a] There was no significant difference in age between both groups determined by a *t*-test. [b] Percentage out of all children attending regular education. [c] Parental reported morbidity.

were invited to participate; which holds 78% of all estimated living 680 children of this birth cohort in The Netherlands (based on an 84% survival rate).[11] A total of 380 parents (72%) agreed to enroll and 337 children provided data for this study (response rate: 63%). Based on the estimated incidence of Down syndrome, our study group represents approximately 48% of all children with Down syndrome in this age-cohort in The Netherlands.[1, 2, 12] In our study, the prevalence of CHD, impaired hearing, eye disease, a diagnosis of asthma and thyroid disease is in accordance with population-based studies in Down syndrome.[1, 13-15] Not all data could be collected for each patient because of practical problems to plan a home visit for psychological testing at the age of 8 years and/or incomplete returned questionnaires.

The presence or absence of RRTI is reported by parents for 325 (96% of 337) children; in 149 of these children RRTI are present ($RRTI^+$-group) and in 176 children RRTI are absent ($RRTI^-$-group). There is no difference between the $RRTI^-$ and $RRTI^+$ children in proportion of males (p=0.09) or mean age (Table 1). The mean age for both subgroups is 8 years and 2 months. The educational career is different between both groups: $RRTI^+$ children with Down syndrome are less likely to primarily start with regular education. Also, the level of education of $RRTI^+$ children with Down syndrome who do attend a regular school is significantly lower. The educational level of girls is higher compared to boys, as described earlier.[16] This being the case in $RRTI^+$ as well as in $RRTI^-$ children.

The prevalence of CHD, a diagnosis of asthma, and impaired hearing are significantly increased in the $RRTI^+$ group. We found no significant differences in other potential confounding factors between $RRTI^+$ and $RRTI^-$ children (Table 1).

Developmental status, behavioral problems and HRQoL

Development of the children, measured by the MSCA was administrated in 270 children (80% of 337); the results are presented in Table 2. Mean scale scores on all domains of the MSCA were significantly lower in $RRTI^+$ children versus RRTI- children. Small effect sizes were found (Cohen's f^2-range: 0.03-0.04). Moreover, in $RRTI^+$ children the mean developmental age was 3 years and 8 months, compared to 4 years and 1 month in $RRTI^-$ children. Thus, the difference in mean developmental age was 5 months. Results of hierarchical regression analysis for developmental age are presented in Table 3. In Step 1, the effect of social economic status, childcare attendance, being breastfed and the presence of siblings was found to be negligible (all not significant), whereas male gender has significantly lower developmental age than female gender, adjusted for the effect of the other variables (Step 2). The results of Step 4 showed a significant effect of RRTI on developmental age, adjusted for the effect of all other variables in the model. Furthermore the effect of RRTI

Table 2. Results of multiple regression analyses for scales scores of the McCarthy Scales of Children's Abilities (MSCA) of 8-year-old Down syndrome children with and without parent-reported recurrent respiratory tract infections (RRTI).

	RRTI+		RRTI-		Regression coefficient[a]	Effect size[b]
	Total (n=130)	Male (n=75) Female (n=55)	Total (n=140)	Male (n=69) Female (n=71)	β	f^2
Verbal	33.06 (19.13)[c]	29.64 (19.96) 37.47 (17.18)	40.68 (16.18)	37.09 (15.72) 44.11 (16.08)	-7.33**	0.04
Perceptual-performance	26.76 (16.58)	21.80 (15.84) 33.33 (15.43)	32.34 (14.90)	28.83 (14.87) 35.93 (14.20)	-5.67**	0.03
Quantitative	9.44 (6.80)	7.86 (6.46) 11.44 (6.75)	12.22 (6.57)	10.46 (6.75) 13.93 (5.99)	-2.61**	0.04
Memory	10.74 (7.77)	9.27 (7.69) 12.64 (7.53)	13.94 (7.37)	11.77 (6.31 16.10 (7.77)	-3.12**	0.04
Motor	23.23 (12.57)	20.03 (12.64) 27.47 (11.32)	27.67 (11.78)	24.99 (11.51) 30.25 (11.62)	-4.63**	0.04
General cognitive score	69.30 (40.25)	59.36 (40.22) 82.29 (36.84)	85.24 (34.43)	76.35 (33.60) 94.00 (33.42)	-15.59**	0.04
Developmental age (SD in months)	3 y 8 m (10.91)	3 y 6 m (10.53) 3 y 11 m (10.66)	4 y 1 m (9.58)	3 y 10 m (9.08) 4 y 3 m (9.30)	-4.05**	0.04

Lower scores indicate more impaired development. RRTI+: children with recurrent respiratory tract infections. RRTI-: children without recurrent respiratory tract infections. [a] β = unstandardized regression coefficient of the effect of RRTI, correcting for the effect of socioeconomic status, childcare attendance, being breastfed (>1 month), siblings, gender, congenital heart defect, diagnosis of asthma, gastrointestinal disease, eye disease, impaired hearing, and thyroid dysfunction. [b] Effect size (f^2): small effect (0.01-0.10), moderate effect (0.10-0.33), large effect (>0.33). [c] Mean scores are presented with standard deviation between brackets. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

is larger than all separate forms of morbidity (Step 3). No interaction effects are present between RRTI and CHD, gender, or impaired hearing.

In total, 317 (94%) CBCL questionnaires were completed (Table 4). The results showed increased behavioral problems in RRTI+ children on the scales of withdrawn, somatic complaints, social problems, thought problems, and attention problems, corrected for the effect of the confounders. Effect sizes were small (Cohen's f^2-range: 0.01-0.03). Although mean scores for both internalizing and externalizing problems were significantly increased in RRTI+ compared to RRTI- children, only the effect

Table 3. Hierarchical regression analysis of developmental age obtained by the McCarthy Scales of Children's Abilities (MSCA) in 8-year-old Down syndrome children (n=270).

	ΔR^2	*p*	β step	*p*	β total	*p*
Step 1	0.006					
Socioeconomic status			0.01		0.23	
Childcare			1.16		1.51	
Being breastfed (>1 month)			1.65		1.32	
Siblings			-0.03		-2.66	
Step 2	0.084	***				
Male gender			-6.15	***	-5.90	***
Step 3	0.038					
Congenital heart disease			-2.66	*	-2.17	
Diagnosis of asthma			-2.20		-1.09	
Gastrointestinal disease			-2.73		-2.47	
Eye disease			0.27		0.41	
Impaired hearing			0.87		1.98	
Thyroid disease			-2.25		-2.61	
Step 4	0.031	**				
Presence of RRTI			-4.05	**	-4.05	**

β = unstandardized regression coefficient; β step is the beta for this variable when it was first entered into the equation; β total is the beta for the variable in the final model including all steps. Negative β means lower developmental age due to this variable.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$, RRTI: recurrent respiratory tract infections

size for internalizing problems is relevant (Cohen's f^2 0.03). There was no main effect or interaction effect present for gender, CHD, and impaired hearing on any of the determined variables (data not shown).

The TACQOL-PF was completed for 323 (96%) children (Table 5). We found a decreased HRQoL in $RRTI^+$ children in 4 of the 7 subscales: the scores for physical wellbeing, motor skills, autonomy, and social functioning were decreased as compared to $RRTI^-$ children, corrected for the effect of the confounders ($p<0.01$). Effect sizes were small (Cohen's f^2-range 0.02-0.03). Mean scores for boys and girls were equal, and no interaction between RRTI and gender or CHD was present (data not shown). A significant interaction effect was found of impaired hearing by RRTI for the scales social problems and negative emotions (p-values respectively 0.05 and 0.02). This interaction effect implied that the effect of having RRTI was – for these two subscales – more pronounced in children with impaired hearing, resulting in a lower HRQoL.

Table 4. Results of multiple regression analyses for the Child Behavior Checklist (CBCL) test scores of 8-year-old Down syndrome children with and without recurrent respiratory tract infections (RRTI).

	RRTI$^+$ (n=145)	RRTI$^-$ (n=172)	Regression coefficient (β)[a]	Effect size (f^2)
Withdrawn	3.17 (2.91)[b]	2.02 (2.30)	1.01**	0.03
Somatic complaints	1.68 (2.20)	1.06 (1.60)	0.42	0.01
Anxious/depressed	0.93 (1.34)	0.85 (1.49)	0.06	0.00
Social problems	4.86 (2.10)	3.98 (2.11)	0.05*	0.01
Thought problems	1.48 (1.87)	0.94 (1.43)	0.58**	0.02
Attention problems	7.34 (3.08)	5.81 (3.07)	1.07**	0.03
Delinquent behavior	1.61 (1.58)	1.34 (1.53)	0.23	0.00
Aggressive behavior	8.06 (5.73)	6.62 (5.64)	1.06	0.01
Sexual problems	0.34 (0.83)	0.38 (0.84)	-0.07	0.00
Total score	**34.59 (17.77)**	**26.25 (17.58)**	**6.40****	**0.03**
Internalizing problems[c]	5.72 (4.85)	3.87 (4.15)	1.49**	0.03
Externalizing problems[d]	9.67 (6.84)	7.95 (6.81)	1.29	0.01

[a] β = unstandardized regression coefficient of the effect of RRTI, correcting for the effect of socioeconomic status, childcare attendance, being breastfed (>1 month), siblings, gender, congenital heart defect, diagnosis of asthma, gastrointestinal disease, eye disease, impaired hearing, and thyroid dysfunction. [b] Mean scores are presented with standard deviation between brackets. [c] Combined from the subscales withdrawn, somatic complaints and anxious/depressed. [d] Combined from the subscales delinquent and aggressive behavior.
Higher scores represent increased behavioral problems. RRTI$^+$: children with respiratory tract infections. RRTI$^-$: children without respiratory tract infections.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$. Effect size (f^2): small effect (0.01-0.10), moderate effect (0.10-0.33), large effect (>0.33).

Table 5. Results of multiple regression analyses for the TACQOL-PF test scores of Down syndrome children with and without parent-reported recurrent respiratory tract infections (RRTI).

	RRTI$^+$ (n=148)	RRTI$^-$ (n=175)	Regression coefficient (β)[a]	Effect size (f^2)
Physical wellbeing	26.43 (3.94)[b]	27.95 (2.88)	-1.02**	0.02
Motor skills	26.96 (4.40)	28.59 (3.32)	-1.35**	0.03
Autonomy	25.42 (3.99)	26.99 (3.06)	-1.33**	0.03
Cognitive functioning	22.71 (3.60)	22.80 (3.50)	0.07	0.00
Social functioning	27.43 (3.74)	28.95 (3.21)	-1.19**	0.03
Positive emotions	14.91 (1.87)	15.18 (1.42)	-0.17	0.00
Negative emotions	11.50 (1.98)	11.90 (1.99)	-0.27	0.00

[a] β = unstandardized regression coefficient of the effect of RRTI, correcting for the effect of socioeconomic status, childcare attendance, being breastfed (>1 month), siblings, gender, congenital heart defect, diagnosis of asthma, gastrointestinal disease, eye disease, impaired hearing, and thyroid dysfunction. [b] Mean scores are presented with standard deviation between brackets. Higher scores represent better health related quality of life. RRTI$^+$: children with recurrent respiratory tract infections. RRTI$^-$: children without recurrent respiratory tract infections. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Effect size (f^2): small effect (0.01-0.10), moderate effect (0.10-0.33), large effect (>0.33).

DISCUSSION

We show that parent-reported RRTI is significantly associated with impaired mental and motor development, behavioral problems, and decreased HRQoL in children with Down syndrome. The mean developmental age of children with Down syndrome in the group with RRTI is 3 years and 8 months. This is 5 months lower compared to the group without RRTI. Hierarchical regression analysis shows that 3.1% of the developmental age is exclusively associated with the presence of RRTI. This is more than the association of CHD with developmental age (2.2%), only gender is more associated (5.9%). Also, behavioral problems and decreased HRQoL are more common in RRTI$^+$ children. Furthermore, school enrollment was less favorable in RRTI+ children.

Since RRTI are often accompanied by impaired hearing, specifically verbal skills could be more influenced compared to perceptual-performance, quantitative, memory and motor development in RRTI$^+$ children with Down syndrome. In our sample, impaired hearing was indeed more common in RRTI$^+$ compared to RRTI$^-$ children (44% vs. 18%, $p<0.001$). However, RRTI$^+$ children had lower scores on all developmental scales, with equal effect sizes ($f^2=0.03$-0.04), and no interaction between RRTI and impaired hearing was observed. So, although hearing loss is a common problem in RRTI$^+$ children with Down syndrome, other domains of development are equally decreased compared to the development of verbal skills. In other words, hearing loss does not lead to selective decrease of verbal skills nor does it enhance developmental delay of RRTI$^+$ children, and so does not seem to be a confounder that explains both RRTI and increased developmental delay.

Although RRTI$^+$ children have decreased scores on mental development in the MSCA-test, the TACQOL cognitive functioning subscore is equal in both groups. This means that decreased levels of mental development do not lead to a decreased HRQoL subscore related to cognitive functioning. An explanation for this may be that RRTI$^+$ children are, according to the results of our study, more likely to attend lower levels of education; maybe they fit in better with their classmates, resulting in a better HRQoL subscore related to cognitive functioning in these children.

The parents more often reported the diagnosis of 'asthma' in the RRTI$^+$ children. Although wheezing is a common feature in young children with Down syndrome,[4] the overall incidence of asthma in adults and children with Down syndrome is decreased compared to the general population.[5, 14, 17-19] Therefore, it is doubtful whether these children really suffer from asthma. Additionally, in Down syndrome total and specific IgE-values are decreased compared to controls, and there is no increased prevalence of positive skin prick tests.[20-22] Therefore, the symptoms

of wheezing and dyspnea in young children with Down syndrome are probably caused by mucosal swelling in constitutionally smaller airways during respiratory tract infections, and not attributable to asthma. Since the subgroup of children with Down syndrome with parent-reported 'asthma' was small, no separate effect sizes could be determined.

Strengths of this study include the large sample size and the consistency of the age level of all children (all studied at 8 years). Also, independent test assistants administrated the psychological tests. However, parental report is used to classify the presence or absence of recurrent airway infections, which is a methodological limitation of our study. In this way 46% of the children were classified with RRTI, which is a comparable proportion to a recent national health survey where parents reported 38% of children with Down syndrome aged 6-10 years as having had a head or chest cold in the previous two weeks.[14] Also, our data are in accordance with earlier studies regarding the overall incidence of CHD,[1, 15] and the co-occurrence of hearing impairment in RRTI+ children with Down syndrome, which were also based on parental report in our study.

For a first exploration of the association between RRTI and development, behavioral problems and HRQoL of children with Down syndrome parent-reported RRTI may suffice to attract attention to the potential importance of this frequently encountered problem. However, further research is needed using medical records and physician-based diagnosis to determine a stricter and probably more validly defined group of children as suffering from RRTI.

Many variables that influence development, behavior, and HRQoL – like gender and CHD – cannot be changed, where RRTI potentially can. Therefore, it is important to investigate whether the observed association is based on a causal relationship. If so, better prevention of RRTI might lead to improved functioning in these children. Several causes for RRTI in Down syndrome have been proposed, such as hypotonia and (micro)aspiration. Other causes include anatomical abnormalities and different physiology of the respiratory tract including ear, nose, and throat. More frequent (chronic) ear disease, rhinorrhea, sinusitis, and obstructive sleep apnea have all been described in Down syndrome and are related to impaired hearing.[23] The co-occurrence of RRTI and impaired hearing in Down syndrome has been described before,[24] and is confirmed by our data. This study also shows that the negative effects of the association between RRTI and the HRQoL scales social problems and negative emotions is increased if these children also have impaired hearing. Probably, impaired hearing influences verbal skills, attention, and social

functioning negatively. Therefore, periodic surveillance and active treatment by an ENT-specialist could be important for children with Down syndrome.

Although CHD is a potential risk factor for hospitalization during respiratory tract infections in children with Down syndrome,[25, 26] it has not been described as a cause for RRTI in earlier studies. In this study, CHD is significantly more common in the RRTI$^+$ children, but there is no significant effect of CHD on development, behavior, and HRQoL additional to the effect of RRTI.

Impairment of the immune system in Down syndrome has been described, and has been related to an increased infection rate.[27-29] Most studies involve development and function of T-lymphocytes.[27] B-lymphocytopenia and decreased response to unconjugated pneumococcal vaccinations are related to RRTI in patients without Down syndrome and have been described in Down syndrome as well.[22, 30] The level of impairment of the humoral immune system and its contribution to RRTI in Down syndrome has not been fully elucidated; more research on this topic is needed.

There are limited studies on pathogens of respiratory tract infections in Down syndrome. Present studies mainly report uncommon pathogens or more extreme course of disease.[3] More insight in causative pathogens may lead to specific preventive interventions, i.e. prophylactic antibiotics or additional immunizations.

Conclusion

Parent-reported RRTI in children with Down syndrome are associated with more impaired development, behavioral problems and HRQoL. Therefore, further studies should focus on the question whether this is a causal relationship. If so, better prevention of RRTI in children with Down syndrome might stimulate development, prevent behavioral problems, improve HRQoL and enable better school enrollment.

ACKNOWLEDGEMENTS

The authors would like to thank parents and patients for their participation in this study. We thank dr. Jeanet Bruil for her contribution to this project and Petra Buijs and Nicolette van Kessel for administrating all psychological tests. We thank the Dutch Down syndrome Foundation for their effort in enabling this study and inviting all participants. This study is financed by the Netherlands Organisation for Health Research and Development Zon-Mw (grant 2200.0061).

REFERENCES

1. Weijerman ME, van Furth AM, Vonk Noordegraaf A, van Wouwe JP, Broers CJ, Gemke RJ. Prevalence, neonatal characteristics, and first-year mortality of Down syndrome: a national study. J Pediatr 2008; 152:15-9.
2. Mohangoo AD, van der Pal-Bruin KM, Buitendijk SE. TNO report on congenital defects in the Netherlands 1997-2008. Leiden: Netherlands Organization for Applied Scientific Research, TNO Quality of Life, 2010.
3. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, van den Ende K, et al. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. Pediatrics 2007; 120:e1076-81.
4. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, Kimpen JL, et al. High incidence of recurrent wheeze in children with down syndrome with and without previous respiratory syncytial virus lower respiratory tract infection. Pediatr Infect Dis J 2010; 29:39-42.
5. McDowell KM, Craven DI. Pulmonary complications of Down syndrome during childhood. J Pediatr 2011; 158:319-25.
6. Van Trotsenburg AS, Heymans HS, Tijssen JG, de Vijlder JJ, Vulsma T. Comorbidity, hospitalization, and medication use and their influence on mental and motor development of young infants with Down syndrome. Pediatrics 2006; 118:1633-9.
7. McCarthy D. Manual of the McCarthy scales of children's abilities. San Antonio, TX: The Psychological Corporation; 1972.
8. Achenbach TM. Manual for the child behavior checklist/4-18 and 1991 profile. Burlington, VT: Dept. of Psychiatry, University of Vermont; 1991.
9. Verrips GH, Vogels AGC., Koopman HM, Theunissen NCM, Kamphuis RP, Fekkes M, Wit JM, Verloove-Vanhorick SP. Measuring health-related quality of life in a child population. Eur J Public Health 1999; 9:188-93.
10. Cohen J. Statistical power analysis for the behavioral sciences. Rev. ed. New York: Academic Press; 1977.
11. Tennant PW, Pearce MS, Bythell M, Rankin J. 20-year survival of children born with congenital anomalies: a population-based study. Lancet 2010; 375:649-56.
12. Anthony S, Dorrepaal CA, Kateman H, van der Pal-Bruin KM. TNO report on congenital defects in The Netherlands 1996-2003. Leiden: Netherlands Organisation for Applied Scientific Research, TNO Quality of Life, 2005.
13. Maatta T, Maatta J, Tervo-Maatta T, Taanila A, Kaski M, Iivanainen M. Healthcare and guidelines: a population-based survey of recorded medical problems and health surveillance for people with Down syndrome. J Intellect Dev Disabil 2011; 36:118-26.
14. Schieve LA, Boulet SL, Boyle C, Rasmussen SA, Schendel D. Health of children 3 to 17 years of age with Down syndrome in the 1997-2005 national health interview survey. Pediatrics 2009; 123:e253-60.

15. Stoll C, Alembik Y, Dott B, Roth MP. Epidemiology of Down syndrome in 118,265 consecutive births. Am J Med Genet Suppl 1990; 7:79-83.
16. Van Gameren-Oosterom HBM, Fekkes M, Buitendijk SE, Mohangoo AD, Bruil J, Van Wouwe JP. Development, Problem Behavior, and Quality of Life in a Population Based Sample of Eight-Year-Old Children with Down Syndrome. PLoS ONE 2011; 6:e21879.
17. Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down's syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-7.
18. Forni GL, Rasore-Quartino A, Acutis MS, Strigini P. Incidence of bronchial asthma in Down syndrome [Editorial comment]. J Pediatr 1990; 116:487.
19. Weijerman ME, Brand PL, van Furth MA, Broers CJ, Gemke RJ. Recurrent wheeze in children with Down syndrome: is it asthma? Acta Paediatr 2011.
20. Lockitch G, Ferguson A. Incidence of bronchial asthma in Down syndrome [reply to editorial comment]. J Pediatr 1990; 116:487-8.
21. Mannan SE, Yousef E, Hossain J. Prevalence of positive skin prick test results in children with Down syndrome: a case-control study. Ann Allergy Asthma Immunol 2009; 102:205-9.
22. Verstegen RH, Kusters MA, Gemen EF, de Vries E. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. Pediatr Res 2010; 67:563-9.
23. Shott SR. Down syndrome: common otolaryngologic manifestations. Am J Med Genet C Semin Med Genet 2006; 142C:131-40.
24. Shott SR, Joseph A, Heithaus D. Hearing loss in children with Down syndrome. Int J Pediatr Otorhinolaryngol 2001; 61:199-205.
25. Medrano Lopez C, Garcia-Guereta L, Grueso J, Insa B, Ballesteros F, Casaldaliga J, et al. Respiratory infection in congenital cardiac disease. Hospitalizations in young children in Spain during 2004 and 2005: the CIVIC Epidemiologic Study. Cardiol Young 2007; 17:360-71.
26. Medrano Lopez C, Garcia-Guereta Silva L, Lirio Casero J, Garcia Perez J. [Respiratory infections, Down's syndrome and congenital heart disease: the CIVIC 21 study]. An Pediatr (Barc) 2009; 71:38-46.
27. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol 2009; 156:189-93.
28. Nisihara RM, Utiyama SR, Oliveira NP, Messias-Reason IJ. Mannan-binding lectin deficiency increases the risk of recurrent infections in children with Down's syndrome. Hum Immunol 2010; 71:63-6.
29. Chaushu S, Yefenof E, Becker A, Shapira J, Chaushu G. A link between parotid salivary Ig level and recurrent respiratory infections in young Down's syndrome patients. Oral Microbiol Immunol 2002; 17:172-6.
30. Costa-Carvalho BT, Martinez RM, Dias AT, Kubo CA, Barros-Nunes P, Leiva L, et al. Antibody response to pneumococcal capsular polysaccharide vaccine in Down syndrome patients. Braz J Med Biol Res 2006; 39:1587-92.

Chapter 4

Intrinsic defect of the immune system in children with Down syndrome

M.A.A. Kusters, R.H.J. Verstegen, E.F.A. Gemen, E. de Vries

Clin Exp Immunol. 2009;156(2):189-93.

ABSTRACT

Down syndrome is the most frequent cause of mental retardation in man. Immunological changes in Down syndrome have already been observed since the 1970's. The neurological system appears to be aging precociously, with early occurrence of Alzheimer disease; until now, the observed immunological differences have been interpreted in the same context. Looking back at past and present results of immunological studies in children with Down syndrome in relation to the clinical consequences they suffer we conclude that it is more likely that the immune system in Down syndrome is intrinsically deficient from the very beginning.

Introduction

Down syndrome, or trisomy 21, is the most frequent genetic cause of mental retardation in man; the incidence is approximately 1 in 750 live births.[1] Consequently, doctors frequently see patients with Down syndrome and encounter their complex medical problems. Individuals with Down syndrome are invariably cognitively impaired, although the severity is highly variable. Characteristic facial features and hypotonia are present in almost all. Around 50% suffer from congenital cardiac anomalies. Congenital cataract, abnormalities of the gastro-intestinal tract and orthopaedic, eye and ear problems occur with increased frequency as compared to individuals without Down syndrome. Histopathologic studies show a small and hypocellular brain, and, by the fourth decade, characteristic features of Alzheimer disease.[2, 3]

Autoimmune phenomena such as acquired hypothyroidism, celiac disease and diabetes mellitus occur in higher frequency compared to subjects without Down syndrome. Leukaemia is estimated to be 15-20 times more frequent in Down syndrome.[4-7] Despite advances in treatment, infections – especially pneumonia – and leukaemia are still major causes of morbidity and mortality in Down syndrome.[7-12] The increased frequency of haematological malignancies, autoimmune diseases, and infections in Down syndrome, and the observed high frequency of hepatitis B surface antigen (HBsAg) carriers, has already led to the hypothesis that Down syndrome is associated with abnormalities of the immune system in the 1970's.[4, 13-16] Indeed, many differences between the immune system of individuals with and without Down syndrome have been found throughout the years, and several hypotheses have been formulated which could have consequences for everyday clinical care in Down syndrome, if true (findings relevant for everyday clinical care are summarized in Table 1).

Higher rates of infections, malignancies, and autoimmune phenomena are normally seen in elderly individuals,[17-20] and Down syndrome is therefore hypothesized to be a form of abnormal precocious aging in various articles.[21-23]

NK-cells and innate immunity

The supposedly higher percentages of natural killer (NK) cells found in Down syndrome seems to support this theory of precocious aging,[21, 24] as high percentages of NK-cells are normally seen with aging. However, these studies have been performed in small groups of Down syndrome individuals with single and double colour flowcytometric staining techniques that could not yet differentiate between NK-cells ($CD3^-$) and NK-marker-bearing T-lymphocytes ($CD3^+$). Our recent study on lymphocyte subpopulations in Down syndrome shows lower absolute numbers

Table 1. Overview of differences relevant to everyday clinical care found between the immune systems of individuals with and without Down syndrome since the 1970's.

Lymphocyte subpopulations		Reference
$CD3^-CD16and/or56^+$ NK-cells	Decreased (abs)	25
$CD19^+$ B-lymphocytes	Decreased (abs; %)	24, 25
$CD3^+$ T-lymphocytes	Decreased/normal (abs)	25
$CD3^+CD4^+$ helper-T-lymphocytes	Decreased (abs; %)	25
$CD3^+CD8^+$ cytotoxic T-lymphocytes	Decreased/normal (abs)	25
$CD4^+CD45RA^+$ cells	Decreased (%)	37, 38
Th1/Th2 ratio	Increased	69
CD4/CD8 ratio	Inverted ratio	22
TCR-$\alpha\beta^+$ T-lymphocytes	Decreased (%)	37
$CD8^+CD57^+$ cells	Increased (%)	21
Immunoglobulins		
IgG	Increased >6yr	15, 28, 30
IgM	Decreased >6yr	15, 28
IgA	Increased >6yr/normal	15, 30
IgG1	Increased/normal	30, 70
IgG2	Decreased/normal	30, 70
IgG3	Increased/normal	30, 70
IgG4	Decreased/normal	30, 70
Response to vaccination		
Pneumococcal polysaccharide vaccine	Decreased/normal	28, 54
Tetanus vaccine	Decreased	74
Pertussis vaccine (acellular)	Decreased	58
Hepatitis B vaccine	Decreased/normal	52, 57, 60
Hepatitis A vaccine	Normal	56
Influenza vaccine	Decreased	74
Polio vaccine (oral)	Decreased	59

Abs = absolute counts; CD = cluster of differentiation; Ig = immunoglobulin; NK = natural killer; TCR = T-cell-receptor; Th = helper-T-lymphocyte; yr = years; % = relative counts

of $CD3^-CD16and/or56^+$ NK-cells in all age groups.[25] Populations with different "NK-activity" have been described in the 1980's, capable of low, intermediate, and high cytotoxicity against the NK-sensitive tumour cell line K562, respectively.[26, 27] Several authors describe a significant increase of cells possessing the low "NK-activity" phenotype in Down syndrome, associated with a significant decrease of cells with the intermediate and high "NK-activity" phenotype.[21, 28] With longevity, however, NK-cells with well-preserved cytotoxic function increase.[29]

Thymus and T-lymphocytes

The thymus is smaller in subjects with Down syndrome, even in newborns, and has an abnormal structure.[16, 26, 28, 30-32] This suggests that T-lymphocytes are the core of the problem in Down syndrome, however, children with congenital heart disease who require cardiac surgery with (partial) thymectomy show rapid and permanent changes in T-lymphocyte numbers,[33, 34] but – unlike in Down syndrome – their frequency of infections and autoimmune diseases is not increased.[35] The thymus in Down syndrome shows a decreased proportion of phenotypically mature thymocytes expressing high levels of the αβ-form of the T-cell-receptor (TCR-αβ) and associated CD3-molecule,[36] and overexpression of TNF-α and IFN-γ cytokines.[27] The overexpression of these cytokines suggests a disregulation in cytokine production in Down syndrome and may provide an explanation for the abnormal thymic anatomy and thymocyte maturation.[27] An increased percentage of peripheral T-lymphocytes expressing the alternative γδ-form of the T-cell receptor (TCR-γδ) has been reported,[26, 37] as well as a lower percentage of $CD4^+CD45RA^+$ 'naïve' cells – they are considered to represent cells that have recently emigrated from the thymus – and a higher percentage of $CD29^+$ 'memory' cells.[26, 38] T-cell-receptor excision circle (TREC) counts are used to estimate recent thymic emigrants (V(D)J recombination events excise intervening stretches of DNA).[39] A significantly lower number of $TREC^+$ peripheral blood cells is found in children with Down syndrome in comparison with healthy control children.[23, 40] These findings could be interpreted as early senescence of the immune system,[26, 38] because naïve helper and cytotoxic T-lymphocytes [29, 41] as well as $TREC^+$ peripheral blood cells [42] decrease with aging, while central and effector memory helper-T-lymphocytes and effector memory and terminally differentiated cytotoxic T-lymphocytes increase.[43] We have recently demonstrated a T-lymphocytopenia in *all* age groups, however, not just in older children with Down syndrome, that concerns $CD4^+$ helper- as well as $CD8^+$ cytotoxic T-lymphocytes with absence of the tremendous expansion that is normally seen in the first year of life, suggesting a deficient reaction to antigenic stimulation.[25, 41, 44] Absolute numbers of T-lymphocyte populations gradually approach those of normal children over time,[25] but it is doubtful whether these cells have normal phenotype and function, having shown a lack of the antigen-driven expansion in earlier years. Functional abnormalities of T-lymphocytes that have been described support this: the *in vitro* proliferative response to phytohemagglutinin (PHA), is markedly below normal in infants as well as adults with Down syndrome.[15, 16, 45-47] In addition, bacterial and viral antigen-induced *in vitro* interleukin-2 (IL-2) production is markedly reduced, although PHA-stimulated IL-2 production is not impaired.[13, 42, 43] An interesting hypothesis is that overexpression of the cell adhesion

molecules LFA-1 and DSCAM – located on chromosome 21 – causes higher affinity between cells leading to abnormal maturation and function,[48, 49] but in most genetic studies in trisomy 21 an overall 150% increase of gene expression is not seen; the genetic overexpression is often specific for a particular organ.[50] Enhanced cell death by apoptosis could also play a role, as transgenic copper-zinc superoxide dismutase mice (in humans located on chromosome 21) show enhanced apoptosis.[51]

B-lymphocytes and antibody production

A considerable hypergammaglobulinemia of IgG and IgA after the age of five, with high levels of IgG1 and IgG3 and low levels of IgG2 and IgG4, is described in Down syndrome,[15, 30, 52] with IgM levels decreasing in adolescence. IgD levels are high.[53] Antibody responses to rabbit erythrocytes and Escherichia coli antigens are low,[28] as are the responses to vaccine antigens such as influenza A, oral polio, acellular pertussis, tetanus and polysaccharide pneumococcal vaccine.[54-59] The frequency of hepatitis B virus carriers is much higher among Down syndrome children compared to age-matched controls, however, normal responses to hepatitis A and B vaccinations are seen, although specific IgG-subclasses can vary.[56, 60] Autoantibodies against human thyroglobulin and gliadin are observed more often in children with Down syndrome,[15, 30, 61] as are high titres against casein and beta-lactoglobulin.[15, 61]

Somewhat paradoxically, we have recently found a profound B-lymphocytopenia in Down syndrome, with absence of the normal enormous expansion in the first year of life.[25] This has been described before,[24, 28, 62, 63] but has not attracted much attention so far. Recent observations even show a significant decrease of B lymphocytes ($CD19^+$) in fetuses with Down syndrome.[64] These abnormalities can either be due to an intrinsic B-lymphocyte defect, or to the consequence of deficient helper-T-lymphocyte function causing inadequate control of B-lymphocyte activation and proliferation. The combination of profound B-lymhocyto*penia* and *hyper*gammaglobulinemia suggests the latter, with the possibility that antibody responses may be oligoclonal and/or inadequate in Down syndrome. We have not found any mono- or oligoclonal M-proteins in 88 Down syndrome children, however (unpublished data). Also, in comparison, patients with DiGeorge syndrome (DGS; 22q11-deletion) show a congenital thymic hypoplasia with a variable degree of T-lymphocyte deficiency in 80% of cases.[65, 66] Like in Down syndrome, $TREC^+$ cell counts are decreased in the periphery, and T-lymphocytes gradually approach normal numbers over time.[39] But – unlike in Down syndrome – B-lymphocytopenia is not seen in DGS.[67, 68]

Helper-T-lymphocyte type 1 cells (Th1) produce cytokines such as IFN-γ, IL-2, and TNF-β which stimulate cytotoxic T-lymphocyte (Tc) responses and IgG1 and IgG3 production, whereas helper-T-lymphocyte type 2 cells (Th2) produce cytokines such as IL-4, IL-5, IL-6, and IL-10 which stimulate antibody responses by B-lymphocytes and the formation of IgG2 and IgG4. In comparison to individuals with mental retardation (no Down syndrome) and healthy controls, adults with Down syndrome have significantly higher percentages of IFN-γ-producing $CD4^+$ and $CD8^+$ cells and a higher Th1/Th2 ratio.[69] This fits the increased levels of IgG1 and IgG3 and decreased levels of IgG2 and IgG4 in Down syndrome, and supports disturbed helper-T-lymphocyte function.[30, 70]

Clinical presentation in relation to immunodeficiency

The clinical presentation of children with Down syndrome – seen in relation to possible immunodeficiency [71] – is dominated by recurrent ear-nose-throat (ENT) and airway infections in their early years, followed by an increasing frequency of autoimmune diseases and lymphoproliferation thereafter. The recurrent ENT and airway infections could fit antibody deficiency, although the macroglossia, hypotonia, and altered anatomy of the upper airways will also play an important role in these infants. The tendency towards autoimmune diseases and lymphoproliferation on the other hand primarily points to immunodisregulation. Partial reduction in the number and function of T-lymphocytes can disturb the tolerogenic balance, generating a combination of immunodeficiency and immune disregulation.[72, 73] Down syndrome children as a group fit the picture of primary immunodeficiency but with apparent individual differences. The relation between the abnormality of immunological values in individual children with Down syndrome and the clinical complications has unfortunately not been extensively studied so far.

Conclusion

All in all, it is much more likely that the immune system in Down syndrome is intrinsically deficient from the very beginning, and not simply another victim of a generalised process of precocious aging. It is not yet clear, but at least possible that – besides the apparent thymus and T-lymphocyte abnormalities in Down syndrome – B-lymphocytes are also intrinsically different.

Further studies are needed to resolve the underlying mechanisms of this immunodeficiency, and to assess the implications thereof for everyday clinical care.

ACKNOWLEDGEMENTS

This study was funded by a grant from the JBZ Research Fund.

REFERENCES

1. Hassold T, Jacobs P. Trisomy in man. Ann Rev Genet 1984; 18:69-97.
2. Lott I. Down's syndrome, aging, and Alzheimer's disease: a clinical review. Ann N Y Acad Sci 1982; 396:15-27.
3. Tolksdorf M, Wiedemann H. Clinical aspects of Down's syndrome from infancy to adult life. Hum genetic suppl 1981; 2:3-31.
4. Miller M. Neoplasia and Down's syndrome. 1970; 171:637.
5. Fraumeni J, Manning M, Mitus W. Acute childhood leukemia: epidemiological study by cell type in 1263 cases at the Children's Cancer Research Foundation in Boston. J Natl Cancer Inst 1971; 46:461-70.
6. Fabia J, Droletter M. malformations and leukemia in children with Down syndrome. Pediatrics 1970; 45:60-70.
7. Goldacre M, Wotton C, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-7.
8. Yang Q, Rasmussen S, Friedman J. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 2002; 359:1019-25.
9. Sanchez-Albisua I, Storm W, Wascher I, Stern M. How frequent is coeliac disease in Down syndrome? Eur J Pediatr 2002; 161:683-4.
10. Karlsson B, Gustafsson J, Hedov G, Ivarsson S, Anneren G. Thyroid dysfunction in Down's syndrome: relation to age and thyroid autoimmunity. Arch Dis Child 1998; 79:242-5.
11. Anwar A, Walker J, Frier B. Type 1 diabetes mellitus and Down's syndrome: prevalence, management and diabetic complications. Diabet Med 1998; 15:160-3.
12. Garrison M, Jeffries H, Christakis D. Risk of death for children with Down syndrome and sepsis. J Pediatr 2005; 147:748-52.
13. Burgio G, Lanzavecchia A, Maccario R, Vitiello A, Plebani A, Ugazio A. Immunodeficiency in Down's syndrome: T-lymphocyte subset imbalance in trisomic children. Clin Exp Immunol 1978; 33:298-301.
14. Oster J, Mikkelsen M, Nielsen A. Mortality and life-table in Down's syndrome. Acta Paediatric 1975; 64:322.
15. Burgio G, Ugazio A, Nespoli L, Marcioni A, Botelli A, Pasquali F. Derangements of immunoglobuline levels, phytohemagglutinin responsiveness and T and B cell markers in Down's syndrome at different ages. Eur J Immunol 1975; 5:600-3.
16. Levin S, Schlesinger M, Handzel Z. Thymic deficiency in Down's syndrome. Pediatrics 1979; 63:80-3.
17. Gatti R, Good R. Aging, immunity and malignancy. Geriatrics 1970; 25:158-68.

18. Ginaldi L, De Martinis M, D'ostilio A, Marini L. The immune system in the elderly:II specific cellular immunity. Immunol Res 1999; 20:109-15.
19. Ram J. Aging and immunological phenomena- a review. J Gerontology 1967; 22:92-107.
20. Pawelec G, Barnett Y, Forsey R, Frascu D, Globerson A. T cells and aging, january 2002 update. Front Biosci 2002; 1:1056-83.
21. Cossarrizza A, Monti D, Montagnani G, Ortolani C, Masi M, Zannotti M, et al. Precocious aging of the immune system in Down syndrome: alteration of B lymphocytes, T lymphocyte subsets, and cells with natural killer markers. Am J Med Genet 1990; 7:213-8.
22. Cuadrado E, Barrena M. Immune dysfunction in Down's syndrome: primary immune deficiency or early senescence of the immune system? Clin Immunol Immunopathol 1996; 78:209-14.
23. Roat E, Prada N, Lugli E. Homeostatic cytokines and expansion of regulatory T cells accompany thymic impairment in children with Down syndrome. Rejuv Res 2008; 11:573-83.
24. Cossarrizza A. Age-related expansion of functionally inefficient cells with markers of natural killer activity in Down's syndrome. Blood 1991; 77:1263-70.
25. De Hingh Y, Van der Vossen P, Gemen E, Mulder A, Hop W, Brus F, et al. Intrinsic abnormalities of lymphocyte counts in children with Down syndrome. J Pediatr 2005; 147:744-7.
26. Murphy M, Epstein L. Down syndrome (trisomy 21) thymuses have a decrease proportion of cells expressing high levels of TCRalpha,beta and CD3. Clin Immunol Immunopathol 1990; 55:453-67.
27. Murphy M, Friend D, Pike-Nobile L, Epstein L. Tumor necrosis factor-alpha and IFN-gamma expression in human thymus. J Immunol 1992; 149:2506-12.
28. Ugazio A, Maccario R, Notarangelo L, Burgio G. Immunology of Down syndrome: a review. Am J Med Genet suppl 1990; 7:204-12.
29. Sansoni P, Vescovini R, Fagnoni F, Basini C, Zanni F. The immune system in extreme longevity. Exp Gerontol 2008; 43:61-5.
30. Nespoli L, Burgio G, Ugazio A, Maccario R. Immunological features of Down's syndrome: a review. J Int Dis Res 1993; 37:543-51.
31. Larocca L, Lauriola L, Raneletti F. Morphological and immunohistochemical study of Down syndrome thymus. Am J Med Genet 1990; 7:225-30.
32. Musiani P, Valitutti S, Castellino F. Intrathymic deficient expansion of T cell precursors in Down syndrome. Am J Med Genet 1990; 7:219-24.
33. Yamaguchi T, Murakami A, Fukahara K, Ueda T, Ichida F, Miyawaki T, et al. Changes in T-cell receptor subsets after cardiac surgery in children. Surg Today 2000; 30:875-8.
34. Habermehl P, Knuf M, Kampmann C, Mannhardt W, Schranz D, Kuroczynski W, et al. Changes in lymphocyte subsets after cardiac surgery in children. Eur J Pediatr 2003; 162:15-21.
35. Eysteinsdottir J, Freysdottir J, Haraldsson A, Stefansdottir J, Skaftadottir I, Helgason H, et al. The influence of partial or total thymectomy during open heart surgery in infants on the immune function later in life. Clin Exp Immunol 2004; 136:349-55.

36. Murphy M, Lempert M, Epstein L. Decreased level of T cell receptor expression by Down syndrome (trisomy 21) thymocytes. Am J Med Genet 1990; 7:234-7.
37. Murphy M, Epstein L. Down syndrome peripheral blood contains phenotypically mature CD3 TCRalphabeta cells but abnormal proportions of TCRgammadelta, TCRaphabeta and CD4+45RA+ cells: evidence for an inefficient release of mature T cells by DS thymus. Clin Immunol Immunopathol 1992; 62:245-51.
38. Barrena M, Echaniz P, Garcia-Serrano C, Cuadrado E. Imbalance of the CD4+ subpopulations expressing CD45RA and CD29 antigens in the peripheral blood of adults and children with Down syndrome. Scand J Immunol 1993; 38:323-6.
39. Lavi R, Kamchaisatian W, Sleasman J, Martin D, Haraguchi S, Day N, et al. Thymic output markers indicate immune dysfunction in Digeorge syndrome. J Allergy Clin Immunol 2006; 118:1184-6.
40. Prada N, Nasi M, Troiano L, Roat E, Pinti M, Nemes E, et al. Direct analysis of thymic function in children with Down's syndrome. Imm Aging 2005; 2:1-8 (not for citation purposes).
41. Saule P, Trauet J, Dutriez V, Lekeux V, Dessaint J-P, Labalette M. Accumulation of memory T cells from childhood to old age: Central and effector memory cells in CD4+ versus effector memory and terminally differentiated memory cells in CD8+ compartment. Mech Ageing Dev 2006; 127:274-81.
42. Junge S, Kloeckener-Gruissem B, Zufferey R, Keisker A, Salgo B, Fauchere J, et al. Correlation between recent thymic emigrants and CD31+ (PECAM-1) CD4+ T cells in normal individuals during aging and in lymphopenic children. Eur J Immunol 2007; 37:3270-80.
43. Cossarizza A, Ortolaini C, Paganelli R, Barbieri D, Monti D, Sansoni P, et al. CD45 isoform expression on CD4+ and CD8+ T cells throughout life, from newborns to centenarians: implications for T cell memory. Mech Ageing Dev 1996; 86:173-95.
44. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: The pediatric AIDS clinical trials group P1009 study. J Allergy Clin Immunol 2003; 112:973-80.
45. Agarwal S, Blumberg B, Gerstley B, London W, Sutnick A, Loeb L. DNA polymerase activity as an index of lymphocyte stimulation: studies in Down's syndrome. J Clin Invest 1970; 49:161-9.
46. Gershwin M, Crinella F, Castles J, Trent J. Immunologic characteristics of Down's syndrome. Ment Def Res 1977; 21:237-49.
47. Rigas D, Elsasser P, Hecht F. Impaired in vitro response of circulating lymphocytes to phytohemagglutinin in Down's syndrome: dose- and time-response curves and relation to cellular immunity. Int Arch Allergy Immunol 1970; 39:587-608.
48. Malago W, Sommer C, Del Cistia Andrade C, Soares-Coasta A, Possik P, Cassago A, et al. Gene expression profile of human down syndrome leukocytes. Croatian Med J 2005; 46:647-56.
49. Sustrova M, Sarikova V. Down's syndrome - effect of increased gene expression in chromosome 21 on the function of the immune and nervous system. Bratisl Lek Listy 1997; 98:221-8.
50. Li C, Guo M, Salas M, Schupf N, Silverman W, Zigman W, et al. Cell type-specific over-expression of chromosme 21 genes in fibroblasts and fetal hearts with trisomy 21. BMC Med Genet 2006; 7.

51. Peled-Kamar M, Lotem J, Okon E, Sachs L, Groner Y. Thymic abnormalities and enhanced apoptosis of thymocytes and bone marrow cells in transgenic mice overexpressing Cu/Zn-superoxide dismutase: implications for Down syndrome. EMBO J 1995; 16:4985-93.
52. Avanzini M, Monafo V, De Amici M, Maccario R, Burgio G, Plebani A, et al. Humoral immunodeficiency in Down syndrome: serum IgG subclass and antibody response to hepatitis B vaccine. Am J Med Genet 1990; 7:231-3.
53. McMillan B, Hanson R, Colubjatnikov G, Sinha S. The effect of institutionalisation on elevated IgD and IgG levels in patients with Down's syndrome. J Ment Def Res 1975; 19:209-23.
54. Costa-Carvalho B, Martinez R, Dias A, Kubo C, Barros-Nunes P, Leiva L, et al. Antibody response to pneumococcal capsular polysaccharide vaccine in Down syndrome patients. Braz J Med Biol Res 2006; 39:1587-92.
55. Epstein L, Philip R. Abnormalities of the immune response to influenza antigen in Down syndrome (trisomy 21). Oncol Immunol Down Syndr 1987:163-82.
56. Ferreira C, Leite J, Taniguchi A, Vieira S, Pereira-Lima J, Reverbel da Silveira T. Immunogenicity and safety of an inactivated Hepatitis A vaccine in children with Down syndrome. J Pediatr Gastroenterol Nutr 2004; 39:337-40.
57. Garcia Bengoechea M, Cortes B. Response to recombinant DNA antihepatitis B vaccine in mentally retarded patients with Down syndrome. A controlled study. Med Clinic 1990; 94:528-30.
58. Livolte S, Mattina A. Safety and effectiveness of an acellular pertussis vaccine in subjects with Down Syndrome. Childs Nerv Syst 1996; 12:100-2.
59. McKay E, Hems G, Massie A, Moffat M, Philips K. Serum antibody to poliovirus in patients in a mental deficiency hospital, with particular reference to Down's syndrome. J Hygiene 1978; 81:25-30.
60. Troisi C, Heidelberg D, Hollinger F. Normal immune response to hepatitis B vaccine in patients with Down's syndrome. JAMA 1985; 254:3196-9.
61. Storm W. Prevalence and diagnostic significance of gliadin antibodies in children with Down syndrome. Eur J Pediatr 1990; 149:833-4.
62. Franceschi C, Licastro F, Paolucci P. T and B lymphocyte subsets in Down's syndrome: a study of non-institutionalized subjects. J Ment Def 1987; 22:179-91.
63. Lockitch G. Age-Related changes in humoral and cell-mediated immunity in Down syndrome children living at home. Pediatr Res 1987; 22:536-40.
64. Zizka Z, Calda P, Fait T. Prenatally diagnosable differences in the cellular immunity of fetuses with Down's and Edwards' syndrome. Fetal Diagn Ther 2006; 21:510-4.
65. Pilliero L, Sanford A, McDonald-McGinn D, Zackai E, Sullivan K. T-cell homeostasis in humans with thymic hypoplasia due to chromosme 22q11.2 deletion syndrome. Blood 2004; 103:1020-5.
66. Chinen J, Rosenblatt H, O'Brian Smith E, Shearer W, Noroski L. Long-term assessment of T-cell populations in DiGeorge syndrome. J Allergy Clin Immunol 2003; 111:573-9.
67. Jawad A, McDonald-McGinn D, Zackai E, Sullivan K. Immunologic features of chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome). J Pediatr 2001; 139:715-23.

68. Kourtis A, Ibegbu C, Nahmias A, Lee F, Clark W, Sawyer M, et al. Early progression of disease in hiv-infected infants with thymus dysfunction. NEJM 1996; 335:1431-6.
69. Franciotta D, Verri A, Zardini E, Andreoni L, De Amici M, Moratti R, et al. Interferon-gamma and Interleukin-4-producing T cells in Down's syndrome. Neurosci Lett 2006; 395:67-70.
70. Barradas C, Charlton J, Mendoca P, Lopes A, Palha M, Trindade J. IgG subclasses serum concentrations in a population of children with Down syndrome: Comparative study with siblings and general population. Allergol Immunopathol 2002; 30:57-61.
71. De Vries E. Patient-centred screening for primary immunodeficiency: a multi-stage diagnostic protocol designed for non-immunologists. Clin Exp Immunol 2006; 145:204-14.
72. Liston A, Enders A, Siggs O. Unravelling the association of partial T-cell immunodeficiency and immune dysregulation. Nat Rev Immunol 2008; 8:545-58.
73. Lopes-da-Silva S, Rizzo L. Autoimmunity in Common Variable Immunodeficiency. J Clin Immunolog 2008; 28:46-55.
74. Philip R, Berger A, McManus N, Warner N, Peacock M, Epstein L. Abnormalities of the in vitro cellular and humoral responses to tetanus and influenza antigens with concomitant numerical alterations in lymphocyte subsets in Down syndrome (trisomy 21). J Immunol 1986; 136:1661-7.

Chapter 5

Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help?

R.H.J. Verstegen, M.A.A. Kusters, E.F.A. Gemen, E. de Vries

Pediatr Res. 2010;67(5):557-62.

ABSTRACT

Down syndrome is known for increased incidence of respiratory infections and autoimmune diseases, indicating impaired immunity. Until now, medical attention has been mainly focused on T-lymphocytes. Therefore, we determined B-lymphocyte subpopulations in 95 children with Down syndrome compared to 33 healthy age-matched controls. Serum immunoglobulin levels of children with Down syndrome were compared to 962 children with recurrent infections but without Down syndrome. The results were combined with clinical data. Transitional and naive B-lymphocytes are profoundly decreased in Down syndrome. This could be caused by an intrinsic B-lymphocyte defect resulting in (partial) failure of B-lymphocyte generation, decreased antigen-induced proliferation and/or increased apoptosis, or by decreased proliferation due to deficient T-lymphocyte help, or a combination of these. The decreased $CD27^{+}$, $CD21^{high}$ and $CD23^{+}$ cells are reminiscent of Common Variable Immunodeficiency and suggestive of disturbed peripheral B-lymphocyte maturation. Immunoglobulin levels in patients with Down syndrome are abnormal – as has been described before – and different from children with recurrent infections but without Down syndrome. We conclude that the humoral immune system is abnormal in Down syndrome, but could not find a relation between B-lymphocyte subset counts, immunoglobulin levels and clinical features of the children with Down syndrome in our cohort, nor could we answer the question whether lymphocytes in Down syndrome are truly intrinsically deficient, or could all findings be explained by deficient T-lymphocyte help.

INTRODUCTION

Down syndrome is associated with recurrent – mainly respiratory – infections,[1, 2] decreased responses to vaccination,[3-8] a higher frequency of hepatitis B surface antigen carriers[3] and autoimmune diseases like celiac disease and hypothyroidism.[9-11] These features are suggestive of immunodeficiency. Until now, medical attention has been mainly focused on the thymic alterations and decreased absolute numbers of T-lymphocytes in peripheral blood.[12, 13] We recently showed that a striking B-lymphocytopenia is present from the very beginning in patients with Down syndrome.[14] This B-lymphocytopenia could either be due to an intrinsic B-lymphocyte defect, to deficient T-lymphocyte help, or a combination of these. An intrinsic B-lymphocyte defect could be due to (partial) failure of B-lymphocyte generation, decreased antigen-induced proliferation and/or increased apoptosis. Deficient T-lymphocyte-help could lead to disturbed B-lymphocyte activation and proliferation. Despite the B-lymphocytopenia, a considerable hypergammaglobulinemia of immunoglobulin (Ig) A and IgG after the age of five, with high levels of IgG1 and IgG3 and low levels of IgG2 and IgG4, are described.[3, 15, 16]

This combination of profound B-lymhocytopenia and hypergammaglobulinemia favors a disturbance in T-lymphocyte help, with the possibility that immunoglobulins are oligoclonal in Down syndrome, and specific T-cell-dependent antibody responses inadequate. The latter has indeed been described.[3, 15] However, the T-cell-independent antibody response to pneumococcal polysaccharide antigen is also decreased in Down syndrome,[4] suggesting an intrinsic B-lymphocyte defect is present as well. We studied B-lymphocyte subpopulations in relation to relevant clinical features in 95 children with Down syndrome, to further unravel this question.

METHODS

Study population

From 95 non-institutionalized children with Down syndrome (49 males; see Figure 1) either visiting the Jeroen Bosch Hospital, 's-Hertogenbosch, or the Rijnstate Hospital, Arnhem, The Netherlands, an extra 3ml of EDTA and 7ml of blood without additive was drawn during routine follow-up of thyroid function after parental informed consent. All children were otherwise healthy at the time of sampling.

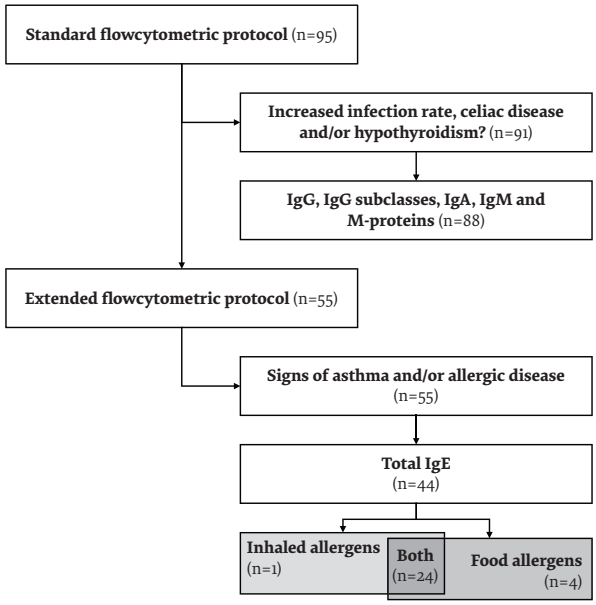


Figure 1. Patient flow diagram. Flow diagram showing group size of Down Syndrome patients in relation to assessed variables.

Leftover EDTA blood from 33 healthy age-matched controls who underwent venipuncture for e.g. pre-operative screening for minor surgery was used as control.

We retrospectively collected the titers of serum IgG, IgA and IgM that were determined for diagnostic purposes in 962 patients without Down syndrome suffering from recurrent infections (<21 years) between January 2006 and July 2008 in the Jeroen Bosch Hospital, 's-Hertogenbosch, and the Bernhoven Hospital, Oss/Veghel, The Netherlands. In 285/962 patients IgG-subclasses were also determined.

The study was approved by the local Medical Ethics Committees of both hospitals.

Immunophenotyping

Three-color flowcytometric immunophenotyping was performed to determine B-lymphocyte subpopulations in both Down syndrome and age-matched controls using the lysed whole-blood method. FITC, phycoerythrin (PE), and PE-cyanin 5 (PE-Cy5) conjugated antibodies were used with the following antigen specificity: CD3 (PE-Cy5; Immunotech, Marseille, France), CD5 (FITC; Becton Dickinson (BD), San Jose, CA, USA), CD10 (FITC; BD), CD16/CD56 (FITC; BD), CD14 (PE; BD), CD15 (FITC; IQProducts, Groningen, The Netherlands), CD19 (PE-Cy5; Immunotech), CD20 (PE; BD), CD21 (PE; BD), CD23 (PE; BD), CD27 (FITC; BD), CD38 (PE; BD), CD45 (PE-Cy5; Immunotech), κ (PE; Dako, Carpinteria, CA, USA), and λ (PE; Dako). In all

children T-lymphocytes ($CD3^+$), B-lymphocytes ($CD19^+$), natural killer (NK) cells ($CD16^+$and/or$CD56^+CD3^-$) and CD21 and CD5 expression on $CD19^+$ B-lymphocytes were determined. An extended protocol was used in the last 55 included children. In this group CD10, κ and λ expression, CD27 and CD20 expression, CD27 and CD38 expression, and CD27 and CD23 expression on $CD19^+$ B-lymphocytes were analyzed as well.

Aliquots were incubated for 15 minutes at room temperature in the dark with different combinations of optimally titrated antibodies. Only in samples which were incubated with anti-κ or anti-λ antibodies the aliquots were washed three times with 0.5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) prior to incubation. Erythrocytes were lysed using FACSLysing solution (BD) according to the manufacturer's protocol. The remaining cells were washed twice with BSA/PBS and analyzed by flow cytometry after calibration with the SPHERO CaliFlow kit (Spherotech, Libertyville, IL, USA) as recommended by the European Society for Clinical Cell Analysis.[17] A FACScan or FACSCalibur flow cytometer (BD) was used in combination with CellQuest or CellQuest Pro software (BD). The lymphocyte-gate was checked with a CD15/CD14/CD45 triple labeling and considered correct if less than 5% contamination was present. T-lymphocytes and NK-cells were used to check whether the 'lymphosum' (B+T+NK) equaled 100±5%. Absolute leukocyte counts were determined with a Sysmex SE-9500 hematology analyzer (Sysmex, Kobe, Japan). Absolute numbers of B-lymphocyte subpopulations were calculated by multiplying the absolute leukocyte count ($x10^9$/l) by the relative total lymphocyte size (%) and relative size of the lymphocyte subpopulation (%).

Immunoglobulins

For 88/95 DS children serum IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM were studied; in 7 children serum was not available. IgG, IgA and IgM were determined by kinetic nephelometry (Beckman Coulter Array 360, Beckman Coulter, Fullerton, CA, USA); IgG-subclasses were assessed by kinetic nephelometry using a human IgG-subclass nephelometry kit (Sanquin Reagents, The Netherlands).

Qualitative M-proteins were assessed by serum electrophoresis on alkaline buffered (pH 9.2) agarose gels by a Hydrasys system (Sebia, GA, USA). In cases of uncertainty, additional serum electrophoresis using immunofixation with monovalent antiserum was performed.

IgE was measured in 44 of the 55 children included in the extended protocol using a sandwich chemiluminescent immunoassay (Immulite 2500, DPC/Siemens, IL, USA); the volume of serum available was insufficient in 11 children. Specific IgE testing (Immulite 2500) of food and inhaled allergens was performed in 28 and 25

children, respectively. When insufficient serum was available, we tested for food allergens only for children aged <2 years and inhaled allergens only for children aged >2 years. The Fp5 food allergen panel (DPC/Siemens) contained egg white, cow's milk, codfish, soya, peanut and wheat allergen. The AlaTOP inhaled allergen panel (DPC/Siemens), contained house mite (Dermatophagoides pteronyssimus), cat dander epithelium, dog dander, Bermuda grass, timothy grass, Penicillium notatum, Alternaria tenuis, birch, Japanese cedar, common ragweed (Ambrosia artemisiifolia), English plantain and Parietaria officinalis allergen. To interpret the IgE results we used our laboratory cut-off values of <50 U/ml for children aged <10 years, and <90 U/ml for children aged >10 years and adults.

Review of medical files

The medical files of 91/95 children with Down syndrome were reviewed retrospectively; 4 files were unavailable. The 91 children were divided into four groups: 1) no increased infection rate, 2) increased infection rate (age at inclusion <8 years), 3) increased infection rate (age at inclusion >8 years), and 4) increased infection rate until, but not after, the age of 8 years. The presence of celiac disease or autoimmune hypothyroidism was noted. Additionally, the 55 patients of the extended protocol were also divided into positive or negative for symptoms of asthma and/or allergic disease (recurrent cough, persistent wheeze, admission on a pediatric ward for asthma exacerbation, clinical response to bronchodilators and/or inhalation corticosteroids, clinical signs of allergic disease).

Statistical analysis

An analysis of variance (completely randomized two-factorial design; $p<0.05$) was applied to the data. For this analysis, we excluded those age groups for which the number of age-matched controls was too low. The two fixed factors in the analysis of variance were age (three age groups: 2-5 years, 5-10 years, and 10-16 years) and the difference between Down syndrome and age-matched controls. Levene's test for equality of error variances was used, results are mentioned in the text only when $p<0.05$. The one sample t-test ($p<0.05$) was used to compare the Ig values of children with Down syndrome and children without Down syndrome suffering from recurrent infections with the mean of age-matched reference values and each other.[18, 19] All analyses were performed with SPSS 16.0 for Windows.

RESULTS

B-lymphocyte subpopulations

The absolute and relative numbers of $CD19^+$ B-lymphocyte subpopulations with results of statistical analysis can be found in Table 1, clinically relevant data are presented in Table 2. We did not find a relation between any of the determined B-lymphocyte subpopulations and the incidence of infections or of allergic complaints and/or asthma in these children with Down syndrome. The values for $CD19^+$ B-lymphocytes were reported before:[14] the $CD19^+$ B-lymphocyte count is decreased significantly in all age groups in Down syndrome as compared to age-matched controls, the enormous expansion which is found in healthy children in the first years of life is lacking.[20] The effect of age on the $CD19^+$ B-lymphocyte count is significantly different between Down syndrome and age-matched controls (interaction for absolute values; Table 1), so this finding is highly significant. The κ/λ ratio is slightly increased in older DS children. $CD5^+$ as well as $CD5^-$ B-lymphocytes follow the pattern of total $CD19^+$ B-lymphocytes in DS children. There is no evident increase of "immature" B-lymphocytes in DS: $CD10^+$ and $CD20^-$ B-lymphocytes do not clearly differ between Down syndrome and age-matched controls.

$CD27^-CD38^{dim}$ naive B-lymphocytes and $CD27^-CD38^+$ transitional B-lymphocytes follow the pattern of total $CD19^+$ B-lymphocytes in DS as well. Unfortunately, we cannot differentiate between $CD27^+CD38^{dim}IgD^+$ marginal zone and $CD27^+CD38^{dim}IgD^-$ memory B-lymphocytes, because the expression of IgD was not determined. Absolute and relative numbers of total $CD27^+$ B-lymphocytes are decreased in Down syndrome as compared to age-matched controls; the absolute numbers show a slight increase during the $CD19^+$ B-lymphocyte expansion in the first years of life, which is less prominent in Down syndrome than in age-matched controls. The $CD27^+CD38^{++}$ plasma cell population is small in peripheral blood, but – unexpectedly – not different between Down syndrome and age-matched controls. The relative and absolute numbers of B-lymphocytes with high expression of CD21 ($CD21^{high}$) are significantly decreased in DS children; the absolute numbers decline with age, but more so in age-matched controls than in children with Down syndrome due to a higher initial peak in the former. The same holds true for CD23. The relative expression of CD23 within the $CD19^+$ B-lymphocyte population shows a far wider range in Down syndrome than in age-matched controls.

Immunoglobulins

The serum levels of IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM found in the children with Down syndrome and in children without Down syndrome but with

Table 1. Absolute and relative numbers of B-lymphocyte subpopulations in Down syndrome children compared to age matched controls.

		9 – 15 months		15 – 24 months		2 – 5 years	
Total $CD19^+$ B-lymphocytes	**DS**	**0.46** (0.19-1.14)		**0.33** (0.16-0.76)		**0.28** (0.13-0.55)	
		18 (9-26)	n=11	**14** (8-24)	n=8	**13** (6-24)	n=16
	AMC	1.47		0.91		0.75 (0.33-0.96)	
		20	n=1	33	n=1	23 (18-34)	n=10
$CD19^+CD5^+$	**DS**	**0.22** (0.06-0.88)		**0.14** (0.04-0.30)		**0.08** (0.04-0.29)	
		49 (31-74)	n=11	**37** (23-55)	n=8	**30** (20-76)	n=16
	AMC	0.64		0.40		0.26 (0.08-0.55)	
		43	n=1	43	n=1	36 (15-59)	n=9
$CD19^+CD10^+$	**DS**	**0.02** (0.01-0.07)		**0.01** (0.01-0.06)		**0.02** (0.00-0.07)	
		0.5 (0.2-2.3)	n=7	**0.4** (0.2-3.4)	n=6	**0.5** (0.1-3.5)	n=7
	AMC	0.03		0.03		0.01 (0.00-0.02)	
		0.4	n=1	0.9	n=1	0.3 (0.1-0.6)	n=10
$CD19^+CD20^-$	**DS**	**0.00** (0.00-0.00)		**0.00** (0.00-0.01)		**0.00** (0.00-0.01)	
		5 (1-8)	n=8	**6** (3-18)	n=6	**5** (1-12)	n=7
	AMC	0.00		0.00		0.00 (0.00-0.01)	
		2	n=1	2	n=1	2 (1-4)	n=10
$CD19^+CD21^-$	**DS**	**0.04** (0.01-0.08)		**0.03** (0.01-0.06)		**0.03** (0.01-0.10)	
		7 (1-16)	n=11	**5** (3-26)	n=8	**9** (4-22)	n=16
	AMC	0.04		0.00		0.05 (0.01-0.08)	
		3	n=1	0	n=1	6 (2-10)	n=9
$CD19^+CD21^{low}$	**DS**	**0.09** (0.03-0.19)		**0.07** (0.04-0.17)		**0.03** (0.02-0.09)	
		22 (6-41)	n=11	**20** (10-29)	n=8	**14** (6-24)	n=16
	AMC	0.22		0.06		0.06 (0.01-0.08)	
		15	n=1	6	n=1	8 (3-18)	n=9

5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
0.19 (0.06-0.45)		**0.11** (0.05-0.58)		**0.11** (0.04-0.12)		p<0.001	p<0.001	p=0.001
9 (5-22)	n=38	**8** (4-20)	n=19	**6** (3-7)	n=3	p<0.001	p<0.001	NS
0.45 (0.32-0.66)		0.32 (0.19-0.60)		0.26 (0.23-0.29)				
17 (12-21)	n=8	16 (11-21)	n=11	14 (10-17)	n=2			
0.06 (0.01-0.15)		**0.03** (0.02-0.22)		**0.02** (0.01-0.02)		p<0.001	p<0.001	p=0.003
29 (9-46)	n=38	**27** (16-47)	n=19	**21** (19-24)	n=3	NS	p=0.007	NS
0.13 (0.1-0.22)		0.08 (0.02-0.28)		0.05 (0.04-0.06)				
29 (21-38)	n=8	24 (7-46)	n=11	20 (13-27)	n=2			
0.01 (0.00-0.02)		**0.00** (0.00-0.02)		**0.01** (0.00-0.01)		NS	p=0.003	p=0.031
0.2 (0.04-1.4)	n=19	**0.1** (0.06-1.1)	n=13	**0.3** (0.04-0.5)	n=2	NS	NS	p=0.048
0.01 (0.00-0.04)		0.01 (0.00-0.02)		0.01 (0.00-0.01)				
0.3 (0.09-1.6)	n=8	0.3 (0.03-0.7)	n=11	0.3 (0.2-0.4)	n=2			
0.00 (0.00-0.01)		**0.00** (0.00-0.00)		**0.00** (0.00-0.00)		NS	NS	NS
4 (2-20)	n=19	**3** (1-9)	n=13	**2** (2-2)	n=2	p<0.001	NS	NS
0.00 (0.00-0.00)		0.00 (0.00-0.01)		0.00 (0.00-0.00)				
2 (1-3)	n=7	1 (0-8)	n=11	4 (3-5)	n=2			
0.02 (0.00-0.17)		**0.01** (0.00-0.05)		**0.00** (0.00-0.02)		NS	p<0.001	NS
11 (2-39)	n=38	**9** (4-20)	n=19	**6** (3-14)	n=3	p<0.001	NS	NS
0.02 (0.00-0.04)		0.01 (0.00-0.02)		0.02 (0.01-0.02)				
3 (1-8)	n=8	2 (1-4)	n=11	5 (5-5)	n=2			
0.03 (0.01-0.09)		**0.01** (0.00-0.07)		**0.01** (0.01-0.02)		p=0.05	p<0.001	NS
15 (5-28)	n=38	**14** (4-20)	n=19	**13** (10-17)	n=3	p<0.001	NS	NS
0.04 (0.02-0.06)		0.02 (0.00-0.05)		0.03 (0.02-0.03)				
6 (2-11)	n=8	6 (5-10)	n=11	9 (7-10)	n=2			

Table 1. (Continued)

		9 – 15 months		15 – 24 months		2 – 5 years	
$CD19^+CD21^{high}$	**DS**	**0.25** (0.15-0.98)		**0.25** (0.09-0.65)		**0.22** (0.11-0.43)	
		69 (48-93)	n=11	**70** (52-86)	n=8	**77** (62-84)	n=16
	AMC	1.22		0.87		0.68 (0.25-0.76)	
		82	n=1	94	n=1	86 (72-95	n=9
$CD19^+CD23^+$	**DS**	**0.36** (0.08-0.56)		**0.23** (0.07-0.53)		**0.24** (0.08-0.37)	
		66 (28-80)	n=8	**73** (26-88)	n=6	**61** (47-98)	n=7
	AMC	1.38		0.60		0.62 (0.29-0.93)	
		92	n=1	64	n=1	82 (66-98)	n=10
$CD19^+CD27^+$	**DS**	**0.04** (0.03-0.06)		**0.04** (0.03-0.06)		**0.05** (0.02-0.08)	
		1.2 (0.9-2.3)	n=8	**1.5** (1.0-2.2)	n=6	**1.9** (1.0-2.6)	n=7
	AMC	0.06		0.08		0.12 (0.05-0.18)	
		0.9	n=1	2.7	n=1	3.9 (1.9-6.1)	n=10
$CD19^+CD27^-CD38^{DIM}$	**DS**	**0.35** (0.15-0.79)		**0.17** (0.08-0.56)		**0.25** (0.08-0.37)	
		64 (41-85)	n=8	**63** (48-82)	n=6	**66** (60-71)	n=7
	AMC	1.07		0.58		0.50 (0.18-0.56)	
		73	n=1	63	n=1	57 (47-67)	n=10
$CD19^+CD27^-CD38^+$	**DS**	**0.13** (0.04-0.24)		**0.06** (0.03-0.09)		**0.06** (0.02-0.08)	
		27 (7-49)	n=8	**17** (8-36)	n=6	**15** (7-21)	n=7
	AMC	0.33		0.23		0.10 (0.05-0.31)	
		22	n=1	25	n=1	18 (7-36)	n=10
$CD19^+CD27^+CD38^{++}$	**DS**	**0.02** (0.00-0.03)		**0.02** (0.00-0.03)		**0.01** (0.00-0.03)	
		3 (0-7)	n=8	**5** (2-15)	n=6	**3** (1-8)	n=7
	AMC	0.01		0.01		0.01 (0.01-0.02)	
		0.4	n=1	1	n=1	1 (1-4)	n=10

5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
0.14 (0.04-0.33)		**0.09** (0.03-0.48)		**0.08** (0.03-0.10)		p<0.001	p<0.001	p=0.006
73 (42-86)	n=38	**77** (60-92)	n=19	**81** (69-88)	n=3	p<0.001	NS	NS
0.42 (0.34-0.62)		0.30 (0.15-0.56)		0.23 (0.21-0.25)				
90 (82-93)	n=8	91 (88-95)	n=11	86 (85-88)	n=2			
0.10 (0.04-0.26)		**0.08** (0.03-0.32)		**0.06** (0.02-0.09)		p<0.001	p<0.001	p=0.017
54 (35-87)	n=19	**75** (39-94)	n=13	**73** (63-83)	n=2	p<0.001	NS	NS
0.38 (0.27-0.60)		0.27 (0.15-0.52)		0.20 (0.16-0.24)				
82 (65-94)	n=8	85 (62-93)	n=11	75 (68-81)	n=2			
0.03 (0.01-0.06)		**0.01** (0.00-0.07)		**0.01** (0.01-0.01)		p<0.001	p<0.001	p=0.041
1.6 (0.6-3.4)	n=19	**1.0** (0.2-2.6)	n=13	**0.6** (0.4-0.8)	n=2	p<0.001	p<0.001	NS
0.08 (0.05-0.11)		0.04 (0.01-0.15)		0.05 (0.04-0.06)				
2.9 (1.7-4.1)	n=8	1.7 (0.8-5.0)	n=11	2.6 (2.5-2.7)	n=2			
0.10 (0.04-0.25)		**0.07** (0.03-0.44)		**0.06** (0.02-0.09)		p<0.001	p<0.001	NS
58 (49-80)	n=19	**67** (55-82)	n=13	**70** (57-82)	n=2	NS	p=0.001	p=0.014
0.31 (0.24-0.42)		0.22 (0.14-0.43)		0.20 (0.18-0.22)				
66 (59-78)	n=8	71 (59-77)	n=11	72 (68-77)	n=2			
0.02 (0.01-0.10)		**0.01** (0.00-0.05)		**0.01** (0.00-0.01)		p<0.001	p<0.001	NS
13 (5-30)	n=19	**8** (5-25)	n=13	**7** (5-9)	n=2	NS	p=0.031	NS
0.05 (0.02-0.15)		0.04 (0.01-0.09)		0.04 (0.04-0.04)				
12 (5-22)	n=8	12 (3-21)	n=11	14 (12-16)	n=2			
0.01 (0.00-0.03)		**0.00** (0.00-0.01)		**0.00** (0.00-0.00)		NS	p<0.001	NS
3 (1-17)	n=19	**2** (0-8)	n=13	**0** (0-1)	n=2	p=0.005	NS	NS
0.01 (0.00-0.01)		0.00 (0.00-0.01)		0.01 (0.00-0.01)				
1 (0-3)	n=8	1 (0-1)	n=11	2 (1-4)	n=2			

Table 1. (Continued)

		9 – 15 months		15 – 24 months		2 – 5 years	
$CD19^+CD27^+CD38^{DIM}$	**DS**	**0.02** (0.01-0.03)		**0.01** (0.01-0.02)		**0.03** (0.01-0.04)	
		3 (2-7)	n=8	**5** (2-7)	n=6	**6** (4-10)	n=7
	AMC	0.07		0.06		0.13 (0.01-0.20)	
		5	n=1	7	n=1	17 (1-22)	n=10
$CD19^+\kappa^+$	**DS**	**0.32** (0.16-0.38)		**0.18** (0.15-0.37)		**0.23** (0.06-0.31)	
		59 (57-64)	n=6	**57** (54-60)	n=4	**60** (50-66)	n=7
	AMC	0.85		0.49		0.44 (0.19-0.55)	
		58	n=1	56	n=1	57 (51-62)	n=9
$CD19^+\lambda^+$	**DS**	**0.19** (0.11-0.25)		**0.13** (0.09-0.25)		**0.12** (0.06-0.24)	
		39 (33-42)	n=6	**40** (37-41)	n=4	**39** (36-45)	n=7
	AMC	0.55		0.41		0.37 (0.12-0.43)	
		39	n=1	46	n=1	45 (33-52)	n=9
κ^+ / λ^+ ratio	**DS**	**1.5** (1.4-2.0)	n=6	**1.4** (1.4-1.6)	n=4	**1.5** (1.1-1.8)	n=7
	AMC	1.5	n=1	1.2	n=1	1.2 (1.0-1.9)	n=9

Grey shaded areas: absolute numbers of B-lymphocyte subpopulations (10^9 cells/l), blank background: relative numbers (%); AMC = age-matched control children; DS = Down syndrome children; NS = not significant; * Analysis of variance, 2 to 16 years, DS versus AMC children. ** Analysis of variance, DS and AMC children together (2 to 16 years), effect of age. † Analysis of variance, interaction effect between DS and AMC children aged 2-16 years.

Table 2. Clinical features.

	9 – 15 months (n=11)	15 – 24 months (n=4)	2 – 5 years (n=16)	5 – 10 years (n=38)	10 – 16 years (n=19)	>16 years (n=3)	Total (n=91)
No increased infection rate (all ages)	n=5		n=6	n=5	n=1	n=2	n=19
Increased - mainly respiratory - infection rate (age at inclusion <8 years)	n=6	n=4	n=10	n=20			n=40
Increased - mainly respiratory - infection rate until, but not after, the age of 8 years				n=6	n=12	n=0	n=18
Increased - mainly respiratory - infection rate (age at inclusion >8 years)				n=7	n=6	n=1	n=14
Celiac disease	0/11*	1/4	1/16	2/38	1/19	0/3	5/91
Auto-immune hypothyroidism	0/11	1/4	0/16	1/38	1/19	0/3	3/91

5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
0.01 (0.00-0.03)		**0.01** (0.00-0.03)		**0.01** (0.00-0.01)		p<0.001	p<0.001	p=0.018
7 (2-11)	n=19	**6** (1-17)	n=13	**9** (5-12)	n=2	p<0.001	NS	NS
0.07 (0.04-0.10)		0.03 (0.01-0.16)		0.02 (0.01-0.03)				
14 (11-20)	n=8	10 (5-28)	n=11	8 (5-11)	n=2			
0.10 (0.03-0.21)		**0.07** (0.04-0.41)		**0.05** (0.02-0.07)		p<0.001	p<0.001	NS
62 (51-68)	n=17	**63** (58-72)	n=10	**62** (58-67)	n=2	p=0.001	NS	NS
0.23 (0.18-0.39)		0.18 (0.10-0.35)		0.15 (0.13-0.16)				
56 (50-64)	n=8	58 (54-65)	n=11	55 (55-55)	n=2			
0.06 (0.02-0.14)		**0.04** (0.02-0.17)		**0.03** (0.01-0.05)		p<0.001	p<0.001	p=0.04
36 (29-48)	n=17	**36** (31-42)	n=10	**38** (35-42)	n=2	p<0.001	NS	NS
0.20 (0.16-0.24)		0.13 (0.08-0.25)		0.11 (0.09-0.12)				
44 (38-50)	n=8	42 (31-45)	n=11	44 (42-46)	n=2			
1.7 (1.2-2.1)	n=17	**1.7** (1.4-2.3	n=10	**1.6** (1.6-1.7)	n=2	p<0.001	NS	NS
1.3 (1.1-1.6)	n=8	1.4 (1.2-2.1)	n=11	1.3 (1.2-1.3)	n=2			

	9 – 15 months (n=8)	15 – 24 months (n=6)	2 – 5 years (n=7)	5 – 10 years (n=19)	10 – 16 years (n=13)	>16 years (n=2)	Total (n=55)
Asthma	4/8**	3/6**	0/7	3/19	0/13	0/2	10/55
Allergy	1/8	1/6	0/7	0/19	0/13	0/2	2/55
Total IgE elevated	0/6	1/3	1/5	2/17	2/12	0/1	6/44
Specific IgE inhaled allergens present	0/2	0/0	0/3	0/12	0/7	0/1	0/25
Specific IgE food allergens present	0/3	0/2	0/4	0/12	0/6	0/1	0/28

*0/11 means 0 out of 11 patients tested, etc. **It is doubtful whether these children will continue to have the diagnosis of asthma in later years.[21]

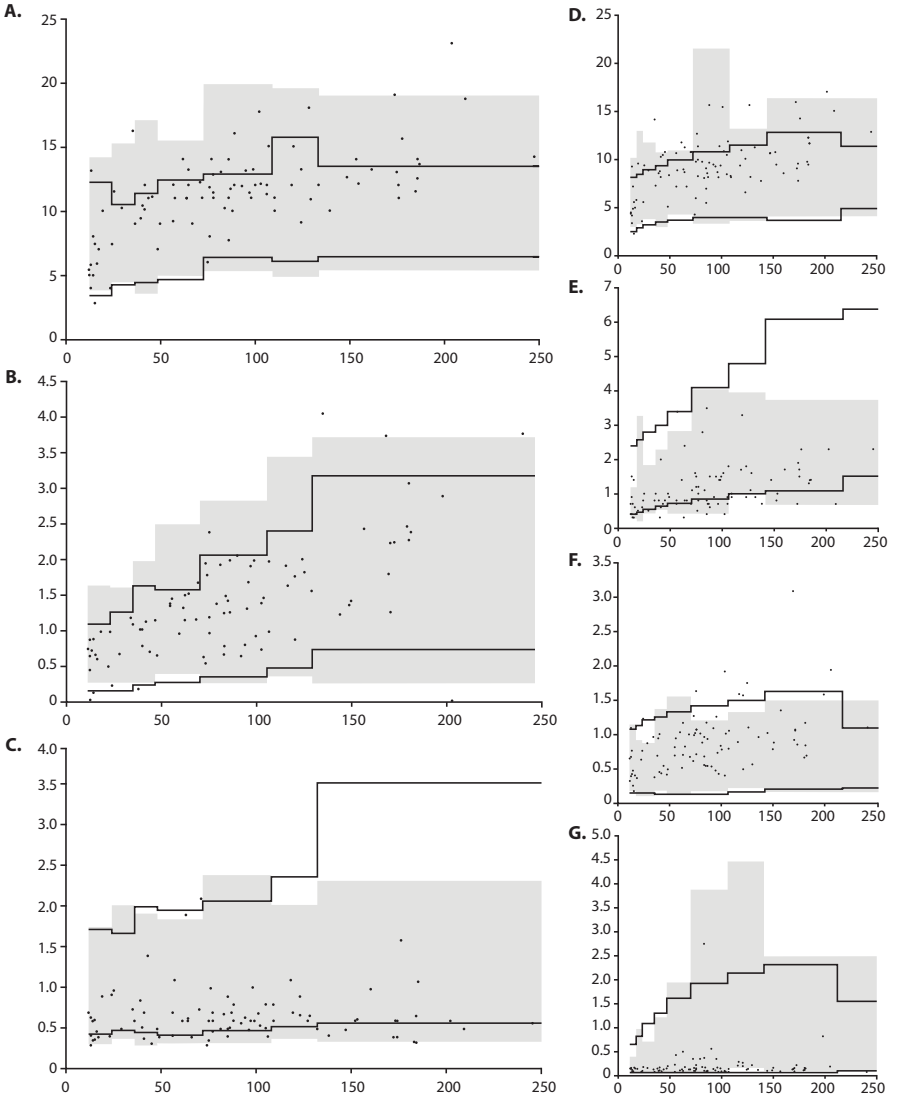


Figure 2. Immunoglobulin values in Down syndrome compared to reference values and children suffering from recurrent infections. Values of IgG (**A**), IgA (**B**), IgM (**C**), IgG1 (**D**), IgG2 (**E**), IgG3 (**F**), and IgG4 (**G**) obtained in 88 DS children are shown as black dots. X-axis age in months; Y-axis immunoglobulin levels (g/l). The grey areas represent the values between the p2.5 and p97.5 of the determined immunoglobulin levels per age group in patients suffering from recurrent infections. Age matched reference values (p2.5 and p97.5) are shown as a black line.[18, 19]

increased infection rates (see Materials & Methods) in comparison to age-matched reference values are shown in Figure 2. In Down syndrome, mean IgG and IgG1 are already higher than the age-matched reference values from the ages of 2 and 3 years onwards, respectively (one-sample t-test; $p<0,05$). Mean IgA and IgG3 are normally distributed, but mean IgM and IgG2 are lower in children with Down syndrome in all age groups. IgG4 values are consistently very low in Down syndrome. Mean Ig serum levels in the children without Down syndrome but with increased infection rates are similar to Down syndrome for IgA and IgG2, but mean IgG is higher in children with Down syndrome in some of the older age groups, and mean IgG1 and IgG3 are higher in Down syndrome from the ages of 3 and 2 years onwards, respectively. Mean IgM and IgG4 are lower in Down syndrome than in the children without Down syndrome but with increased infection rates in the older age groups. We did not find any mono- or oligoclonal M-proteins in the 88 children with Down syndrome tested. IgE is increased in 6 of the 44 children with Down syndrome tested; 5 show high relative percentages of $CD23^+$ B-lymphocytes which are within the range of the age-matched controls (one with asthma; data not shown). Specific IgE testing of food and inhaled allergens is negative in all children tested.

DISCUSSION

The profound B-lymphocytopenia in children with Down syndrome, with decreased transitional and naive B-lymphocytes as compared to age-matched controls, is the most striking result of our study. There are no indications for release of unusual numbers of "immature" B-lymphocytes from the bone marrow ($CD10^+$, $CD20^-$), the cells show the normal phenotype of the transitional and naive stages of peripheral B-lymphocyte development.[22] As stated before, this could be caused by decreased B-lymphocyte proliferation due to a disturbance in T-lymphocyte help, an intrinsic B-lymphocyte defect, or a combination of these.

Interestingly, the distribution of B-lymphocyte subpopulations is reminiscent of the situation found in common variable immunodeficiency (CVID) patients: $CD27^+$ cells, $CD21^{high}$ cells and $CD23^+$ cells are decreased in absolute and relative numbers in the children with Down syndrome.[23-25] These findings are suggestive of an intrinsic defect in B-lymphocyte maturation in the periphery.

CD21 is the complement type 2 receptor; it has a role in the response to polysaccharide antigens like pneumococcal capsular elements. These antigens form a complex with CD21 on B-lymphocytes causing a T-cell-independent response. The lower response to unconjugated pneumococcal vaccination and the

increased rate of respiratory infections in Down syndrome could be related to this decreased expression of CD21. Interestingly, a subgroup of CVID patients with relatively increased $CD21^{low}$ B-lymphocytes are more likely to develop splenomegaly, auto-immune diseases and lower respiratory tract infections;[26] the latter two are frequently found in Down syndrome as well.

CD23 is the low-affinity IgE-receptor (FcεRII), it is a ligand of CD21. Together, they stimulate B-lymphocyte proliferation and differentiation.[27] CD23-expression is increased just before the class-switch from IgM to IgG, IgA or IgE.[27] Besides, CD23 is involved in both positive and negative feedback-loops for IgE-homeostasis.[27] Interestingly, both asthma incidence (RR 0.4, 95% CI 0.2-0.6) and IgE-levels are decreased in Down syndrome,[28,29] which is consistent with our findings. Our results suggest that increased IgE production is associated with a higher level of CD23 expression in children with Down syndrome.

The serum Ig values in children with Down syndrome – with or without recurrent infections – and children without Down syndrome but with recurrent infections are both abnormal, but differ from each other. Decreased IgG2 is a well-known abnormality in children with recurrent infections; this coincides with the findings in Down syndrome. However, the increased IgG, IgG1, and IgG3, and decreased IgM and IgG4 are only found in the children with Down syndrome.

In conclusion, we found that humoral immunity is disturbed in children with Down syndrome. We could not differentiate between an intrinsic B-lymphocyte defect and disturbed T-lymphocyte help as the most important cause based on our present data. This question remains unanswered, and further studies are needed to solve it.

ACKNOWLEDGMENTS

We would like to thank patients and parents for their participation in this study and Mr. Jeroen Ooms for technical assistance in preparing Figure 2. This study was supported financially by the JBZ Research Fund and the Irene Foundation.

REFERENCES

1. Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down's syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-1017.

2. Garrison MM, Jeffries H, Christakis DA. Risk of death for children with down syndrome and sepsis. J Pediatr 2005; 147:748-752.
3. Avanzini MA, Monafo V, De AM, Maccario R, Burgio GR, Plebani A, et al. Humoral immunodeficiencies in Down syndrome: serum IgG subclass and antibody response to hepatitis B vaccine. Am J Med Genet Suppl 1990; 7:231-233.
4. Costa-Carvalho BT, Martinez RM, Dias AT, Kubo CA, Barros-Nunes P, Leiva L, et al. Antibody response to pneumococcal capsular polysaccharide vaccine in Down syndrome patients. Braz J Med Biol Res 2006; 39:1587-1592.
5. Ferreira CT, Leite JC, Taniguchi A, Vieira SM, Pereira-Lima J, da Silveira TR. Immunogenicity and safety of an inactivated hepatitis A vaccine in children with Down syndrome. J Pediatr Gastroenterol Nutr 2004; 39:337-340.
6. Epstein LB, Philip R. Abnormalities of the immune response to influenza antigen in Down syndrome (trisomy 21). Prog Clin Biol Res 1987; 246:163-182.
7. Philip R, Berger AC, McManus NH, Warner NH, Peacock MA, Epstein LB. Abnormalities of the in vitro cellular and humoral responses to tetanus and influenza antigens with concomitant numerical alterations in lymphocyte subsets in Down syndrome (trisomy 21). J Immunol 1986; 136:1661-1667.
8. McKay E, Hems G, Massie A, Moffat MA, Phillips KM. Serum antibody to poliovirus in patients in a mental deficiency hospital, with particular reference to Down's syndrome. J Hyg (Lond) 1978; 81:25-30
9. Karlsson B, Gustafsson J, Hedov G, Ivarsson SA, Anneren G. Thyroid dysfunction in Down's syndrome: relation to age and thyroid autoimmunity. Arch Dis Child 1998; 79:242-245.
10. Sanchez-Albisua I, Storm W, Wascher I, Stern M. How frequent is coeliac disease in Down syndrome? Eur J Pediatr 2002; 161:683-684.
11. Storm W. Prevalence and diagnostic significance of gliadin antibodies in children with Down syndrome. Eur J Pediatr 1990; 149:833-834.
12. Burgio GR, Lanzavecchia A, Maccario R, Vitiello A, Plebani A, Ugazio AG. Immunodeficiency in Down's syndrome: T-lymphocyte subset imbalance in trisomic children. Clin Exp Immunol 1978; 33:298-301.
13. Levin S, Schlesinger M, Handzel Z, Hahn T, Altman Y, Czernobilsky B, Boss J. Thymic deficiency in Down's syndrome. Pediatrics 1979; 63:80-87.
14. De Hingh YC, van der Vossen P, Gemen EF, Mulder AB, Hop WC, Brus F, et al. Intrinsic abnormalities of lymphocyte counts in children with down syndrome. J Pediatr 2005; 147:744-747.
15. Burgio GR, Ugazio AG, Nespoli L, Marcioni AF, Bottelli AM, Pasquali F. Derangements of immunoglobulin levels, phytohemagglutinin responsiveness and T and B cell markers in Down's syndrome at different ages. Eur J Immunol 1975; 5:600-603.
16. Nespoli L, Burgio GR, Ugazio AG, Maccario R. Immunological features of Down's syndrome: a review. J Intellect Disabil Res 1993; 37 (Pt 6):543-551.

17. Kraan J, Gratama JW, Keeney M, D'Hautcourt JL. Setting up and calibration of a flow cytometer for multicolor immunophenotyping. J Biol Regul Homeost Agents 2003; 17:223-233.
18. Jolliff CR, Cost KM, Stivrins PC, Grossman PP, Nolte CR, Franco SM, et al. Reference intervals for serum IgG, IgA, IgM, C3, and C4 as determined by rate nephelometry. Clin Chem 1982; 28:126-128.
19. Vlug A, Nieuwenhuys EJ, van Eijk RV, Geertzen HG, van Houte AJ. Nephelometric measurements of human IgG subclasses and their reference ranges. Ann Biol Clin (Paris) 1994; 52:561-567.
20. Comans-Bitter WM, de Groot R., van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1979; 130:388-393.
21. Martinez FD. Development of wheezing disorders and asthma in preschool children. Pediatrics 2002; 109:362-367.
22. Van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med 2007; 204:645-655.
23. Warnatz K, Wehr C, Drager R, Schmidt S, Eibel H, Schlesier M, Peter HH. Expansion of CD19(hi) CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia. Immunobiology 2002; 206:502-513.
24. Moratto D, Gulino AV, Fontana S, Mori L, Pirovano S, Soresina A, et al. Combined decrease of defined B and T cell subsets in a group of common variable immunodeficiency patients. Clin Immunol 2006; 121:203-214.
25. Warnatz K, Denz A, Drager R, Braun M, Groth C, Wolff-Vorbeck G, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 2002; 99:1544-1551.
26. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood 2008; 111:77-85.
27. Gould HJ, Beavil RL, Reljic R, Shi J, Ma CW, Sutton BJ, Ghirlando R 1997 IgE homeostasis: is CD23 the safety switch? In: Vercelli D (ed) IgE Regulation: Molecular Mechanisms. John Wiley and Sons, New York, pp 35-59.
28. Forni GL, Rasore-Quartino A, Acutis MS, Strigini P. Incidence of bronchial asthma in Down syndrome *(Editorial comment)*. J Pediatr 1990; 116:487
29. Lockitch G, Ferguson A. Incidence of bronchial asthma in Down syndrome *(Reply to editorial comment)*. J Pediatr 1990; 116:487-488.

Chapter 6

Defective B-cell memory in patients with Down syndrome

R.H.J. Verstegen, G.J. Driessen, S.J.W. Bartol, C.J.M. van Noesel, L. Boon, M. van der Burg, J.J.M. van Dongen, E. de Vries, M.C. van Zelm

J Allergy Clin Immunol. 2014;134(6):1346-53.

ABSTRACT

Background

Patients with Down syndrome carry immunological defects, as evidenced by the increased risks for autoimmune diseases, hematological malignancies and respiratory tract infections. Moreover, the low numbers of circulating B cells suggest impaired humoral immunity.

Objective

We sought to study how the immunodeficiency in Down syndrome results from immunological defects in the B-cell compartment.

Methods

We studied the blood B-cell subset composition, replication history, somatic hypermutation status and class switch recombination in 17 children with Down syndrome. Germinal center and plasma cells were studied in tonsils from 4 additional children with Down syndrome.

Results

Blood transitional B-cell numbers were normal, but naive mature and memory B-cell numbers were reduced despite slightly increased serum BAFF levels. Germinal centers and plasma cells in tonsil appeared normal, as were serum Ig levels. $CD27^+IgD^+IgM^+$ "natural effector" B cells showed reduced proliferation and somatic hypermutation levels, while these were normal in $CD27^+IgD^-$ memory B cells. Furthermore, IgM^+ and IgA^+, but not IgG^+, memory B cells showed impaired molecular signs for antigen selection. The B-cell pattern was highly similar to that of common variable immunodeficiency patients with a defect in B-cell activation and proliferation.

Conclusion

Children with Down syndrome seem capable of normal germinal center and plasma cell formation. Still, blood memory B-cell numbers were reduced and showed impaired molecular maturation of IgA and IgM, which are important for mucosal immunity. The observed molecular defects in circulating IgA and IgM B-cell memory could reflect impaired local responses, which underlie the increased susceptibility to respiratory tract infections of patients with Down syndrome.

INTRODUCTION

Down syndrome is the most common genetic cause of developmental delay in humans and is associated with health issues that include hypotonia, congenital heart disease and gastro-intestinal malformations. Furthermore, high incidences of recurrent respiratory tract infections, hematological malignancies and autoimmune diseases are reported,[1-3] which suggest an immune defect. Indeed, laboratory studies have demonstrated abnormalities in the blood B- and T-cell compartments, as well as decreased IgM, IgG2 and IgG4 levels and poor Ig responses to vaccines.[4, 5]

Upon antigen stimulation, naive mature B cells differentiate into memory B and plasma cells. Activated B cells induce somatic hypermutations (SHMs) in the variable regions of their Ig heavy and light chains. The mutated Ig molecules are subsequently selected for antigen-affinity. In addition, the B cells can induce class-switch recombination to change the IgH isotype region from IgM into IgG, IgA or IgE. Based on their IgH isotype and expression of CD27, six memory B-cell subsets can be identified in blood that have been derived from three distinct pathways (Figure 1A).[6]

Some of the clinical and immunological features found in Down syndrome resemble common variable immunodeficiency (CVID). CVID is a primary immunodeficiency, characterized by sinopulmonary infections and idiopathic hypogammaglobulinemia.[7] CVID has a heterogeneous pathophysiology, which can be visualized by flowcytometric analysis of the blood B-cell compartment.[8] In addition to memory B-cell defects, a subset of CVID patients carry increased $CD21^{low}CD38^{-}$ B cells. These $CD21^{low}$ B cells are mostly naive, express highly autoreactive antibodies, are more prone to die by apoptosis and display functional anergy.[9]

To study how the immunodeficiency in children with Down syndrome results from immunological defects in the B-cell compartment, we performed detailed cellular and molecular analysis of their B lymphocytes. The results were compared with age-matched healthy controls and the previously described CVID subgroups.[8]

METHODS

Patients

Clinical data, blood samples and tonsils were collected from children with Down syndrome after written informed consent was obtained from their parents. In addition, we collected blood from healthy age-matched controls and tonsils from

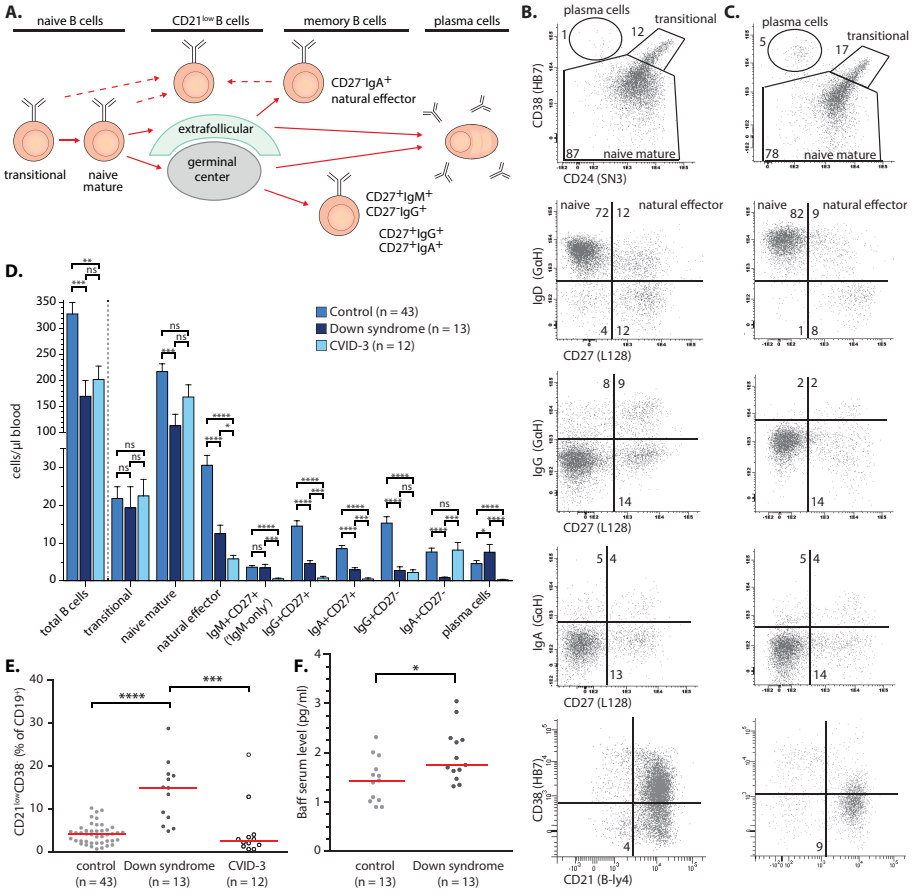


Figure 1. Composition of the blood B-cell compartment in children with Down syndrome. **A**, Model for B-cell maturation. **B and C,** Flow cytometric gating strategy to determine the described B-cell subsets in a representative control (**B**) and a patient with Down syndrome (**C**). **D**, Absolute numbers of B-cell subsets. **E**, Frequencies of $CD21^{low}CD38^{-}$ B cells. **F**, BAFF serum levels. Panels **D-F** include Down syndrome patients (P1, P4-P8, P11-P17). Statistical analysis with Mann-Whitney test: ns, not significant; *, $p<.05$; **, $p<.01$; ***, $p<.001$; ****, $p<.0001$.

Table 1. PCR primers and UPL FAM-labeled hydrolysis probes for gene expression analysis

gene	forward primer (5'- 3')	position (exon)	reverse primer (5'-3')	position (exon)	UPL probe #
AID [12]	ACTCTGGACACCACTATGGACA	1	GCGGACATTTTTGAATTGGT	2	69
PRDM1	ACGTGTGGGTACGACCTTG	1	CTGCCAATCCCTGAAACCT	2	67
IRF4	GTTCCTGAGGGAGCCAAAA	3	GGTGGCTCATGGACATCTG	4	65
XBP1	GGAGTTAAGACAGCGCTTGG	3	CACTGGCCTCACTTCATTCC	4	37

UPL, universal probe library (Assay Design Center; Roche)

otherwise healthy children undergoing tonsillectomy for medical reasons. This study was performed according to the Declaration of Helsinki and the guidelines of the Medical Ethics Committees employed by the Jeroen Bosch Hospital and the Erasmus MC.

Flow cytometric analysis of lymphocytes in blood and tonsil

Absolute counts of blood T, B and natural killer cells were obtained with a diagnostic lyse-no-wash protocol. Furthermore, 8-color flow cytometric immunophenotyping was performed as described before to detect transitional, naive mature, $CD21^{low}$, 6 memory B-cell subsets and plasma cells on a 3-laser FACS LSRII (BD Biosciences; Figure 1B and C).[6] Germinal center B cells ($CD19^{+}CD38^{+}IgD^{-}$) and plasma cells in tonsil ($CD38^{hi}$) were defined as previously described.[10]

Transitional, naive mature, natural effector and $CD27^{+}IgD^{-}$ memory B cells were high-speed cell sorted to >95% purity on a FACSAria I (BD Biosciences) as described before.[10]

Histology and immunohistochemistry of tonsils

The tonsil specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Immunohistochemistry was performed according to standard procedures involving monoclonal antibodies against CD3 (SP7), CD21 (2G9, both Thermo Scientific), CD20 (L26), CD138 (MI15, both DAKO) and PD1 (NAT105, Abcam).

Quantification of BAFF serum levels

BAFF serum levels were measured by ELISA and analyzed in duplicate according to the manufacturer's instructions (R&D Systems).

In vitro plasma cell differentiation of purified B cells from tonsils

Resting B cells from tonsils were purified and cultured with combinations of anti-IgM F(ab')2, anti-CD40, CpG ODN2006 and IL-21 as described earlier.[12] Cells were harvested after a 6-day culture for TaqMan-based quantitative RT-PCR on a StepOnePlus (Applied Biosystems). Target gene expression levels were determined with intron-spanning primes and fluorogenic probes (Table 1), corrected for *ABL* expression levels and expressed as fold difference from uncultured naive cells.[17] All quantitative RT-PCR reactions were performed in duplicate.

Molecular analysis of replication history and Ig gene rearrangements

DNA was isolated from each sorted subset for analysis of the replication history with the Kappa-deleting Recombination Excision Circles (KREC) assay and the Igκ restriction enzyme hot-spot mutation assay (IgκREHMA) as described previously.[10, 11]

IGA and *IGG* transcripts were amplified and cloned from cDNA prepared from mononuclear cells, using *IGHV3* and *IGHV4* leader primers and consensus Cα or Cγ reverse primers.[6, 12] In addition, *IGHV3/4-IGHJ* rearrangements were amplified from DNA of sorted natural effector B cells.[13] Sequences were generated on an ABIPRISM 3130XL and analyzed with the IMGT (http://imgt.cines.fr/), JoinSolver (http://joinsolver.niaid.nih.gov) and BASELINe programs (http://selection.med.yale.edu/baseline/).[14-16]

Statistics

Statistical analyses were performed using the Mann-Whitney test (SPSS version 18.0), or $\chi 2$ test as indicated in Figure legends. A P-value <0.05 was considered statistically significant.

RESULTS

Clinical and basic immunological characterization

Blood samples were obtained from 17 patients (6 male), aged 7-17 years, at their routine annual visit to the outpatient clinic for Down syndrome. Tonsils were obtained from 4 children (3 male, 3-5 years old) with karyotype confirmed diagnosis of Down syndrome following scheduled tonsillectomy. Basic clinical and immunological information of these patients is shown in Table 2. Patients were selected on the absence of malignancies and serious respiratory tract infections that required admission to a pediatric intensive care unit. Ear, nose and throat (ENT)-problems, viral induced wheezing and autoimmune mediated hypothyroidism were common, as expected.

Circulating T and NK cells were in the low-normal range, whereas B-cell numbers were below the 5th percentile of the normal range in 10/17 patients (Table 2). The distribution of Ig serum levels was altered as previously described in Down syndrome.[4, 5, 18] Thus, the clinical and basic immunological parameters of the patients in our study population were in line with previous studies and representative for children with Down syndrome.[1, 4, 5, 19, 20]

Composition of the blood B-cell compartment in children with Down syndrome

To study the nature of reduced total B-cell numbers, we performed detailed flowcytometric analysis in 13 Down syndrome patients and compared these with 43 age-matched healthy controls (Figure 1B, C and D). The children with Down syndrome had normal numbers of $CD27^{-}IgM^{+}IgD^{+}CD24^{high}CD38^{high}$ transitional B cells, whereas $CD27^{-}IgM^{+}IgD^{+}CD24^{dim}CD38^{dim}$ naive mature B cells were significantly decreased (Figure 1D). Since soluble BAFF is a critical survival factor for mature B cells,[21] we quantified serum BAFF levels. These were slightly increased in Down syndrome, rather than declined, as compared with age-matched controls (Figure 1F). This indicates that serum BAFF levels are not limiting and might even be increased as a result of reduced usage by the low numbers of mature B cells.

Of the 6 memory B-cell subsets, $CD27^{+}IgD^{-}IgM^{+}$ 'IgM-only' B cells were normally present, but both natural effector B cells and $CD27^{+}IgG^{+}$, $CD27^{+}IgA^{+}$, $CD27^{-}IgG^{+}$ and $CD27^{-}IgA^{+}$ class-switched memory B-cell numbers were significantly lower in Down syndrome patients than in healthy controls. In contrast, circulating plasma cell numbers were increased in Down syndrome patients. Together with normal to high levels of serum IgG and IgA (Tables 2 and 3), this indicates that Ig responses can take place in patients with Down syndrome, but these patients seem defective in generation and/or maintenance of T-cell dependent and T-cell independent memory B cells.

Of the 13 Down syndrome patients, 10 had natural effector and/or $CD27^{+}IgD^{-}$ memory B-cell numbers that were below the 5^{th} percentile of the normal range of age-matched controls (Table 4). These 10 patients could be assigned to one of the previously identified B-cell patterns in CVID patients: 4 patients displayed pattern 2 (defect in early B-cell maturation or survival; 2 patients with pattern 3 (defect in B-cell activation and proliferation); 4 patients with pattern 4 (defect in germinal center function). The other 3 patients showed a normal B-cell composition (pattern 5).[8] There was no association between age and type of B-cell pattern. The average B-cell subset composition of Down syndrome patients was most similar to CVID pattern 3 (CVID-3; Figure 1D) with the exception of the additional reduction in $CD27^{-}IgA^{+}$ B cells and the normal numbers of IgM-only B cells and circulating plasma cells.

Interestingly, $CD21^{low}CD38^{low}$ B cells were increased in all patients with Down syndrome, irrespective of age (Figure 1E). This clearly contrasted the CVID-3 group, which showed mostly normal frequencies of $CD21^{low}$ B cells.[8] Thus, patients with Down syndrome seem to carry defects in B-cell activation, similar to CVID-3, but their B-cell phenotype is unique, especially with regards to circulating plasma cells and $CD21^{low}$ B cells.

Table 2. Clinical and basic immunological characteristics of patients with Down syndrome

Patient	Gender	Age (yrs)	Recurrent respiratory infections	Prophylactic antibiotics	History of inhaled medication*	Tympano-stomy tubes	Adeno-tonsillectomy	Hypothyroid disease†	Lymphocyte subsets (cells/μL)			Immunoglobulin levels (g/L)						
									T cells	B cells	NK cells	IgG	IgG1	IgG2	IgG3	IgG4	IgA	IgM
Blood																		
P1	M	7	Yes	Yes	No	Yes	No	No	1,830	296	340	11.0	8.3	0.93	0.45	0.19	0.84	0.76
P2	M	7	Yes, until age 6 yrs	No	No	No	No	No	1,160	290	150	11.5	9.4	1.25	0.81	0.23	1.23	0.99
P3	F	7	Yes	No	Yes	Yes	No	Yes	1,100	270	120	9.9	8.6	**0.67**	0.45	0.03	0.76	0.63
P4	M	7	No	No	No	No	No	No	ND	310	ND	11.8	8.7	1.09	*1.53*	0.12	1.54	1.03
P5	F	8	No	No	Yes	No	No	No	ND	**153**	ND	11.4	8.5	**0.87**	1.21	0.03	1.99	**0.48**
P6	F	8	Yes	Yes	Yes	No	No	No	ND	219	ND	*14.2*	9.6	2.68	1.50	0.14	1.71	**0.48**
P7	M	9	Yes, until age 6 yrs	No	Yes	No	No	No	800	**87**	100	*14.8*	*10.8*	1.71	0.89	0.03	1.26	1.36
P8	F	10	Yes, until age 6 yrs	No	Yes	No	No	No	ND	**126**	ND	11.3	8.1	1.56	1.02	0.23	1.28	**0.41**
P9	F	10	Yes, until age 8 yrs	No	No	Yes	No	No	980	**140**	200	10.2	8.1	**0.74**	0.74	0.12	1.64	**0.43**
P10	F	11	Yes, until age 8 yrs	No	No	Yes	No	Yes	950	**110**	110	9.7	7.1	**0.63**	0.72	0.08	1.84	0.57
P11	F	13	No	No	No	No	Yes	No‡	1,330	406	210	12.3	8.3	2.08	1.31	0.28	0.97	0.53
P12	M	14	No	No	No	No	No	No	ND	**120**	ND	10.5	7.3	2.17	1.43	0.11	2.39	0.63
P13	M	15	Yes, until age 10 yrs	No	Yes	Yes	Yes	No	**560**	**50**	80	11.7	8.1	2.02	1.24	0.30	2.43	0.45
P14	F	15	No	No	No	No	No	No‡	ND	**68**	ND	12.5	7.6	3.19	0.52	0.42	1.69	0.58
P15	F	17	No	No	No	Yes	No	Yes	ND	167	ND	12.9	9.0	2.30	0.93	0.37	1.76	0.99
P16	F	17	No	No	No	No	No	No‡	1,520	**60**	100	***18.1***	***14.7***	1.79	0.66	<0.01	1.46	0.47
P17	F	17	No	No	No	Yes	No	Yes	1,110	**68**	**80**	16.0	***10.7***	2.79	1.23	0.44	1.84	**0.22**
Tonsils																		
T1	F	4	Yes	Yes	Yes	No	Yes	No	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T2	M	4	Yes	Yes	No	No	Yes	No‡	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T3	M	5	Yes	No	No	Yes	Yes	No	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T4	M	3	Yes	No	No	No	Yes	No‡	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

← **Table 2.** Clinical and basic immunological characteristics of patients with Down syndrome
None of the children were ever admitted to a Pediatric Intensive Care Unit due to serious respiratory tract infections or showed signs of celiac disease, diabetes mellitus, hematological malignancies, asthma or allergy. * History of use of inhaled β2-adrenergic receptor agonists and/or corticoids. † Hypothyroid disease for which thyroid hormone replacement therapy was started and anti-TPO values were increased (>25U/L). ‡ Patients with normal thyroid function but increased anti-TPO values (>25U/L). Values of lymphocyte subsets and immunoglobulin levels below and above age related normal values are marked in bold and italic font, respectively. For normal values of immunoglobulin levels see Table 3. For normal values of lymphocyte subsets see Comans-Bitter *et al.*[20]

Table 3. Normal values of serum immunoglobulin levels

Age group	IgA (g/L)	IgM (g/L)	IgG (g/L)	IgG1 (g/L)	IgG2 (g/L)	IgG3 (g/L)	IgG4 (g/L)
7-12 year	0.3-2.0	0.5-2.0	6.0-12.3	3.8-10.0	0.9-5.0	0.15-1.5	0.03-2.1
>12 year	0.7-4.0	0.4-2.3	7.0-16.0	3.8-10.0	0.9-5.0	0.15-1.5	0.03-2.1

Data represent 5 and 95 percentiles and are obtained from De Vries *et al.*[18]

Table 4. Absolute numbers of blood B-cell subsets and designation to CVID patterns

Patient	age (yr)	total B cells (cells/μl)	Transitional (cells/μl)	Naive mature (cells/μl)	Natural effector (cells/μl)	$CD27^+IgD^-$ memory (cells/μl)	plasma cells (cells/μl)	CVID pattern*
P1	7	296	45.6	206.5	18.0	**12.7**	3.5	4
P4	7	310	43.7	167.8	33.7	25.1	*30.3*	5
P5	8	**153**	21.7	**103.4**	**6.9**	**9.5**	8.4	2
P6	8	219	33.5	142.7	**10.5**	14.2	10.3	3
P7	9	**87**	**8.7**	**52.7**	**12.5**	**5.5**	1.2	2
P8	10	**126**	12.9	**87.6**	**7.7**	**5.1**	8.8	2
P11	11	406	61.3	309.9	13.8	**4.5**	6.9	4
P12	14	**120**	4.9	90.6	**4.2**	**7.8**	8.4	3
P13	15	40	2.9	21.1	**2.9**	**5.5**	2.6	2
P14	17	**68**	**1.6**	**34.9**	12.0	**10.8**	6.4	4
P15	17	167	5.7	115.7	20.1	16.2	7.9	5
P16	17	**60**	3.9	100.4	10.2	22.4	2.4	5
P17	17	**68**	4.9	**39.8**	10.1	**5.0**	1.9	4
controls (5-95 percentiles)	*5-10*		*11-77*	*111-486*	*15-88*	*13-100*	*1-15*	–
controls (5-95 percentiles)	*11-16*		*4-108*	*87-390*	*7-90*	*10-76*	*0.5-20*	–
controls (5-95 percentiles)	*>16*		*3-50*	*57-447*	*9-88*	*13-122*	*1-23*	–

Subnormal values are shown in bold font. Increased numbers are shown in italics.
* Definition of CVID subgroups: 2, Decreased naive mature, natural effector and $CD27^+IgD^-$ memory; 3, normal naive mature, decreased natural effector and $CD27^+IgD^-$ memory; 4, normal naive mature and natural effector, decreased $CD27^+IgD^-$; 5, normal numbers of all indicated B-cell subsets.[8]

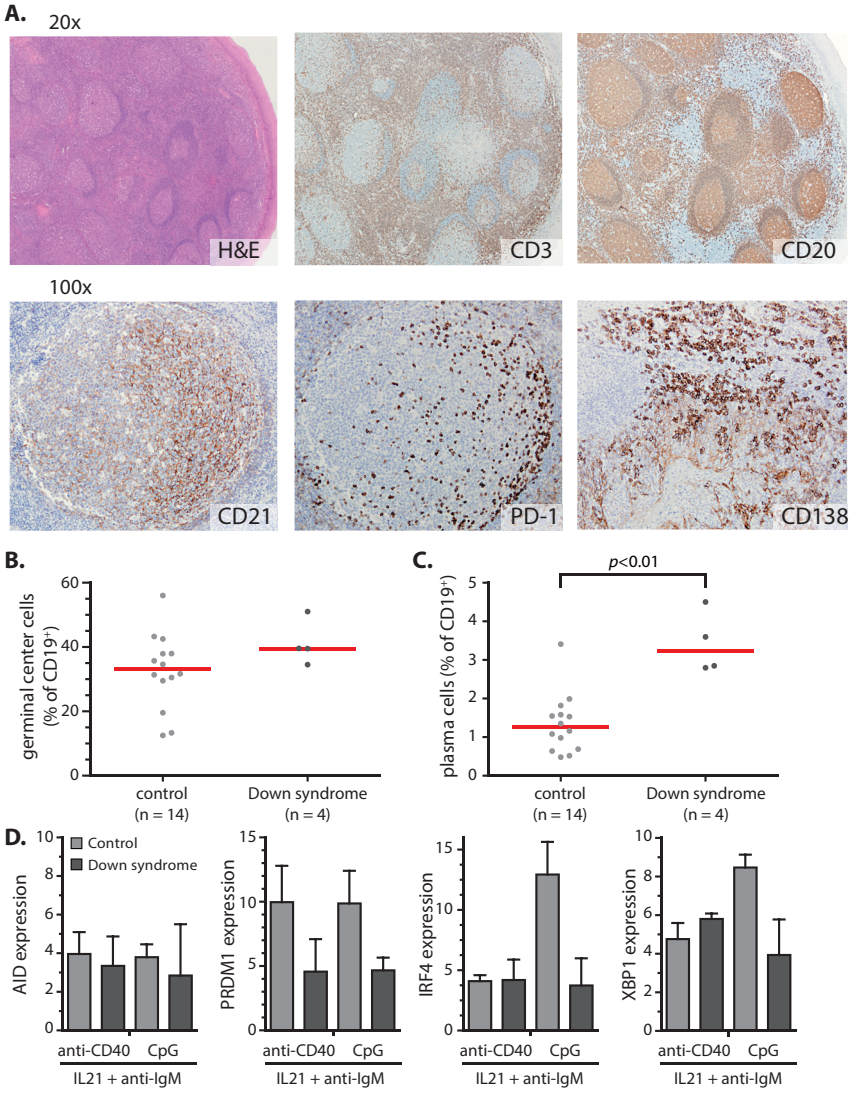


Figure 2. Germinal center composition and plasma cell maturation. **A**, Tonsil sections of a representative patient with Down syndrome. **B,** Frequencies of $CD38^{+}IgD^{-}$ germinal center B cells and **C,** $CD38^{hi}$ plasma cells **D,** Gene expression levels in naive B cells following 6-day stimulation, expressed as fold difference from uncultured naive cells. Statistical analysis with the Mann-Whitney test.

Germinal center formation and plasma cell maturation

To study whether the defects in blood memory B cells resulted from impaired immune responses, we analyzed tonsils of children with Down syndrome. The tonsil sections were unremarkable and demonstrated a normal make-up of the lymphoid tissue with variable numbers of well-proportioned secondary lymph

follicles (Figure 2A, B and C). The germinal centers harbored light zones and dark zones containing proliferating B cell blasts, as well as networks of $CD21^+$ follicular dendritic cells and $PD\text{-}1^+$ follicular helper T cells.

Total plasma cell frequencies were slightly increased (Figure 2C), and in each tonsil, subsets expressing of IgM, IgA or IgG were found (not shown). To study B-cell activation and plasma cell differentiation, naive B cells obtained from tonsils were stimulated with anti-IgM and IL-21 in combination with anti-CD40 or CpG to mimic T-cell help and T-cell independent TLR signaling respectively. B cells from children with Down syndrome normally upregulated *AID* gene expression levels. Anti-CD40 stimulation resulted in normal upregulation of *IRF4* and *XBP1*, while *PRDM1* transcription was lower than in controls. CpG induced more transcription of all three genes in controls, but not in Down syndrome patients (Figure 2D). Thus, naive B cells in patients with Down syndrome can induce plasma cell differentiation, although this may be slightly less efficient than controls, especially following T-cell independent stimuli.

Distinct phenotypic alterations in IgM^+, IgA^+ and IgG^+ memory B cells

To further study defects in B-cell memory, we first analyzed expression of typical memory markers on B-cell subsets of the patients. Naive mature B cells from Down syndrome patients seemed normal with low expression of CD80, CD95 and TACI (Figure 3). In contrast to $CD27^+IgG^+$ memory B cells, natural effector and $CD27^+IgA^+$ memory B cells showed impaired upregulation of CD80. All three memory subsets show increased upregulation of CD95. TACI was higher on natural effector B cells of Down syndrome patients than of controls, but it was normally upregulated on IgG and IgA memory B cells. These phenotypic profiles demonstrate that IgM memory, represented by natural effector B cells, is mostly affected in Down syndrome patients, while IgA and IgG are less affected.

Molecular analysis of antigen-driven B-cell maturation in Down syndrome and CVID-3

In addition to phenotypic analysis of memory B-cell subsets, we performed molecular analysis of their replication history, somatic hypermutations and Ig class-switch profiles. Transitional B cells of Down syndrome patients, CVID-3 and controls showed no proliferation or SHM (Figure 4A and B), confirming their status of recent bone marrow emigrants.[10] Naive mature B cells of Down syndrome and CVID-3 patients showed homeostatic proliferation of 1-2 cell divisions in absence of SHM, which was also not significantly different from the healthy controls.

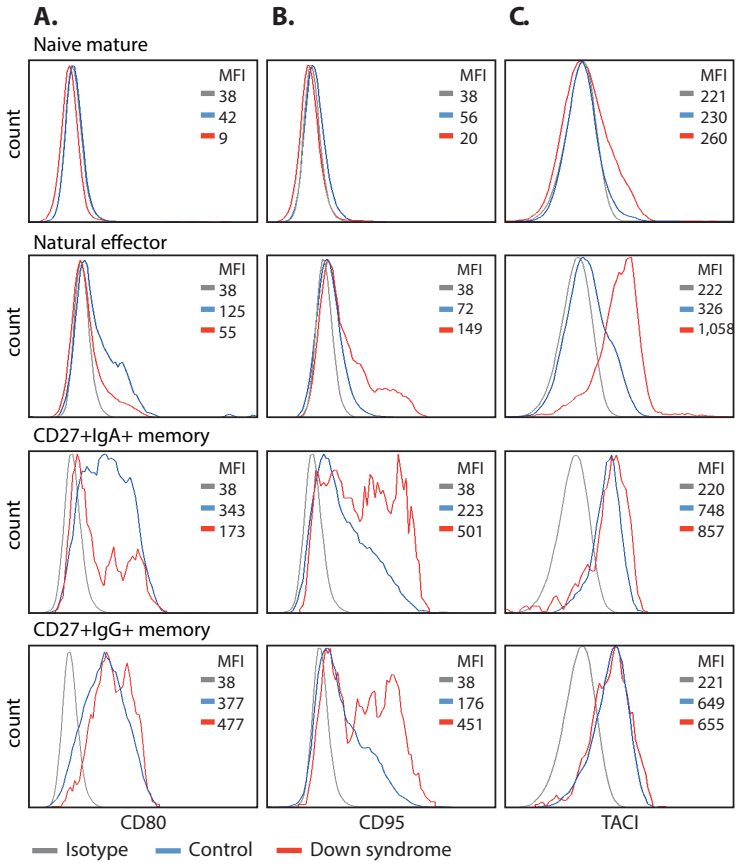


Figure 3. Abnormal immunophenotypes of memory B-cell subsets of Down syndrome patients. Expression levels of **A,** CD80 (L307.4), **B,** CD95 (DX2) and **C,** TACI (goat polyclonal) on naive mature, natural effector, $CD27^+IgA^+$ and $CD27^+IgG^+$ memory B cells. MFI, median fluorescence intensity. Data shown from 4 patients with Down syndrome (P7, P13, P16, P17) and 4 healthy controls.

Natural effector B cells of Down syndrome and CVID-3 patients showed proliferation in conjunction with SHM. Still, these levels were significantly lower than healthy controls. Proliferation of $CD27^+IgD^-$ B cells of Down syndrome and CVID-3 patients was similar to controls, but these cells showed reduced frequencies of mutated IGKV3-20 alleles in patients with Down syndrome. IgA and IgG subclass analysis of rearranged *IGH* transcripts of Down syndrome patients revealed no difference in usage as compared to healthy controls. The normal use of *IGHM*-downstream IgG2, IgG4 and IgA2 indicates that Ig class switching to downstream constant regions was not impaired (Figure 5). The effects on proliferation, SHM and class switching were not age-related. Thus, antigen-dependent B-cell maturation is

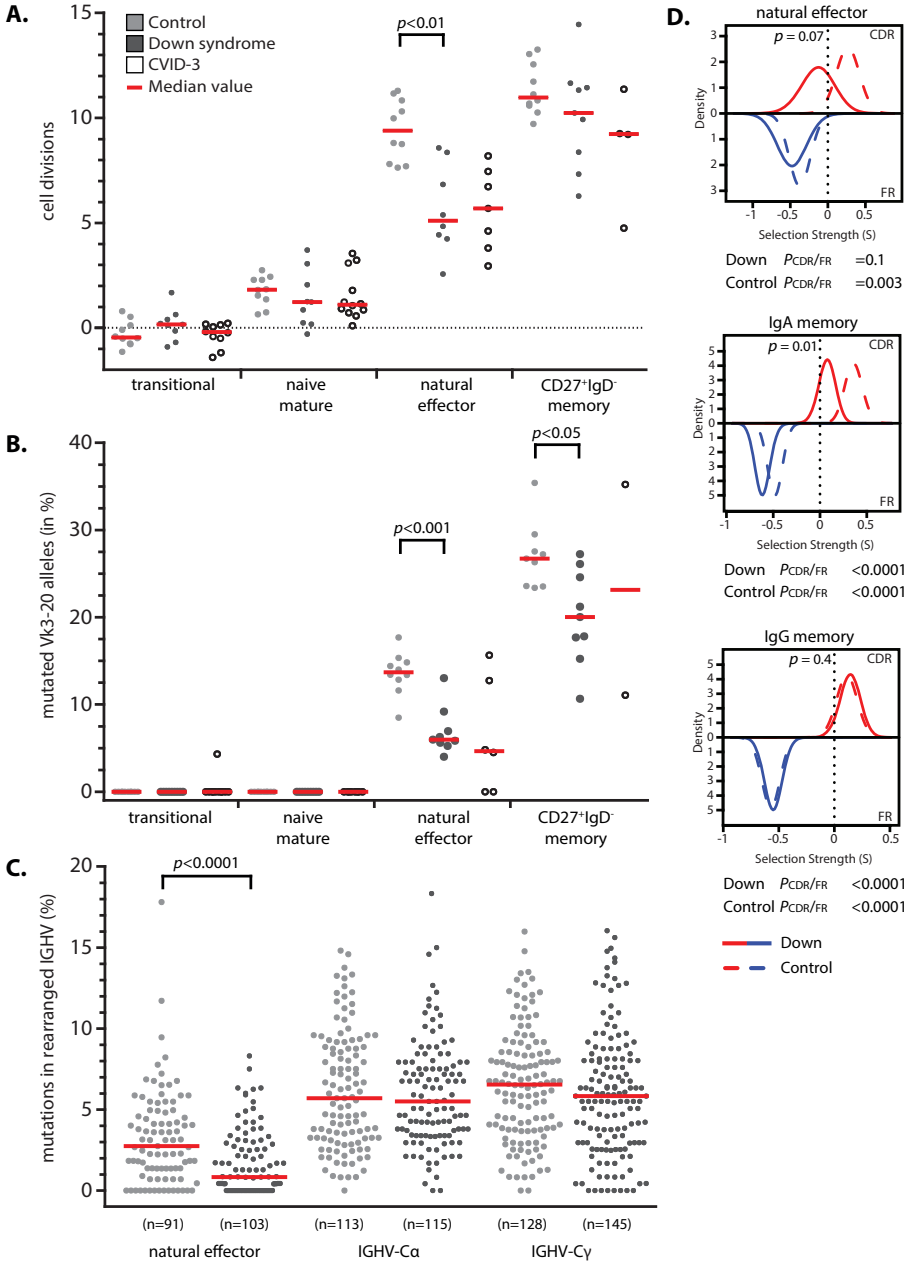


Figure 4. Replication history and somatic hypermutation. **A,** The *in vivo* replication history and **B,** frequencies of mutated IGKV3-20 alleles in B-cell subsets (patients P1, P4-6, P8, P11-12, P14-15). **C,** SHM frequencies in rearranged *IGHV* genes. **D,** selection for replacement mutations in CDR (red) and FR regions (blue).[14, 15] Each subset includes 18-52 sequences from 4-6 individuals. Statistical analysis in panels A-C with Mann-Whitney test.

clearly impaired in patients with Down syndrome, especially in natural effector B cells.

Targeting of somatic hypermutations

More detailed mutation analysis in *IGHV* genes showed decreased SHM frequencies in natural effector B cells (Figure 4C). In contrast to the IgκREHMA assay, SHM frequencies were normal in IgA and IgG memory B cells (Figure 4C), and no differences were seen in SHM frequencies for the various IgA and IgG subclasses (Figure 5B and C).

To study whether the SHM processes were induced normally in memory B cells of Down patients, we analyzed targeting of mutations to sequence motifs.[22] Natural effector B cells of Down patients had significantly decreased targeting of the RGYW DNA motifs (R = purine, Y = pyrimidine, and W = A or T) that are direct targets of Activation-Induced Cytidine Deaminase (AID) (Table 5). IgA and IgG transcripts showed normal RGYW targeting. Furthermore, natural effector and Ig-class switched memory B cells showed normal transition/transversion ratios, as well as normal WA/TW targeting. Thus, memory B cells of Down syndrome patients showed normal repair of AID-induced lesions, and the only defect appears to be reduced AID activity in natural effector B cells.

Molecular analysis of Ig selection processes

In healthy individuals, the use of inherently autoreactive IGHV4-34 genes and long complementarity determining regions in IGH (IGH-CDR3) are counter-selected in memory B cells and therefore less frequent than in naive B cells.[6, 23-26] Natural effector B cells of controls and Down syndrome patients showed smaller IGH-CDR3 than naive mature B cells of healthy controls (Figure 6A). However, we did not observe decreased use of IGHV4-34 in natural effector B cells of either controls or Down syndrome patients (Figure 6B). IGH-CDR3 sizes and IGHV4-34 use were decreased in IgA and IgG transcripts of both controls and patients as compared with naive mature B cells (Figure 6). Still, the median IGH-CDR3 size of IgA transcripts in Down syndrome patients was significantly larger than in controls. Furthermore, the use of IGHV4-34 was slightly, but not significantly increased in IgA and IgG transcripts of patients with Down syndrome as compared with controls. Thus, despite minor differences with healthy controls, natural effector and Ig class switched memory B cells of patients with Down syndrome showed normal molecular signs of Ig repertoire selection.

Both controls and Down syndrome patients revealed increased replacement/silent mutation (R/S) ratios in regions as compared to FR in (Table 5 and Figure 7).[14, 15]

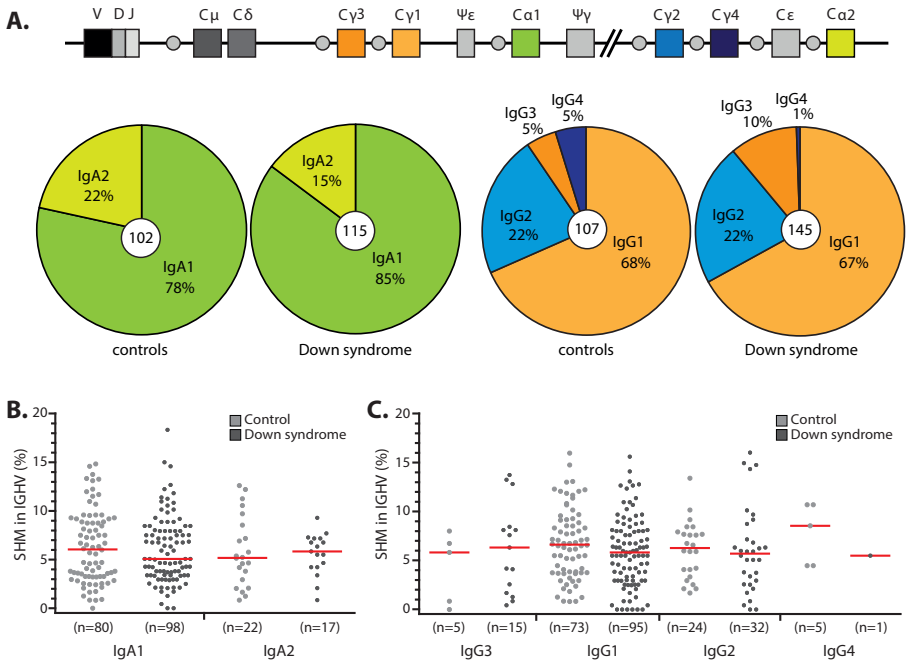


Figure 5. IGHV mutation frequencies of distinct IgA and IgG subclass transcripts. **A,** Distribution of IgA and IgG subclass usage in *IGH* transcripts. The total number of analyzed sequences is indicated in the center of each plot. No significant differences in subclass usage. The frequency of mutated nucleotides in *IGHV* genes are shown for the two IgA subclasses (**B**) and IgG subclasses (**C**). Each dot represents a transcript from a healthy control (light grey) or a patient with Down syndrome (dark grey), with red lines indicating the median value for each category. The total number of analyzed transcripts is shown in brackets. 27-45 sequences from 4 patients (P1, P9-11) and 18-52 sequences from 4 controls were obtained for IgA and IgG transcripts.

However, the increased R/S ratios in CDR vs FR do not necessarily reflect selection processes, because the codon usage in CDR differs from FR in their nature to be more susceptible to replacement mutations.[27,28] Therefore, we analyzed the *IGH* sequences with the BASELINe program that determines whether the mutation patterns differed from what can be expected from random targeting.[14] Similar to previous observations, we found positive selection for CDR and negative selection for FR in natural effector, IgA and IgG memory B cells in healthy controls (Figure 4D; dotted lines). The differences between selection in CDR and FR were highly significant. In contrast, natural effector B cells of Down syndrome patients did not show positive selection of replacement mutations in CDR. While IgA and IgG transcripts of Down syndrome patients showed significant selection for replacement mutations in CDR, the selection strength of IgA was significantly lower than in healthy controls. Thus, in addition to the phenotypic profiles, natural effector and IgA memory B cells

Table 5. Targeting and selection of individual mutations in rearranged *IGHV*

	Natural Effector				IgA Memory				IgG Memory			
	Control (n=120)		Down (n=98)		Control (n=112)		Down (n=115)		Control (n=129)		Down (n=145)	
Mutated rearrangements (%)	100/120	(83.3)	76/98	(77.6)	111/112	(99.1)	113/115	(98.3)	126 /129	(97.7)	139/145	(95.9)
Transitions (%)	504/909	(55.4)	281/552	(50.9)	997/1850	(53.9)	921/1792	(51.4)	1241/2438	(50.9)	1191/2296	(51.9)
Transversions (%)	405/909	(44.6)	271/552	(49.1)	853/1850	(46.1)	871/1792	(48.6)	1197/2438	(49.1)	1105/2296	(48.1)
Transitions at C·G (%)	298/543	(54.9)	164/311	(52.7)	563/1076	(52.3)	553/1083	(51.1)	743/1432	(51.9)	728/1401	(52.0)
Targeting of C·G (%)	543/909	(59.7)	311/552	(56.3)	1076/1850	(57.7)	1083/1792	(60.4)	1432/2438	(58.7)	1401/2296	(61.0)
RGYW (%)	244.1/909	(26.9)	106.5/552	(19.3) **	483.3/1850	(26.1)	486.4/1792	(27.1)	613.7/2438	(25.2)	606/2296	(26.4)
WRCY (%)	132/909	(14.5)	79.7/552	(14.4)	264.6/1850	(14.3)	250.6/1792	(14.0)	351.7/2438	(14.4)	321.3/2296	(14.0)
WA (%)	131.7/909	(14.5)	74.8/552	(13.5)	252.3/1850	(13.6)	207.3/1792	(11.6)	303.7/2438	(12.5)	303.8/2296	(13.2)
TW (%)	45.2/909	(5.0)	39.1/552	(7.1)	151.9/1850	(8.2)	143.7/1792	(8.0)	159.0/2438	(6.5)	137.9/2296	(6.0)
FR (R/S)	379/212	(1.8)	229/135	(1.7)	719/460	(1.6)	677/494	(1.4)	1065/618	(1.7)	903/600	(1.5)
CDR (R/S)	259/59	(4.4)	145/41	(3.5)	535/134	(4.0)	485/136	(3.6)	596/159	(3.7)	642/151	(4.3)

FR indicates framework region; CDR, complementarity determining region; R/S is the ratio between replacement (R) and silent mutations (S); the number of analyzed sequences is indicated in brackets next to the population name. All analyses were performed with the JOINSOLVER™ program and the differences between controls and patients were analyzed with the $\chi 2$ test. Significant differences ($p<0.01$, also after adjustment for multiple comparisons) are indicated with **.

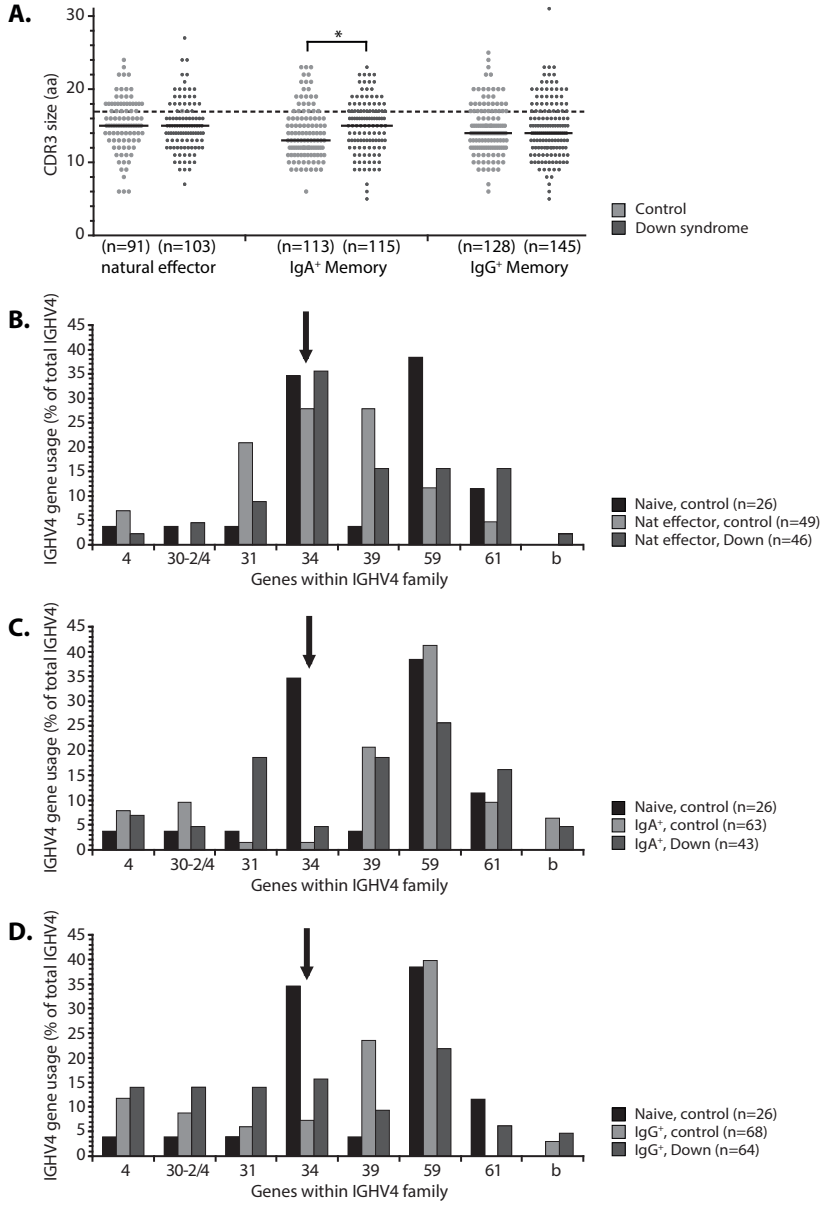


Figure 6. Selection against IGHV4-34 usage and long IGH-CDR3 in natural effector, IgA and IgG memory B cells.
(**A**) IGH-CDR3 size distributions. Black lines represent median values; the dashed line represents median value for centroblasts (n=67). Analysis with student's t-test. *p<0.05. (**B-D**) IGHV4 gene usage in natural effector (**B**), IgA (**C**) and IgG (**D**) memory B cells of Down syndrome patients. The arrows indicate IGHV4-34 gene usage. The total numbers of analyzed transcripts are shown in brackets. Panel A-D include 23-28 sequences from 4 patients (P1, P8, P11 and P12) and 10-18 from 6 controls for natural effector B cells, and 27-45 sequences from 4 patients (P1, P9-11) and 18-52 sequences from 4 controls for IgA and IgG transcripts.

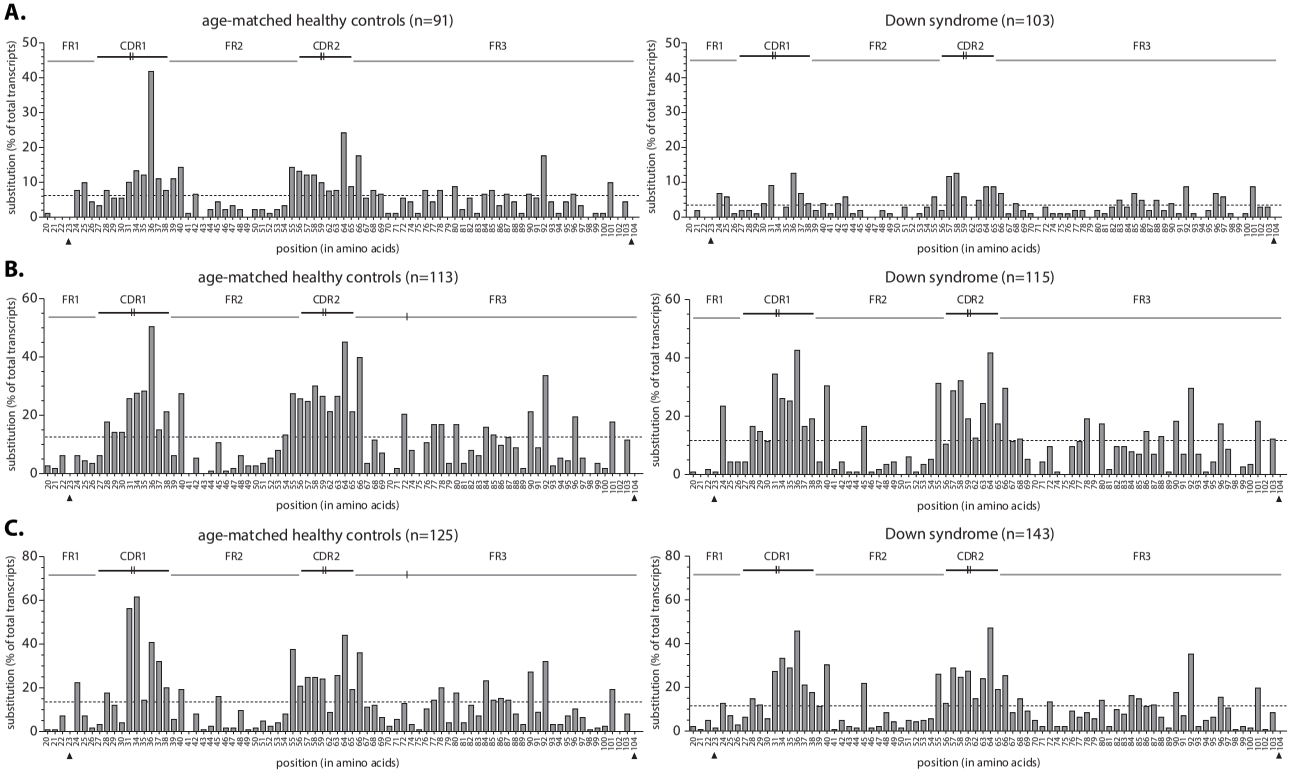


Figure 7. Distribution of replacement mutations in rearranged IGHV genes in memory B-cell subsets. Distribution of replacement mutations in rearranged *IGHV* genes are determined for natural effector B cells (**A**), IgA$^+$ memory B cells (**B**), and IgG$^+$ memory B cells (**C**) from controls (left) and patients with Down syndrome (right). Each bar represents the frequency of replacement mutations at each amino acid position starting from 20 (first codon following primer sequence) to 104 (last codon of the FR3 region). FR denotes framework region and CDR denotes complementarity determining region. The total numbers of analyzed transcripts are shown in brackets. Panels A-D include 23-28 sequences from 4 patients (P1, P8, P11 and P12) and 10-18 from 6 controls for natural effector B cells, and 27-45 sequences from 4 patients (P1, P9-11) and 18-52 sequences from 4 controls for IgA and IgG transcripts.

showed defects in molecular maturation, whereas IgG memory B cells appeared normal.

DISCUSSION

Through cellular and molecular analysis of the B-cell compartment, we showed specific defects in B-cell memory in patients with Down syndrome. $CD27^+$ memory B cells were reduced in number and displayed impaired proliferation and antibody maturation. The B-cell pattern was reminiscent of a subgroup of CVID patients with a potential defect in B-cell activation (CVID pattern 3).[8] Still, in contrast to CVID, patients with Down syndrome had normal germinal centers, high numbers of plasma cells and normal Ig serum levels. Thus, in addition to their anatomical and physiological abnormalities of the respiratory tract, Down syndrome patients carry B-cell memory defects that may contribute to the increased frequency of respiratory tract infections.

Our detailed analysis of the blood B-cell compartment in patients with Down syndrome revealed a decrease in naive mature B cells. This decrease can be the result of reduced output from bone marrow, reduced homeostatic proliferation or impaired survival. Patients with multisystem DNA-repair disorders such as Nijmegen Breakage Syndrome or Ataxia Telangiectasia display a humoral immunodeficiency with reduced B-cell output due to impaired DNA repair during V(D)J recombination in bone marrow.[29-31] This is reflected by reduced transitional B cells and increased homeostatic proliferation of naive mature B cells, reminiscent of CVID pattern 1.[8] However, none of our Down syndrome patients showed this B-cell pattern. It is therefore less likely that their reduced B-cell compartment is the result of impaired bone marrow output.

Homeostatic proliferation of naive mature B cells in Down syndrome seemed normal with ~2 cell cycles in absence of SHM. This leaves impaired survival as the most likely cause of the reduced naive B-cell compartment. This is supported by previous studies that showed increased apoptosis of B cells in patients with Down syndrome.[32, 33] However, we found that the critical cytokine for naive B-cell survival, BAFF, was slightly increased in serum of patients. Unfortunately, we were unable to study BAFF-R expression on B cells. Low BAFF-R expression could contribute to the observed decreased survival. B-cell survival is also dependent on macrophage migration–inhibitory factor (MIF) produced by bone marrow-resident dendritic cells.[34] Circulating dendritic cells are decreased in patients with Down syndrome,[35] which could be associated with impaired production of MIF and underlie the reduced naive B-cell numbers in Down syndrome.

On top of decreased naive mature B-cell numbers, circulating $CD27^+$ memory B cells were also reduced in patients with Down syndrome. Both natural effector and IgD^- memory B-cell subsets showed increased expression levels of the FAS receptor (CD95), which is also known as the death receptor that induced apoptosis.[36] Increased CD95 expression levels can tip the balance between BCR-induced survival and CD95-induced cell death, thereby negatively affecting memory B-cell numbers.[37, 38]

In addition to more CD95 expression, natural effector B cells showed impaired proliferation and SHM levels, and defective selection for replacement mutations in CDR. Thus, on top of a potential survival defect, Down syndrome patients are defective in generation of IgM^+ B-cell memory. Since IgM responses are important for clearing blood borne pathogens,[39] this defect could underlie the increased susceptibility of patients with Down syndrome to blood borne infections.[40] Children with genetic defects in myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP) and interleukin 1 receptor-associated kinase 4 (IRAK4) Toll-like receptor signaling experience invasive bacterial infections.[41] Recently, MyD88-TIRAP-IRAK4–dependent signaling was found to be critical for generation of homeostasis of natural effector B cells.[42] However, the natural effector B cells in these immunodeficient children carry normal SHM frequencies.[41] Thus, patients with Down syndrome might have a TLR signaling defect that could contribute to the observed deficiency in natural effector B cells in patients with Down syndrome, but it is unlikely that this is the single cause.

A specific subset of neutrophils was recently shown to support splenic marginal zone B-cell responses.[43] Since neutrophil abnormalities have been described in Down syndrome,[44, 45] it is possible that these contribute to our observed defects in circulating natural effector B cells. Because these B-helper neutrophils do not circulate in blood, this would require further study of spleen tissue, which we were unable to obtain.

In addition to natural effector B cells, IgA and IgG memory B cells were reduced in patients with Down syndrome. Despite normal SHM levels, IgA transcripts showed impaired molecular selection. Although serum IgA and IgM levels were found normal, these defects might impair local mucosal immunity considering their role in the respiratory and intestinal tract. Despite reduced numbers, IgG B-cell memory seemed quite normal in phenotype and molecular maturation in the patients that we studied. This fits in with the generally mildly affected responses to vaccinations in patients with Down syndrome.[4, 46-49] These features might at least partly explain why patients in this age range (7-17 years) are less susceptible to recurrent respiratory tract infections than very young children of <5 years old.[5, 50]

We identified increased CD21^{low} B-cell numbers in patients with Down syndrome. Since these cells are also increased in autoimmune diseases as well as CVID patients with autoimmune phenomena,[9, 51] this could be related to the increased risk for autoimmune disease in Down syndrome. However, we did not observe increased IGHV4-34 usage or long IGH-CDR3 in memory B cells of our patients, indicating that not all autoimmunity selection checkpoints are affected in B cells of Down syndrome patients.[24, 25]

Most of the Down syndrome patients we studied showed abnormalities in their blood B-cell compartment that fitted with one of the previously published CVID patterns.[8] Despite the memory B-cell defects, germinal centers, plasma cells and serum Ig levels were normal in Down syndrome patients. Because defects in Ig levels are the hallmark of CVID, Down syndrome patients do not meet the CVID criteria. Still, the reduced memory B cell numbers in Down syndrome patients were suggestive of a defect in B-cell activation and proliferation (CVID pattern 3). Together with the impaired molecular maturation of IgM and IgA memory, these defects in B-cell memory might have implications for subsequent encounters with the same pathogen and hence the susceptibility to recurrent infections. Thus, our study indicates that analysis of immune-competence in Down syndrome patients should include analysis of (mucosal) IgA and IgM responses.

ACKNOWLEDGEMENTS

We are grateful for the patients their parents for their willingness to contribute to this study. We thank dr. Martijn Alles (Elkerliek Hospital, Helmond, the Netherlands), dr. Noortje Puts and dr. Vinus Bok (Maximá Medical Center, Veldhoven, the Netherlands) and dr. Ronald Admiraal (Radboud University Medical Center, Nijmegen, the Netherlands) for providing tonsil material of children with Down syndrome, and dr. Peet Nooijen (Jeroen Bosch Hospital, 's-Hertogenbosch) for his support at the laboratory of Clinical Pathology.

REFERENCES

1. Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down's syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-7.

2. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, van den Ende K, et al. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. Pediatrics 2007; 120:e1076-81.
3. Roizen NJ, Patterson D. Down's syndrome. Lancet 2003; 361:1281-9.
4. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol 2009; 156:189-93.
5. Verstegen RH, Kusters MA, Gemen EF, de Vries E. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. Pediatr Res 2010; 67:563-9.
6. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood 2011; 118:2150-8.
7. Park MA, Li JT, Hagan JB, Maddox DE, Abraham RS. Common variable immunodeficiency: a new look at an old disease. Lancet 2008; 372:489-502.
8. Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood 2011; 118:6814-23.
9. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood 2010; 115:5026-36.
10. Van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med 2007; 204:645-55.
11. Andersen P, Permin H, Andersen V, Schejbel L, Garred P, Svejgaard A, et al. Deficiency of somatic hypermutation of the antibody light chain is associated with increased frequency of severe respiratory tract infection in common variable immunodeficiency. Blood 2005; 105:511-7.
12. Van Zelm MC, Bartol SJW, Driessen GJ, Mascart F, Reisli I, Franco JL, et al. Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation. J Allergy Clin Immunol 2014.
13. Van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17:2257-317.
14. Yaari G, Uduman M, Kleinstein SH. Quantifying selection in high-throughput Immunoglobulin sequencing data sets. Nucleic Acids Res 2012; 40:e134.
15. Uduman M, Yaari G, Hershberg U, Stern JA, Shlomchik MJ, Kleinstein SH. Detecting selection in immunoglobulin sequences. Nucleic Acids Res 2011; 39:W499-504.
16. Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, et al. IMGT, the international ImMunoGeneTics information system. Nucleic Acids Res 2009; 37:D1006-12.

17. Van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest 2010; 120:1265-74.
18. De Vries E, Kuijpers TW, van Tol MJ, van der Meer JW, Weemaes CM, van Dongen JJ. [Immunology in medical practice. XXXV. Screening of suspected immunodeficiency: diagnostic protocols for patients with opportunistic or recurrent severe infections, wasting and failure to thrive]. Ned Tijdschr Geneeskd 2000; 144:2197-203.
19. Shott SR. Down syndrome: common otolaryngologic manifestations. Am J Med Genet C Semin Med Genet 2006; 142C:131-40.
20. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997; 130:388-93.
21. Kreuzaler M, Rauch M, Salzer U, Birmelin J, Rizzi M, Grimbacher B, et al. Soluble BAFF levels inversely correlate with peripheral B cell numbers and the expression of BAFF receptors. J Immunol 2012; 188:497-503.
22. Longo NS, Satorius CL, Plebani A, Durandy A, Lipsky PE. Characterization of Ig gene somatic hypermutation in the absence of activation-induced cytidine deaminase. J Immunol 2008; 181:1299-306.
23. Wu YC, Kipling D, Leong HS, Martin V, Ademokun AA, Dunn-Walters DK. High-throughput immunoglobulin repertoire analysis distinguishes between human IgM memory and switched memory B-cell populations. Blood 2010; 116:1070-8.
24. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. Science 2003; 301:1374-7.
25. Pugh-Bernard AE, Silverman GJ, Cappione AJ, Villano ME, Ryan DH, Insel RA, et al. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. J Clin Invest 2001; 108:1061-70.
26. Pascual V, Victor K, Lelsz D, Spellerberg MB, Hamblin TJ, Thompson KM, et al. Nucleotide sequence analysis of the V regions of two IgM cold agglutinins. Evidence that the VH4-21 gene segment is responsible for the major cross-reactive idiotype. J Immunol 1991; 146:4385-91.
27. Dunn-Walters DK, Spencer J. Strong intrinsic biases towards mutation and conservation of bases in human IgVH genes during somatic hypermutation prevent statistical analysis of antigen selection. Immunology 1998; 95:339-45.
28. Bose B, Sinha S. Problems in using statistical analysis of replacement and silent mutations in antibody genes for determining antigen-driven affinity selection. Immunology 2005; 116:172-83.
29. Piatosa B, van der Burg M, Siewiera K, Pac M, van Dongen JJ, Langerak AW, et al. The defect in humoral immunity in patients with Nijmegen breakage syndrome is explained by defects in peripheral B lymphocyte maturation. Cytometry A 2012; 81:835-42.

30. Van der Burg M, Pac M, Berkowska MA, Goryluk-Kozakiewicz B, Wakulinska A, Dembowska-Baginska B, et al. Loss of juxtaposition of RAG-induced immunoglobulin DNA ends is implicated in the precursor B-cell differentiation defect in NBS patients. Blood 2010; 115:4770-7.
31. Driessen GJ, Ijspeert H, Weemaes CM, Haraldsson A, Trip M, Warris A, et al. Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity. J Allergy Clin Immunol 2013; 131:1367-75 e9.
32. Gemen EF, Verstegen RH, Leuvenink J, de Vries E. Increased circulating apoptotic lymphocytes in children with Down syndrome. Pediatr Blood Cancer 2012; 59:1310-2.
33. Elsayed SM, Elsayed GM. Phenotype of apoptotic lymphocytes in children with Down syndrome. Immun Ageing 2009; 6:2.
34. Sapoznikov A, Pewzner-Jung Y, Kalchenko V, Krauthgamer R, Shachar I, Jung S. Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches. Nat Immunol 2008; 9:388-95.
35. Bloemers BL, van Bleek GM, Kimpen JL, Bont L. Distinct abnormalities in the innate immune system of children with Down syndrome. J Pediatr 2010; 156:804-9, 9 e1-9 e5.
36. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998; 281:1305-8.
37. Rathmell JC, Townsend SE, Xu JC, Flavell RA, Goodnow CC. Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. Cell 1996; 87:319-29.
38. Wang J, Lobito AA, Shen F, Hornung F, Winoto A, Lenardo MJ. Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. Eur J Immunol 2000; 30:155-63.
39. Kruetzmann S, Rosado MM, Weber H, Germing U, Tournilhac O, Peter HH, et al. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. J Exp Med 2003; 197:939-45.
40. Garrison MM, Jeffries H, Christakis DA. Risk of death for children with down syndrome and sepsis. J Pediatr 2005; 147:748-52.
41. Von Bernuth H, Picard C, Puel A, Casanova JL. Experimental and natural infections in MyD88- and IRAK-4-deficient mice and humans. Eur J Immunol 2012; 42:3126-35.
42. Weller S, Bonnet M, Delagreverie H, Israel L, Chrabieh M, Marodi L, et al. IgM+IgD+CD27+ B cells are markedly reduced in IRAK-4-, MyD88-, and TIRAP- but not UNC-93B-deficient patients. Blood 2012; 120:4992-5001.
43. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol 2012; 13:170-80.
44. Akinci O, Mihci E, Tacoy S, Kardelen F, Keser I, Aslan M. Neutrophil oxidative metabolism in Down syndrome patients with congenital heart defects. Environ Mol Mutagen 2010; 51:57-63.
45. Yamato F, Takaya J, Yasuhara A, Teraguchi M, Ikemoto Y, Kaneko K. Elevated intracellular calcium in neutrophils in patients with Down syndrome. Pediatr Int 2009; 51:474-7.

46. Costa-Carvalho BT, Martinez RM, Dias AT, Kubo CA, Barros-Nunes P, Leiva L, et al. Antibody response to pneumococcal capsular polysaccharide vaccine in Down syndrome patients. Braz J Med Biol Res 2006; 39:1587-92.
47. Joshi AY, Abraham RS, Snyder MR, Boyce TG. Immune evaluation and vaccine responses in Down syndrome: evidence of immunodeficiency? Vaccine 2011; 29:5040-6.
48. Kusters MA, Jol-van der Zijde CM, van Tol MJ, Bolz WE, Bok LA, Visser M, et al. Impaired avidity maturation after tetanus toxoid booster in children with Down syndrome. Pediatr Infect Dis J 2011; 30:357-9.
49. Kusters MA, Manders NC, de Jong BA, van Hout RW, Rijkers GT, de Vries E. Functionality of the pneumococcal antibody response in Down syndrome subjects. Vaccine 2013; 31:6261-5.
50. Hilton JM, Fitzgerald DA, Cooper DM. Respiratory morbidity of hospitalized children with Trisomy 21. J Paediatr Child Health 1999; 35:383-6.
51. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol 2004; 113:161-71.

Chapter 7

Increased circulating apoptotic lymphocytes in children with Down syndrome

E.F.A. Gemen, R.H.J. Verstegen, J. Leuvenink, E. de Vries

Pediatr Blood Cancer. 2012;59(7):1310-2.

ABSTRACT

Down syndrome resembles immunodeficiency with increased infections, auto-immune diseases and haematological malignancies. Until now, immunological studies in Down syndrome mainly focused on T-lymphocytes. We recently described a profound B-lymphocytopenia in children with Down syndrome. This could be caused by increased apoptosis. Therefore, we determined expression of flowcytometric markers for apoptosis (Annexin-V and propidium iodide) on peripheral lymphocytes in 72 children with Down syndrome and 32 age-matched controls. Within the total lymphocyte compartment, apoptosis was more pronounced in Down syndrome; it increased with age. Moreover, apoptosis was highest within the B-lymphocyte compartment which may be a contributing factor to the B-lymphocytopenia found in Down syndrome.

INTRODUCTION

Down syndrome, or trisomy 21, is associated with increased, mainly respiratory, infections, auto-immune diseases and haematological malignancies, suggesting impaired immunity. Indeed, many alterations of the cellular and humoral immune system have been found in patients with Down syndrome.[1] Whereas the function and number of T-lymphocytes have been the focus of immunological studies in Down syndrome for many years, we recently showed that children with Down syndrome also have a striking B-lymphocytopenia which is more profound than the decreased number of T-lymphocytes.[2]

Since apoptosis plays an important role in lymphocyte homeostasis, we studied the expression of flowcytometric apoptosis markers on peripheral lymphocytes and lymphocyte subpopulations in a large cohort of children with Down syndrome.

METHODS

Study population

After parental informed consent, an extra 3ml of EDTA blood was drawn during routine follow-up of thyroid function in 72 otherwise healthy children with Down syndrome at the Jeroen Bosch Hospital, 's-Hertogenbosch, and the Rijnstate Hospital, Arnhem, the Netherlands. Leftover EDTA blood of 32 healthy age-matched controls who underwent a regular venipuncture for pre-operative screening served as control. The Medical Ethics Commitees of both hospitals approved the study.

Immunophenotyping

Within 6 hours after sample collection, three-color flowcytometric immunophenotyping was performed on a FACSCan or FACSCalibur flow cytometer (BD) to determine relative lymphocyte subpopulations of $CD3^+$ T-lymphocytes, $CD3^+CD4^+$ helper T- (Th-) lymphocytes, $CD3^+CD8^+$ cytotoxic T-(Tc-) lymphocytes, $CD19^+$ B-lymphocytes and CD16and/or$CD56^+CD3^-$ NK-cells in both children with Down syndrome and age-matched controls children as described earlier.[3, 4] Annexin-V fluoresceïne-isothiocyanate (AV; FITC, Becton Dickinson Pharmingen (BD), San Jose, CA, USA) and propidium iodide (PI; Sigma-Aldrich, St. Louis, USA) staining took place after mononuclear cell separation on a Ficoll density gradient (Amersham Biosciences, Uppsala, Sweden). The stages of apoptosis were determined within the lymphogate and defined as AV^-/PI^- viable cells, AV^+/PI^- early apoptotic cells, AV^+/PI^+ late apoptotic cells and AV^-/PI^+ dead cells.

Isolated mononuclear cells were resuspended in 10% bovine serum albumin/ Rosewell Park Memorial Institute (RPMI) medium to a concentration of $0.25x10^9$ cells/L and cultured for 16 hours in an incubator (37°C, 5% CO_2). After culture, the sample was divided in several aliquots. The first aliquot was used for flowcytometric analysis of AV/PI. For the AV/PI staining, unlabeled cells resuspended in AV-buffer were stained with AV-FITC and PI. The other aliquots were used for determining lymphocyte subpopulations. Within these subpopulations AV-expression was measured. Labeled cells were washed with PBS, resuspended in AV-binding buffer (2-8°C; BD) and incubated with AV (FITC or PE; BD) for 15 minutes in ice water for this purpose. After AV-staining, the cells were washed with AV-binding buffer and immediately analyzed. Due to the small sample sizes we were not able to perform all tests for each patient.

Statistics

The independent-samples t-test ($p<0.05$) was used to compare the results between Down syndrome and age-matched controls and assess differences between clinical features. Comparison of values at t=0 and t=16 hours was performed using the paired-samples t-test. Bi-variate correlation between age and expression of AV and PI was assessed using Pearson's correlation coefficients. All analyses were performed with SPSS 17.0 for Mac OS X.

RESULTS

The absolute numbers of total lymphocytes as well as T- and B-lymphocytes, are decreased in children with Down syndrome compared to age-matched controls ($p=0.001$, $p=0.013$ and $p<0.001$, respectively), as described earlier (Table 1).[2, 5] At t=0 (Down syndrome n=58 and age-matched controls n=28) the percentage of viable lymphocytes in children with Down syndrome is decreased ($p<0.001$) and the percentage of early and late apoptotic cells is increased ($p<0.001$) compared to age-matched controls (Figure 1). The distribution of AV/PI-staining is similar in all ages in age-matched controls, whereas in children with Down syndrome the percentage of viable lymphocytes decreases with increasing age at t=0 ($r=-0.388$, $p=0.003$). Also, the percentages of early apoptotic and dead lymphocytes correlate positively with increasing age at t=0 ($r=0.268$, $p=0.04$ and $r=0.335$, $p=0.01$, respectively). For age-matched controls, there is a significant correlation between the absolute number of lymphocytes and the percentage of viable cells at T=0 ($r=0.499$, $p=0.007$). Compared to t=0, both Down syndrome and age-matched controls (Down syndrome n=53 and

Table I. Absolute numbers of lymphocyte subpopulations in children with Down syndrome compared to age matched controls.

		9 – 15 months		15 – 24 months		2 – 5 years		5 – 10 years		10 – 16 years		>16 years		Total	
Total lymphocytes	**DS**	**2.82** (1.42-6.20)	n=6	**2.58** (1.89-3.21)	n=4	**2.00** (1.26-4.19)	n=12	**1.88** (0.96-3.70)	n=36	**1.59** (0.93-2.89)	n=12	**1.81** (1.53-2.09)	n=2	**1.91** (0.93-6.20)	n=72
	AMC	3.80	n=1		n=0	2.90 (1.80-3.60)	n=9	2.70 (2.20-3.30)	n=9	2.10 (1.40-3.70)	n=11	1.95 (1.70-2.20)	n=2	2.60 (1.40-3.80)	n=32
T lymphocytes $CD3^+$	**DS**	**1.91** (0.98-4.29)		**1.86** (1.37-2.32)		**1.43** (0.81-3.07)		**1.39** (0.62-2.67)		**1.14** (0.54-2.45)		**1.41** (1.25-1.56)		**1.39** (0.54-4.29)	
	AMC	2.28				1.89 (1.24-2.38)		1.86 (1.49-2.38)		1.62 (0.83-2.41)		1.36 (1.07-1.65)		1.65 (0.83-2.41)	
Helper T lymphocytes $CD3^+CD4^+$	**DS**	**1.28** (0.69-2.97)		**1.14** (0.70-1.59)		**0.77** (0.40-1.67)		**0.63** (0.30-1.40)		**0.60** (0.34-1.16)		**0.87** (0.81-0.92)		**0.66** (0.30-2.97)	
	AMC	0.82				0.74 (0.46-0.98)		0.71 (0.60-1.16)		0.61 (0.29-0.91)		0.66 (0.48-0.84)		0.70 (0.29-1.16)	
Cytotoxic T lymphocytes $CD3^+CD8^+$	**DS**	**0.52** (0.21-1.21)		**0.76** (0.42-0.85)		**0.54** (0.28-1.42)		**0.71** (0.27-1.46)		**0.50** (0.18-1.16)		**0.56** (0.33-0.79)		**0.57** (0.18-1.47)	
	AMC	0.46				0.43 (0.32-0.74)		0.37 (0.27-0.56)		0.30 (0.18-0.67)		0.28 (0.19-0.36)		0.38 (0.18-0.74)	
B lymphocytes $CD19^+$	**DS**	**0.52** (0.19-1.20)		**0.36** (0.17-0.76)		**0.26** (0.18-0.48)		**0.20** (0.00-0.01)		**0.13** (0.07-0.60)		**0.12** (0.12-0.12)		**0.20** (0.06-1.20)	
	AMC	1.21				0.69 (0.31-0.96)		0.44 (0.35-0.63)		0.31 (0.19-0.59)		0.24 (0.21-0.27)		0.43 (0.19-1.21)	
NK cells $CD3^-/CD16^+$ and/ or 56^+	**DS**	**0.20** (0.19-0.54)		**0.22** (0.11-0.33)		**0.17** (0.11-0.47)		**0.18** (0.05-1.05)		**0.19** (0.10-0.52)		**0.22** (0.17-0.26)		**0.19** (0.05-1.05)	
	AMC	0.15				0.20 (0.09-0.34)		0.22 (0.12-0.48)		0.24 (0.09-0.56)		0.22 (0.22-0.22)		0.22 (0.09-0.56)	

Absolute numbers (x 10^9/L), * median, ** range; AMC = age-matched controls; DS = Children with Down syndrome. Age groups are based on Comans-Bitter et al.[5]

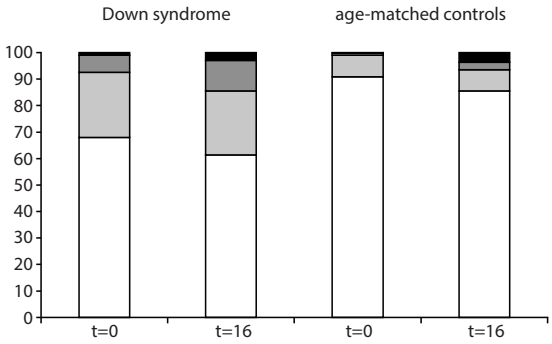


Figure 1. Stages of apoptosis in Down syndrome and age matched controls at inclusion (t=0; Down syndrome n=58 and age-matched controls n=28) and after 16 hours of culture (t=16; Down syndrome n=53 and age-matched controls n=26) divided in AV-/PI- viable cells (white), AV+/PI- early apoptotic cells (light grey), AV+/PI+ late apoptotic cells (dark grey) and AV-/PI+ dead cells (black).

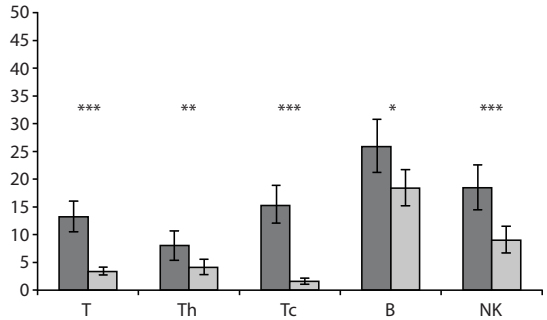


Figure 2. Relative expression of Annexin V (AV) on lymphocytes after 16 hours of culture in Down syndrome (black bars) and age-matched controls (grey bars) divided in T-lymphocytes (T), helper T-lymphocytes (Th), cytotoxic T-lymphocytes (Tc), B-lymphocytes (B) and NK-cells (NK) (Down syndrome: n=55, 57, 58, 42, 53 and age-matched controls: n=26). Results of independent t-tests between Down syndrome and age-matched controls are presented: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The error bars represent the 95% confidence interval.

age-matched controls n=26) show a similar decreased percentage of viable cells and increased percentage of late apoptotic and dead lymphocytes at t=16.

The percentage of apoptotic cells (AV^+) within the T- and B-lymphocyte and NK-cell subpopulations at t=16 is increased in children with Down syndrome compared to age-matched controls ($p<0.001$, $p=0.011$ and $p<0.001$, respectively; Figure 2). In both Down syndrome and age-matched controls, the percentage of AV^+ cells is highest within the B-lymphocyte subpopulation. In Down syndrome, the percentage of apoptotic AV^+ cells is higher in cytotoxic T-lymphocytes than in helper-T-lymphocytes, whereas in age-matched controls the opposite results are found. There was no correlation between absolute numbers of lymphocyte subsets and AV expression at T=16. Between t=0 and t=16, the relative number of B-lymphocytes

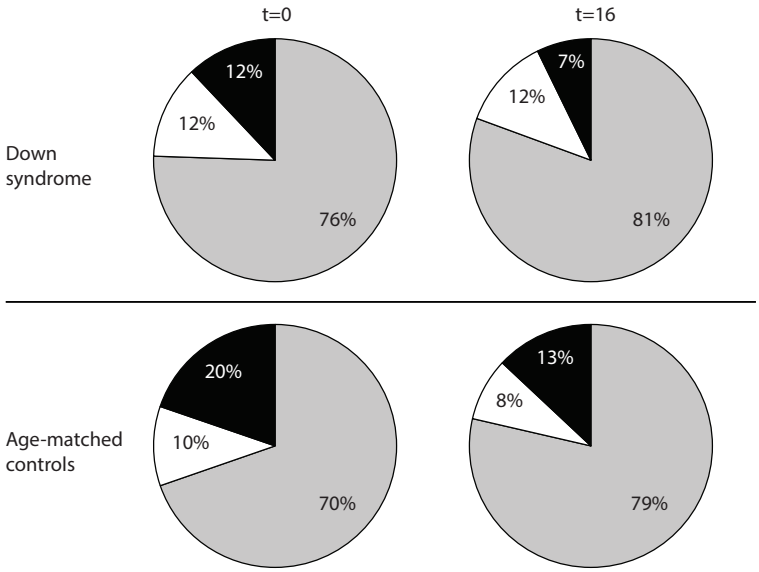


Figure 3. Relative distribution of T-lymphocytes (light grey), B-lymphocytes (black) and NK-cells in Down syndrome and age-matched controls at inclusion (t=0) and after 16 hours (t=16). Between t=0 and t=16 the relative number of B-lymphocytes decreases and the relative number of T-lymphocytes increases (p<0.001) in Down syndrome and age-matched controls. Only in age-matched controls, the percentage of NK-cells also decreased significantly between t=0 and t=16 (p=0.011).

decreases and the relative number of T-lymphocytes increases (p<0.001) in Down syndrome and age-matched controls. Only in age-matched controls, the percentage of NK-cells also decreased significantly between t=0 and t=16 (p=0.011; Figure 3). Moreover, an increasing CD4/CD8 ratio is found in Down syndrome (t=0: 1.33, t=16: 1.80, p<0.001) as well as age-matched controls (t=0: 1.82, t=16: 2.39, p<0.001).

DISCUSSION

We found increased circulating apoptotic lymphocytes in children with Down syndrome in comparison to age-matched controls. Likewise, Antonucci *et al.* published increased apoptosis of peripheral blood cells in Down syndrome in 1997; this was evaluated by electron microscopy, *in situ* nick translation, and agarose gel electrophoresis.[6] Since then, flow cytometric techniques have been introduced. With these, Elsayed *et al.* also found increased relative numbers of early apoptotic T- and B-lymphocytes in 17 children with Down syndrome (aged 4 months – 14 years),[7] and Corsi *et al.* described increased early apoptosis of T-lymphocytes in Down syndrome.[8] Bloemers *et al.* reported normal fractions of apoptotic naive $CD4^+$ and $CD8^+$ T-lymphocytes in children with Down syndrome,[9] which suggests that the

presence of apoptosis may be different between specific circulating T-lymphocyte subpopulations.

In contrast to Elsayed *et al.* we collected data from a large group of children with Down syndrome, which allowed us to determine the effect of age. We found that with increasing age, less circulating viable lymphocytes are present in Down syndrome. In both Down syndrome and age-matched controls, highest rates of apoptosis were found in B-lymphocytes. At last, we determined markers of apoptosis at two time-points. In Down syndrome, apoptosis was most increased at the beginning of the experiment (t=0), reflecting *in vivo* apoptosis. The difference of apoptosis between both time-points is caused by *in vitro* apoptosis, which is equal in Down syndrome and age-matched controls. Therefore, it is suggestive that the increased apoptosis in Down syndrome is more caused by increased induction of apoptosis rather than an increased susceptibility to apoptosis.

Thus, Down syndrome is not only associated with an absolute lymphocytopenia, which has been described earlier,[2] but also with a larger fraction of circulating apoptotic lymphocytes. The cause of the increased rate of lymphocyte apoptosis in Down syndrome is unclear. However, it may be a contributing factor to the profound B-lymphocytopenia in Down syndrome, but further studies are needed to elucidate this.

ACKNOWLEDGMENTS

We would like to thank patients and parents for their participation in this study, and Natasja Runderkamp for her technical assistance in analyzing the bloodsamples.

REFERENCES

1. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol 2009; 156:189-93.
2. De Hingh YC, van der Vossen PW, Gemen EF, Mulder AB, Hop WC, Brus F, et al. Intrinsic abnormalities of lymphocyte counts in children with down syndrome. J Pediatr 2005; 147:744-7.
3. Kusters MA, Gemen EF, Verstegen RH, Wever PC, de Vries E. Both normal memory counts and decreased naive cells favor intrinsic defect over early senescence of Down syndrome T lymphocytes. Pediatr Res 2010; 67:557-62.
4. Verstegen RH, Kusters MA, Gemen EF, de Vries E. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. Pediatr Res 2010; 67:563-9.

5. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997; 130:388-93.
6. Antonucci A, Di Baldassarre A, Di Giacomo F, Stuppia L, Palka G. Detection of apoptosis in peripheral blood cells of 31 subjects affected by Down syndrome before and after zinc therapy. Ultrastruct Pathol 1997; 21:449-52.
7. Elsayed SM, Elsayed GM. Phenotype of apoptotic lymphocytes in children with Down syndrome. Immun Ageing 2009; 6:2.
8. Corsi MM, Ponti W, Venditti A, Ferrara F, Baldo C, Chiappelli M, et al. Proapoptotic activated T-cells in the blood of children with Down's syndrome: relationship with dietary antigens and intestinal alterations. Int J Tissue React 2003; 25:117-25.
9. Bloemers BL, Bont L, de Weger RA, Otto SA, Borghans JA, Tesselaar K. Decreased thymic output accounts for decreased naive T cell numbers in children with Down syndrome. J Immunol 2011; 186:4500-7.

Chapter 8

Impact of Down syndrome on the performance of neonatal screening assays for severe primary immunodeficiency diseases

R.H.J. Verstegen, S. Borte, L.A. Bok, P.H.Th. van Zwieten, U. von Döbeln, L. Hammarström, E. de Vries

J Allergy Clin Immunol. 2014;133(4):1208-11.

ABSTRACT

The rate of abnormal results in neonatal screening programs for SCID and XLA, related to the T- and B-lymphocytopenia in newborns with Down syndrome, depends on the cut off ranges used and might be reduced by implementation of second-tier tests.

TO THE EDITOR

Neonatal screening programs for severe combined immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA) have recently been established based on molecular quantitation of the levels of T-cell receptor excision circles (TRECs) for SCID and kappa-deleting recombination excision circles (KRECs) for XLA in dried blood spot samples (DBSS) obtained from regular Guthrie cards.[1-4] This technique features a remarkable sensitivity for the identification of newborns characterized by severe T- and/or B-lymphocytopenia at birth. However, after testing a second punch from the primary Guthrie card ('re-test rate'), ~1 in 500 samples require a second Guthrie card ('re-run rate'), which suggests that diseases or conditions other than SCID or XLA yield abnormal test results in the combined TREC/KREC assay.[2] For example, it has previously been shown that immaturity of the immune system in preterm neonates, as well as inflammatory conditions (e.g. sepsis and lymphocyte extravasation), and 22q11 deletion syndromes (velocardiofacial and DiGeorge syndrome, OMIM 192430 and 188400, respectively) have negative impact on target-disease recall rates.[3]

Throughout life, patients with Down syndrome (trisomy 21; OMIM 190685) have decreased numbers of T- and B-lymphocytes,[5, 6] and are thus likely to have lower TREC and KREC copy numbers. Clinicians identify the vast majority of these newborns by clinical examination, yet this information is often not made available to the screening lab. Given a Down syndrome birth rate of about 1 in 600-900 newborns, we hypothesized that this condition may thus contribute to the overall re-run rate of the TREC/KREC assay. Given that there is no cohort data available on the copy numbers of TRECs and KRECs in Guthrie cards from children diagnosed with Down syndrome, we studied original stored DBSS of children with Down syndrome and compared them to healthy newborns. Moreover, we developed a second-tier test for Down syndrome and 22q11 deletion syndromes to streamline the workflow of abnormal TREC/KREC results in newborn screening laboratories. Depending on local legislation and screening logistics, this contribution may be useful to improve screening efficiency, speed, as well as notification of the parents.

This study was approved by the Medical Ethical Committee "METOPP" employed by the Jeroen Bosch Hospital. Following parental consent, we collected 3.2 mm punches from the original stored Guthrie cards of 82 Dutch children with Down syndrome, prepared ~3-5 days after birth. The DBSS had been stored at room temperature for 6 to 68 (median 46) months.

Freshly collected Guthrie cards from 1119 anonymized healthy Swedish children and 5 children with 22q11 deletion syndromes, as well as 110 Dutch storage-time matched samples had been collected, which served as controls and were partly published earlier.[2, 3] DNA extraction from DBSS and quantitative triplex real-time qPCR for TRECs, KRECs and β-actin (ACTB) was performed on a ViiA7 real-time PCR system (Applied Biosystems) as previously described.[2] The qPCR procedure was optimized based on custom reagents provided by Affymetrix (Santa Clara, CA, USA). Amplification of ACTB was used to assess the success of DNA extraction from the Guthrie cards and to standardize TREC and KREC copy numbers. The TREC and KREC copy numbers measured in DBSS from 28 children with Down syndrome and 41 storage-time matched controls were normalized on the average of 1700 ACTB copies, as calculated from the other patient samples. Abnormal TREC/KREC screening results were defined as <15 TRECs/μL and <10 KRECs/μL of dried blood based on cut off values reported previously.[2] According to current prospective trials in Germany and Sweden additional cut off values were applied (<8 TRECs/μL and <6 KRECs/μL). The re-run rate would decrease from ~1 in 500 to ~1 in 1500 when the lower cut-off values are used. All abnormal results for TREC and/or KREC copy numbers were confirmed by retesting of a second punch from the original card or from a second Guthrie card, when available.

In order to potentially improve the diagnostic workflow of abnormally tested samples, we applied a second triplex real-time qPCR reaction using the original DNA eluates from the Guthrie cards in order to assess markers located on chromosomes 19 (*PTBP1* gene), 21 (*KCNE1 gene*) and 22 (*TBX1* gene). The real-time qPCR reactions were performed in a final volume of 20 μL containing 2x VeriQuest Probe qPCR Master Mix (Affymetrix), 8μM of primers and 3μM of probes as further specified in Table 1. Real-time qPCR conditions were adapted from those previously described to allow simultaneous execution of the triplex TREC-KREC-ACTB and the PTBP1-KCNE1-TBX1 assays in one test plate.[2] The relative allelic marker gene expression on chromosomes 21 (*KCNE1*) and 22 (*TBX1*) was calculated based on the assumption of two allelic copies of the *PTBP1* gene on chromosome 19. Thus, patients with Down syndrome would be expected to carry more than two *KCNE1* copies, while patients with 22q11 deletion syndromes are characterized by hemizygous deletions of the TBX1 gene region.

All DBSS from children with Down syndrome generally presented with lower levels of TRECs and KRECs compared to healthy newborns and storage-time matched controls (median 25.5 vs 222.2 and 76.8 TRECs/μL, $p<0.0001$ and median

Table 1. Primer and probe sequences used for the trisomy 21 / 22q11 deletions second-tier RT-qPCR assay

Marker gene (Chromosome location)	Oligonucleotide sequences 5`-3`	Primer direction / MGB probe reporter
PTBP1 (19p13.3)	GTTCCCGTAACTGAAACATCAATG	Forward
	AAGGAACCTGAGGATGCTGTGT	Reverse
	CAGGCTCAGCTGGT	NED
KCNE1 (21q22.12)	GGGATTCTTCGGCTTCTTCAC	Forward
	CGTTGAATGGGTCGTTCGA	Reverse
	CATGCTGAGCTACATCCGCTCCAAGA	VIC
TBX1 (22q11.21)	GATGAGGCCAAATGACTCGATT	Forward
	CACCTCTTGCATGCACACTTG	Reverse
	AGGTTTCAGAGCCCAAG	6-FAM

17.9 vs 109.5 and 45.2 KRECs/µL, $p<0.0001$, both assessed by Mann-Whitney test; Figure 1A). As suspected, the values of KRECs and TRECs in stored cards are slightly lower compared to fresh cards. The gestational age did not influence the TREC and KREC results in Down syndrome DBSS (data not shown). The overall trend towards lower TREC and KREC copy numbers in newborns with Down syndrome indicates that the choice of suitable cut off values for SCID and XLA critically influences the re-test, as well as the re-run rate of the TREC-KREC assay.

Although the diagnosis of Down syndrome is increasingly being suspected prenatally or shortly after birth, genetic confirmation is commonly not available to the screening lab at the time the Guthrie cards are collected. Moreover, 22q11 deletion syndrome will generally not be suspected at birth. The application of a second-tier test strategy to exclude newborns with Down syndrome or 22q11 deletion syndromes might therefore be a useful approach to sharpen the screening workflow for SCID and XLA before a follow-up card is ordered or a patient is called in for examination as all patients in this study would have been identified correctly (Figure 1B).

These results fit in with other immunological features of patients with Down syndrome consistent with immunodeficiency, and offers new evidence that B-lymphocytes in Down syndrome also feature an intrinsic defect since KRECs are already abnormally low at birth. Starting in the 1970s, research on the immune system in Down syndrome has been performed with emphasis on the development of T-lymphocytes.[6] The thymus of children with Down syndrome is smaller and shows structural abnormalities, and low absolute numbers of T-lymphocytes have been frequently reported.[6, 7] In addition, TRECs are decreased in children with Down syndrome, resembling decreased thymic output.[8] Only during the past few

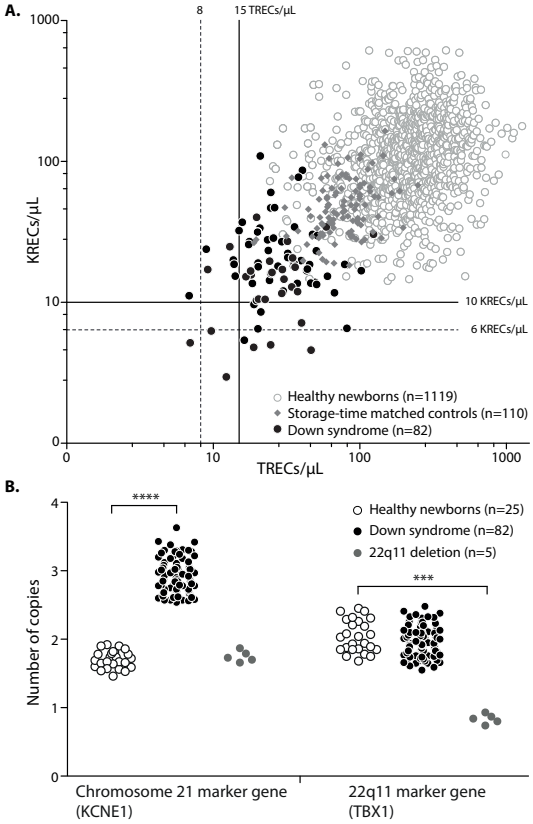


Figure 1. A. TREC and KREC copy numbers in dried blood spots samples (DBSS) from newborns with Down syndrome, storage-time matched controls and healthy newborns. Lines represent different applicable cut off values.[2] **B.** Relative expression of chromosome 21 and 22q11 marker genes in DBSS from healthy newborns, children with Down syndrome and children with 22q11 deletion syndrome. *** Mann-Whitney test $p<0.001$; **** paired t-test $p<0.0001$.

years, a stronger focus has been put on B-lymphocytes. The absolute number of B-lymphocytes is decreased in patients with Down syndrome and decreased expression of CD21, CD23 and CD27 on B-lymphocytes is reminiscent of common variable immunodeficiency.[9] Immunization responses to several protein and polysaccharide antigens is abnormal,[6] without an increase of adverse effects in live-attenuated vaccines, as seen in patients with SCID.[10] Our data show that these findings are probably not exclusively caused by a disturbed T-B interaction due to insufficient T-cell help, as has been suggested until now.

Newborns with Down syndrome present with T- and B-lymphocytopenia. Accordingly, these patients with Down syndrome may lead to re-testing and initiation of follow-up processes in neonatal TREC/KREC screening programs for

severe primary immunodeficiencies; the magnitude of this problem depends on the chosen cut offs.

ACKNOWLEDGEMENTS

We thank the parents and patients for their contribution to this study, as well as W.E.A. Bolz,† F. Dikken, W. Rijnvos, E. van Leer, and A.M. van Wermeskerken for pediatric consultancy and the Dutch National Institute for Public Health and the Environment (RIVM) for supplying stored Guthrie cards.

REFERENCES

1. Baker MW, Grossman WJ, Laessig RH, Hoffman GL, Brokopp CD, Kurtycz DF, et al. Development of a routine newborn screening protocol for severe combined immunodeficiency. J Allergy Clin Immunol 2009; 124:522-7.
2. Borte S, von Dobeln U, Fasth A, Wang N, Janzi M, Winiarski J, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood 2012; 119:2552-5.
3. Borte S, Wang N, Oskarsdottir S, von Dobeln U, Hammarstrom L. Newborn screening for primary immunodeficiencies: beyond SCID and XLA. Ann N Y Acad Sci 2011; 1246:118-30.
4. Nakagawa N, Imai K, Kanegane H, Sato H, Yamada M, Kondoh K, et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. J Allergy Clin Immunol 2011; 128:223-5 e2.
5. Thilaganathan B, Tsakonas D, Nicolaides K. Abnormal fetal immunological development in Down's syndrome. Br J Obstet Gynaecol 1993; 100:60-2.
6. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol 2009; 156:189-93.
7. Kusters MA, Gemen EF, Verstegen RH, Wever PC, de Vries E. Both normal memory counts and decreased naive cells favor intrinsic defect over early senescence of Down syndrome T lymphocytes. Pediatr Res 2010; 67:557-62.
8. Roat E, Prada N, Lugli E, Nasi M, Ferraresi R, Troiano L, et al. Homeostatic cytokines and expansion of regulatory T cells accompany thymic impairment in children with Down syndrome. Rejuvenation Res 2008; 11:573-83.
9. Verstegen RH, Kusters MA, Gemen EF, de Vries E. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. Pediatr Res 2010; 67:563-9.
10. Bakare N, Menschik D, Tiernan R, Hua W, Martin D. Severe combined immunodeficiency (SCID) and rotavirus vaccination: reports to the Vaccine Adverse Events Reporting System (VAERS). Vaccine 2010; 28:6609-12.

Chapter 9

Discussion

GENERAL DISCUSSION

Down syndrome is a heterogeneous condition that is characterized by developmental delay and increased incidences of numerous medical problems. In The Netherlands, it is advised that children with Down syndrome are treated by specialized health care workers who use an evidence based medical guideline.[1] Children as well as adults suffer from recurrent respiratory tract infections that cause high morbidity as well as mortality. Therefore, the studies in this thesis investigated how respiratory tract infections influence the lives of children with Down syndrome and focussed on defects in humoral immunity as a potential cause for their increased susceptibility to these infections.

Respiratory symptoms in Down syndrome

KiDS diary study

Respiratory tract infections are the main reason for doctor visits and for hospitalizations in children with Down syndrome.[2-5] To gain more insight in the respiratory symptoms that parents observe in their children, we have designed the prospective KiDS diary study that is currently ongoing (**Chapter 2**).

This study design is different from other studies because we include respiratory symptoms, not medical diagnoses. Because many symptoms do not require a visit to the doctor, our study will provide a better estimate of the incidence of respiratory symptoms throughout the year in children with Down syndrome. Additionally, we will be able to determine the difference in incidence and severity in the various age groups, and we can study how these symptoms will evolve in the course of 2 years of follow-up. Potential influence of co-morbidity can be assessed in the statistical analysis. At last, it will provide insight on the impact of these symptoms on school attendance and parental absence from work. These data are useful to support studies on preventive and therapeutic modalities.

Wheezing

Wheezing is defined as a high-pitched lung sound produced by expiratory airflow through narrowed airways. It is a common symptom in pre-school children. Approximately 50% of all children will develop at least one episode of wheezing before the age of 6 years.[6] Although many different causes can underlie wheezing, viral-induced wheezing and asthma are most common in childhood.[7-9] It is desirable to distinguish viral-induced wheezing and 'true' asthma as different treatment and follow-up strategies are needed. For example, in children that have wheezing

related to asthma, inhaled and systemic corticosteroids are effective treatment options, whereas their effect is limited in children with viral-induced wheezing.[10-13] Moreover, in asthma symptoms are more likely to persist into adulthood, whereas viral-induced wheezing generally subsides with increasing age.[7] Unfortunately, the distinction between viral induced wheezing and asthma is difficult to make.[7,8] First, both diseases present in an identical manner and have identical findings at physical examination. Second, pre-school children are not able to perform spirometry, which is required for a diagnosis of asthma.

In our studies (**Chapters 5, 6 and 7**), we have observed that many children with Down syndrome have recurrent wheezing for which inhaled bronchodilators as well as inhaled corticosteroid drugs are prescribed. However, none of the children used these drugs after the age of 8 years. Current literature shows a lower prevalence of asthma and allergic diseases (i.e. conjunctivitis, rhinitis) in Down syndrome compared to individuals without Down syndrome.[14-16] Thus, it is more likely that viral induced wheezing is the underlying cause of wheezing in children with Down syndrome. The presence of wheezing over time will be studied in the KiDS diary study (**Chapter 2**). We believe this will provide more information on the incidence and how these symptoms evolve over time. This may lead to support for parents and treating physicians to limit the use of inhaled corticosteroid drugs in Down syndrome. Especially since inhaled corticosteroid drug have an immunosuppressive effect on the mucosal immunity in the lungs,[17-20] children with Down syndrome might become even more susceptible to respiratory tract infections when using these drugs.

The impact of respiratory symptoms on daily life

Development and behavioural status

The impact of medical complications in Down syndrome is generally determined based on severe adverse events such as hospitalization, admission to an Intensive Care Unit and mortality. Outcome measures that give more insight in the impact of medical complications on daily life are also important. This can be performed by assessing development and behaviour, including practical and social skills, and also quality of life. Focus on these parameters allows further improvement of everyday life for individuals with Down syndrome.

Many parents report that the respiratory tract infections negatively affect the developmental status of their child. In **Chapter 3** we confirmed this in a large cohort of 8-year-old children with Down syndrome. Moreover, we found that parent reported recurrent respiratory tract infections have a negative impact on the child's

behaviour. They show more attention and internalizing problems, such as being withdrawn. The question remains how development and behaviour in childhood relates to the level of independence they will achieve as adults. Recently, Van Gameren-Oosterom *et al.* have performed a follow-up study in the same cohort we have described in **Chapter 3**.[21] The authors determined to what extent the children in their adolescent years (aged 16 to 19 years) were able to perform practical and social skills, such as cooking, taking a bus or making a telephone call. It would be very interesting to correlate development and behaviour at the age of 8 years to the level of functioning in adulthood. This proposed study would answer the question whether recurrent respiratory tract infections in children with Down syndrome result in less practical and social skills when they grow up.

The current paradigm is that academic and social skills in children with Down syndrome improve when they attend regular education.[22, 23] However, it could be that these outcomes are more related to the level of intellectual disability as this highly affects regular school placement and attainment of academic skills.[22-24] Thus, in future studies on adolescents, the influence of attending regular education on practical and social skills should be determined and taken into account when the effect of recurrent respiratory tract infections is studied, especially because these infections are associated with more children attending special education.

Health Related Quality of Life

In our study we have shown that Health Related Quality of Life (HRQoL) in children with Down syndrome is negatively affected by the presence of recurrent respiratory tract infections. Unfortunately, there is no HRQoL assessment tool that is specifically designed and validated for children or adults with Down syndrome. The currently used questionnaire contains questions regarding activities that are related to development, such as reading and writing. Some other questions focus on the child's behaviour, which is often affected in Down syndrome.[25] Since children with Down syndrome are compared to normative data, they have scores very much outside of the reference values for the subscales of gross motor skills, autonomy, cognitive functioning and social functioning, which makes interpretation difficult.[25] We therefore believe that there is a need to develop specific questionnaires to assess HRQoL in individuals with Down syndrome. These should include questions that take the effect of development and behaviour into account.

It has become clear that respiratory tract infections have a negative impact on development, behaviour and HRQoL of young children with Down syndrome. However, further studies are needed to determine if these effects persists

throughout adolescence. If so, this would further stress the importance of more effective preventive and treatment modalities of respiratory tract infections in children with Down syndrome.

Humoral immunity in Down syndrome

Naive B-cell numbers

Children with Down syndrome suffer from increased incidences of respiratory infections, auto-immunity and haematological malignancies that suggest impaired immunity. We have studied the composition and function of the B-cell compartment in Down syndrome (Figure 1). In accordance with previous studies,[26, 27] we found that children with Down syndrome have decreased blood B-cell numbers that are more profound than the decrease of T cells (**Chapters 5 and 6**). As discussed in **Chapter 6**, impaired bone marrow output and impaired homeostatic proliferation were excluded as potential causes of low naive B-cell numbers. This leaves decreased survival as the most likely cause. Because naive B cells depend on B-cell activating factor (BAFF) for their survival,[28-30] we studied serum BAFF levels in patients with Down syndrome and showed that these were slightly increased (**Chapter 6**). Thus, serum BAFF is no limiting factor for B-cell survival. In **Chapter 7** we studied

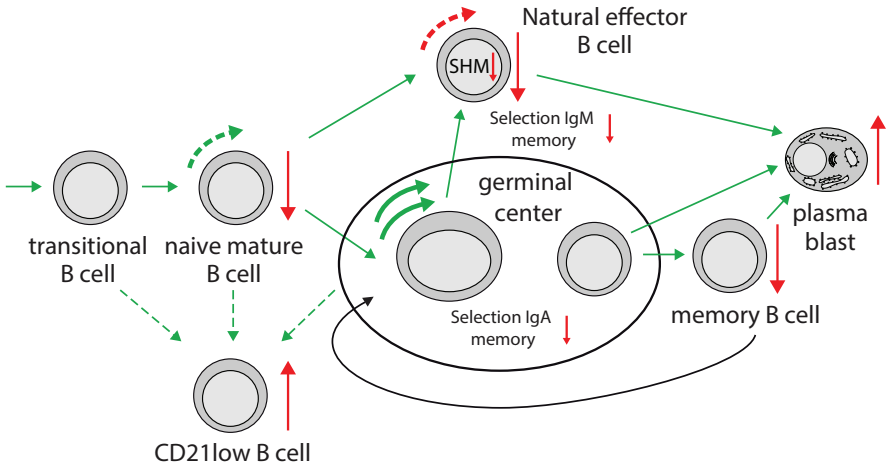


Figure 1. Characteristics of the peripheral B-cell compartment in Down syndrome.
Patients with Down syndrome have normal numbers of transitional B cells but decreased naive mature B cells, without increased homeostatic proliferation. The germinal center composition is normal but affinity maturation is impaired in IgA memory. Generation of IgG memory is normal. However, the absolute number of memory B cells is decreased. Natural effector B cells show decreased proliferation, somatic hypermutations (SHM) and impaired selection. Higher numbers of plasma blasts are found in Down syndrome. The aberrant development of $CD21^{low}$ B cells is increased in Down syndrome. All abnormalities are shown in red.

whether apoptosis was altered in Down syndrome and negatively affects the size of the B-cell compartment. In this study we demonstrated that apoptosis of B cells was increased in children with Down syndrome. We therefore conclude that this is probably the most important contributor to low blood B-cell numbers in Down syndrome.

Multiple intra- and extracellular mechanisms regulate apoptosis of B cells. Extracellular signals can induce apoptosis by interacting with CD95 (FAS). Therefore, we have studied CD95 expression and showed that this was normal in naive mature B cells. This suggests that this pathway is not responsible for the increased apoptosis. We therefore hypothesize that mitochondria may result in increased apoptosis in Down syndrome. In each living cell, mitochondria are responsible for energy supply. Moreover, they determine cell fate upon cellular stress, such as DNA damage and hypoxia.[31] It is known that mitochondrial morphology as well as function is impaired in Down syndrome.[32] This results in increased oxidative stress and subsequent down-regulation of energy metabolism.[32] In addition, it is known that susceptibility of mitochondria to damaging agents is increased in Down syndrome, but their effect on B cells was not studied.[33, 34] Therefore, future studies could include analysis of mitochondrial function and their relation to apoptosis in B cells. It would be important to investigate how the mitochondria react upon B-cell activation since this will increase the metabolic need.

B-cell antigen receptor repertoire

Patients with Down syndrome have a restricted B-cell repertoire, as a result of low B-cell numbers. However, the question remains whether the repertoire is further restricted by impaired V(D)J recombination. Multiple diseases have impaired V(D)J recombination. For example, Nijmegen Breakage Syndrome (NBS) and Ataxia Telangectasia (AT) are autosomal recessive diseases that result from inherited genetic defects in the *NBN* and *ATM* genes, respectively. The encoded proteins are part of the DNA damage response, which is activated during V(D)J recombination when double-stranded DNA breaks are induced. These patients not only have decreased bone marrow output, but also have a restricted B-cell repertoire.[35, 36] In Down syndrome, Morawiec *et al*. have demonstrated that peripheral blood lymphocytes are prone to DNA damage in conjunction with impaired DNA repair.[37] Therefore, it cannot be excluded that the B-cell repertoire in Down syndrome is further restricted as a consequence of impaired DNA repair. We believe that repertoire analysis by next generation sequencing would be interesting to investigate. IGH sequences can be obtained from multiple independent PCR reactions. Subsequently, the number

of coincidences, meaning the same sequences in independent PCR reactions, can be used as a measure for repertoire restriction.[38]

Antibody response and generation of B-cell memory

The clinical phenotype of Down syndrome is suggestive for impaired adaptive immunity. Because patients have normal serum Ig values and mildly affected vaccination responses, there defects do not correspond to well-defined antibody deficiencies. However, in **Chapter 6** we found impaired generation of B-cell memory. We showed decreased memory B-cell numbers, that might be the result of upregulated CD95 expression as demonstrated. Moreover, we showed that affinity maturation was impaired in IgM and IgA memory, but not IgG memory.

Patients with Down syndrome seem to have normal T-cell dependent co-stimulatory signalling. Genetic mutations that affect CD40-CD40L and ICOS-ICOSL signalling as well as AID result in class switch disorders. These patients have abnormal germinal center configurations and lack serum IgG.[39] In our study we have demonstrated that patients with Down syndrome have normal germinal centers with normal presence of follicular helper T cells. Moreover, serum IgG levels and affinity maturation of IgG was normal (**Chapter 6**).

The identified defects in IgM memory B cells, might suggest impaired signalling from T-cell independent co-stimulation. In the absence of T-cell help, secondary activation signals come from BCR crosslinking or pattern recognition receptors, which include Toll-like receptors (TLR). TLR signalling is mainly depending on the MyD88-TIRAP-IRAK4 pathway that has been found to be critical for natural effector homeostasis.[40] However, impairment of this pathway does not result in decreased somatic hypermutations that was found in Down syndrome.[41] Therefore, this pathway may be altered but cannot explain all findings in natural effector B cells.

Mutations in the transmembrane activator and CAML interactor (TACI) are associated with CVID and autoimmunity by impairing the central B-cell tolerance checkpoint as well as B-cell activation through TLR7 and TLR9.[42] In Down syndrome, the expression of TACI was increased rather than decreased in natural effector B cells (**Chapter 6**). Unfortunately, it is unknown what mechanisms drive the upregulation of TACI in health and disease.

Thus, T-cell independent co-stimulation might be affected in Down syndrome. However, the results do not fit in with known abnormalities of these signalling pathways. In order to understand how B-cell activation might be altered in Down syndrome, it is first required that the co-stimulatory signals in T-cell independent reactions are studied.

Especially young children with Down syndrome are subject to recurrent respiratory tract infections. This does fit in with the impaired generation of B-cell memory. However, it can be hypothesized that immune responses improve during life, perhaps due to repetitive exposure. For this reason, it would be interesting to study antibody responses in younger children with Down syndrome (<4 years of age) during infections and upon immunization. Because alterations of the B-cell compartment were found in affinity maturation of IgM and IgA memory, it is important to include specific IgM and IgA antibodies. It would be most useful to focus on the immunizations that are part of the national vaccination program, such as the pneumococcal vaccine as the children might benefit from the results themselves. In case a decreased response is found to immunization for this respiratory pathogen, it might results in extra immunizations to boost the immune response.

Despite normal serum IgA levels, patients with Down syndrome have defects in generation of IgA memory. It can therefore be suggested that this result in functional IgA deficiency. And indeed, the clinical phenotype of patients with Down syndrome shows similarities when compared to patients with selective IgA deficiency. These patients also have higher numbers of respiratory tract infections as well as autoimmune diseases.[43,44] However, in contrast to selective IgA deficiency there are fewer individuals with Down syndrome that have allergic disease.[43] To further understand whether clinical features in Down syndrome are related to IgA dysfunction, affinity of specific IgA antibodies should be studied.

Clinical implications of this thesis and future perspectives

Respiratory tract infections

Many variables contribute to the increased susceptibility to respiratory tract infections in Down syndrome. To this date, it is unknown to what extent the immune system of Down syndrome plays a role in increasing the risk for respiratory tract infections. In this thesis we describe multiple alterations in humoral immunity. Unfortunately, we were unable to correlate our findings to the presence of infections in our patients (**Chapters 5 and 6**). This is probably due to multicausality of respiratory tract infections in Down syndrome. In order to determine the influence of humoral immunity on respiratory tract infections in Down syndrome a longitudinal study is needed that correlates blood analysis to clinical disease.

We have demonstrated the impact of recurrent respiratory tract infections on development, behaviour and HRQoL in children with Down syndrome. These

findings support clinicians to evaluate children who suffer from recurrent infections and determine whether treatable causes for recurrent infections are present. As mentioned earlier, it has to be determined whether the impact of infections remains as children grow up. Future studies could include preventive as well as therapeutic modalities. Preventive measures might include prophylactic antibiotics or additional vaccinations. The use of palivizumab decreases disease severity in RSV-infection,[45, 46] but it remains a question whether it also limits wheezing in Down syndrome as it does in prematurity.[47] In addition, earlier surgical treatments by ear, nose and throat (ENT) specialists might be a therapeutic intervention that is worthwhile to study.

Auto-immune diseases

Patients with Down syndrome are more likely to develop autoimmune diseases that especially include hypothyroidism, celiac disease and type 1 diabetes mellitus. These diseases have in common that they are associated with the production of specific autoantibodies. Moreover, in patients without Down syndrome who received B-cell depletion therapy (i.e. rituximab) for other reasons, clinical improvement of their autoimmune diseases and decreased autoantibody production was observed.[48-52] This demonstrates the important role of B cells in these diseases.

In **Chapters 5 and 6** we have described the existence of an increased population of $CD21^{low}$ B cells, which is present in other autoimmune diseases as well, such as rheumatoid arthritis, Sjögren syndrome, systemic lupus erythematosus and a CVID subpopulation with autoimmunity.[53-57] Functional analysis of this subset in other diseases has shown that these cells are unresponsive to antigen stimulation and are more prone to apoptosis.[56] Unfortunately, little is known regarding their origin and role in autoimmune diseases. To give more insight in their origin, future studies could perform transcript analysis to determine whether these cells are derived from naive B cells or memory B cells that have undergone somatic hypermutation and class switch recombination.

Neonatal screening

The decreased numbers of B and T cells in Down syndrome result in a higher false-positive test result in neonatal screening on SCID and X-linked agammaglobulinemia (XLA) (**Chapter 8**). If current cut-off values were used, approximately 25% of children would have been classified as having a positive test result for SCID or XLA. However, in a recent large population study the number of children with Down syndrome with a false-positive SCID screening was very low (~0.005%).[58] In addition, we showed lower TREC and KREC values in stored Guthrie

cards from controls as compared to those obtained in fresh cards. It is therefore hypothesized that DNA extraction efficiency has to be taken into account when test results from older Guthrie cards are analysed. This can be studied by including an extra RQ-PCR reaction to determine the amount of a control gene (i.e. ALB, ACTB).

At this moment it is uncertain how decreased B and T cells at birth relate to disease later in life. For example, a retrospective study in patients with 22q11 deletion showed that a lower TREC count at birth was associated with more viral infections in the first years of life.[59] Thus, it would be interesting to perform a follow-up study to correlate the level of morbidity experienced in the first years of life to B and T cell counts in neonates with Down syndrome.

Final remarks

Every year approximately 250 children with Down syndrome are born in The Netherlands. Moreover, due to the increased survival in Down syndrome, the total population has increased to ~12,000 individuals. Because these children and adults have higher medical needs, it remains important that healthcare further improves to limit healthcare costs and increase quality of life by focussing on prevention and early detection of complications.

In this thesis we have described how recurrent respiratory tract infections have their impact on children with Down syndrome. Furthermore, we have shown that humoral immunity is impaired in these patients, this especially affects T-cell independent responses. This might – at least partly – be responsible for the increased susceptibility for respiratory infections.

We suggest that future studies on the humoral immune system in Down syndrome include studies of the IgM, IgA and IgG immune response in vitro upon stimulation and during infections and after immunization, especially in young children. Furthermore, the prevention and treatment of these infections have to be studied, including their effect on HRQoL as determined in a Down syndrome-specific manner.

REFERENCES

1. Borstlap R, van Gameren-Oosterom HBM, Lincke C, Weijerman ME, van Wieringen H, van Wouwe JP. An update of the multidisciplinary guideline for the medical treatment of children with Down syndrome [Een update van de multidisciplinaire richtlijn voor de medische begeleiding van kinderen met Downsyndroom]. 2011.
2. Tenenbaum A, Hanna RN, Averbuch D, Wexler ID, Chavkin M, Merrick J. Hospitalization of children with down syndrome. Front Public Health 2014; 2:22.
3. Fitzgerald P, Leonard H, Pikora TJ, Bourke J, Hammond G. Hospital admissions in children with down syndrome: experience of a population-based cohort followed from birth. PLoS One 2013; 8:e70401.
4. Jama-Alol KA, Moore HC, Jacoby P, Bower C, Lehmann D. Morbidity due to acute lower respiratory infection in children with birth defects: a total population-based linked data study. BMC Pediatr 2014; 14:80.
5. Greenbaum AH, Chen J, Reed C, Beavers S, Callahan D, Christensen D, et al. Hospitalizations for severe lower respiratory tract infections. Pediatrics 2014; 134:546-54.
6. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 1995; 332:133-8.
7. Henderson J, Granell R, Heron J, Sherriff A, Simpson A, Woodcock A, et al. Associations of wheezing phenotypes in the first 6 years of life with atopy, lung function and airway responsiveness in mid-childhood. Thorax 2008; 63:974-80.
8. Brand PL, Caudri D, Eber E, Gaillard EA, Garcia-Marcos L, Hedlin G, et al. Classification and pharmacological treatment of preschool wheezing: changes since 2008. Eur Respir J 2014; 43:1172-7.
9. Brand PL, Baraldi E, Bisgaard H, Boner AL, Castro-Rodriguez JA, Custovic A, et al. Definition, assessment and treatment of wheezing disorders in preschool children: an evidence-based approach. Eur Respir J 2008; 32:1096-110.
10. Krawiec M, Strzelak A, Krenke K, Modelska-Wozniak I, Jaworska J, Kulus M. Fluticasone or Montelukast in Preschool Wheeze: A Randomized Controlled Trial. Clin Pediatr (Phila) 2014.
11. Panickar J, Lakhanpaul M, Lambert PC, Kenia P, Stephenson T, Smyth A, et al. Oral prednisolone for preschool children with acute virus-induced wheezing. N Engl J Med 2009; 360:329-38.
12. Jartti T, Lehtinen P, Vanto T, Vuorinen T, Hartiala J, Hiekkanen H, et al. Efficacy of prednisolone in children hospitalized for recurrent wheezing. Pediatr Allergy Immunol 2007; 18:326-34.
13. Vuillermin P, South M, Robertson C. Parent-initiated oral corticosteroid therapy for intermittent wheezing illnesses in children. Cochrane Database Syst Rev 2006:CD005311.
14. Forni GL, Rasore-Quartino A, Acutis MS, Strigini P. Incidence of bronchial asthma in Down syndrome. J Pediatr 1990; 116:487-8.
15. Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Cancers and immune related diseases associated with Down's syndrome: a record linkage study. Arch Dis Child 2004; 89:1014-7.

16. Mannan SE, Yousef E, Hossain J. Prevalence of positive skin prick test results in children with Down syndrome: a case-control study. Ann Allergy Asthma Immunol 2009; 102:205-9.
17. Langhoff E, Ladefoged J, Dickmeiss E. The immunosuppressive potency of various steroids on peripheral blood lymphocytes, T cells, NK and K cells. Int J Immunopharmacol 1985; 7:483-9.
18. Rudiger JJ, Gencay M, Yang JQ, Bihl M, Tamm M, Roth M. Fast beneficial systemic anti-inflammatory effects of inhaled budesonide and formoterol on circulating lymphocytes in asthma. Respirology 2013; 18:840-7.
19. O'Sullivan S, Cormican L, Burke CM, Poulter LW. Fluticasone induces T cell apoptosis in the bronchial wall of mild to moderate asthmatics. Thorax 2004; 59:657-61.
20. Melis M, Siena L, Pace E, Gjomarkaj M, Profita M, Pirazzoli A, et al. Fluticasone induces apoptosis in peripheral T-lymphocytes: a comparison between asthmatic and normal subjects. Eur Respir J 2002; 19:257-66.
21. Van Gameren-Oosterom HB, Fekkes M, Reijneveld SA, Oudesluys-Murphy AM, Verkerk PH, Van Wouwe JP, et al. Practical and social skills of 16-19-year-olds with Down syndrome: independence still far away. Res Dev Disabil 2013; 34:4599-607.
22. De Graaf G, van Hove G, Haveman M. More academics in regular schools? The effect of regular versus special school placement on academic skills in Dutch primary school students with Down syndrome. J Intellect Disabil Res 2013; 57:21-38.
23. Turner S, Alborz A, Gayle V. Predictors of academic attainments of young people with Down's syndrome. J Intellect Disabil Res 2008; 52:380-92.
24. Van Wouwe JP, van Gameren-Oosterom HB, Verkerk PH, van Dommelen P, Fekkes M. Mainstream and special school attendance among a Dutch cohort of children with Down Syndrome. PLoS One 2014; 9:e91737.
25. Van Gameren-Oosterom HB, Fekkes M, Buitendijk SE, Mohangoo AD, Bruil J, Van Wouwe JP. Development, problem behavior, and quality of life in a population based sample of eight-year-old children with Down syndrome. PLoS One 2011; 6:e21879.
26. De Hingh YC, van der Vossen PW, Gemen EF, Mulder AB, Hop WC, Brus F, et al. Intrinsic abnormalities of lymphocyte counts in children with down syndrome. J Pediatr 2005; 147:744-7.
27. Cossarizza A, Ortolani C, Forti E, Montagnani G, Paganelli R, Zannotti M, et al. Age-related expansion of functionally inefficient cells with markers of natural killer activity in Down's syndrome. Blood 1991; 77:1263-70.
28. Kreuzaler M, Rauch M, Salzer U, Birmelin J, Rizzi M, Grimbacher B, et al. Soluble BAFF levels inversely correlate with peripheral B cell numbers and the expression of BAFF receptors. J Immunol 2012; 188:497-503.
29. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science 2001; 293:2111-4.

30. Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci U S A 2009; 106:13945-50.
31. Green DR, Galluzzi L, Kroemer G. Cell biology. Metabolic control of cell death. Science 2014; 345:1250256.
32. Helguera P, Seiglie J, Rodriguez J, Hanna M, Helguera G, Busciglio J. Adaptive downregulation of mitochondrial function in down syndrome. Cell Metab 2013; 17:132-40.
33. Roat E, Prada N, Ferraresi R, Giovenzana C, Nasi M, Troiano L, et al. Mitochondrial alterations and tendency to apoptosis in peripheral blood cells from children with Down syndrome. FEBS Lett 2007; 581:521-5.
34. Dogliotti G, Galliera E, Dozio E, Vianello E, Villa RE, Licastro F, et al. Okadaic acid induces apoptosis in Down syndrome fibroblasts. Toxicol In Vitro 2010; 24:815-21.
35. Driessen GJ, Ijspeert H, Weemaes CM, Haraldsson A, Trip M, Warris A, et al. Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity. J Allergy Clin Immunol 2013; 131:1367-75 e9.
36. Van der Burg M, Pac M, Berkowska MA, Goryluk-Kozakiewicz B, Wakulinska A, Dembowska-Baginska B, et al. Loss of juxtaposition of RAG-induced immunoglobulin DNA ends is implicated in the precursor B-cell differentiation defect in NBS patients. Blood 2010; 115:4770-7.
37. Morawiec Z, Janik K, Kowalski M, Stetkiewicz T, Szaflik J, Morawiec-Bajda A, et al. DNA damage and repair in children with Down's syndrome. Mutat Res 2008; 637:118-23.
38. Boyd SD, Marshall EL, Merker JD, Maniar JM, Zhang LN, Sahaf B, et al. Measurement and clinical monitoring of human lymphocyte clonality by massively parallel VDJ pyrosequencing. Sci Transl Med 2009; 1:12ra23.
39. Van Zelm MC, Bartol SJ, Driessen GJ, Mascart F, Reisli I, Franco JL, et al. Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation. J Allergy Clin Immunol 2014; 134:135-44 e7.
40. Weller S, Bonnet M, Delagreverie H, Israel L, Chrabieh M, Marodi L, et al. IgM+IgD+CD27+ B cells are markedly reduced in IRAK-4-, MyD88-, and TIRAP- but not UNC-93B-deficient patients. Blood 2012; 120:4992-5001.
41. Von Bernuth H, Picard C, Puel A, Casanova JL. Experimental and natural infections in MyD88- and IRAK-4-deficient mice and humans. Eur J Immunol 2012; 42:3126-35.
42. Romberg N, Chamberlain N, Saadoun D, Gentile M, Kinnunen T, Ng YS, et al. CVID-associated TACI mutations affect autoreactive B cell selection and activation. J Clin Invest 2013; 123:4283-93.
43. Jorgensen GH, Gardulf A, Sigurdsson MI, Sigurdardottir ST, Thorsteinsdottir I, Gudmundsson S, et al. Clinical symptoms in adults with selective IgA deficiency: a case-control study. J Clin Immunol 2013; 33:742-7.
44. Jorgensen GH, Thorsteinsdottir I, Gudmundsson S, Hammarstrom L, Ludviksson BR. Familial aggregation of IgAD and autoimmunity. Clin Immunol 2009; 131:233-9.

45. Yi H, Lanctot KL, Bont L, Bloemers BL, Weijerman M, Broers C, et al. Respiratory syncytial virus prophylaxis in Down syndrome: a prospective cohort study. Pediatrics 2014; 133:1031-7.
46. Paes B, Mitchell I, Yi H, Li A, Lanctot KL, Investigators C. Hospitalization for respiratory syncytial virus illness in Down syndrome following prophylaxis with palivizumab. Pediatr Infect Dis J 2014; 33:e29-33.
47. Yoshihara S, Kusuda S, Mochizuki H, Okada K, Nishima S, Simoes EA. Effect of palivizumab prophylaxis on subsequent recurrent wheezing in preterm infants. Pediatrics 2013; 132:811-8.
48. Nikiphorou E, Hall FC. First report of improvement of coeliac disease in a patient with Sjogren's syndrome treated with rituximab. Rheumatology (Oxford) 2014.
49. Palazzo C, Nicaise-Roland P, Palazzo E. Rituximab: an effective treatment for rheumatologic and digestive symptoms of celiac disease? Joint Bone Spine 2012; 79:422-3.
50. Raterman HG, Simsek S, Lems WF, Meesters EW, Dijkmans BA, Nurmohamed MT. Rituximab and thyroid function. Arch Intern Med 2009; 169:1073-4.
51. Pescovitz MD, Greenbaum CJ, Bundy B, Becker DJ, Gitelman SE, Goland R, et al. B-lymphocyte depletion with rituximab and beta-cell function: two-year results. Diabetes Care 2014; 37:453-9.
52. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, et al. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009; 361:2143-52.
53. Rakhmanov M, Gutenberger S, Keller B, Schlesier M, Peter HH, Warnatz K. CD21low B cells in common variable immunodeficiency do not show defects in receptor editing, but resemble tissue-like memory B cells. Blood 2010; 116:3682-3.
54. Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, Driessen G, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. Proc Natl Acad Sci U S A 2009; 106:13451-6.
55. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol 2004; 113:161-71.
56. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood 2010; 115:5026-36.
57. Saadoun D, Terrier B, Bannock J, Vazquez T, Massad C, Kang I, et al. Expansion of autoreactive unresponsive CD21-/low B cells in Sjogren's syndrome-associated lymphoproliferation. Arthritis Rheum 2013; 65:1085-96.
58. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA 2014; 312:729-38.
59. Lingman Framme J, Borte S, von Dobeln U, Hammarstrom L, Oskarsdottir S. Retrospective analysis of TREC based newborn screening results and clinical phenotypes in infants with the 22q11 deletion syndrome. J Clin Immunol 2014; 34:514-9.

Chapter 10

Summary
Nederlandse samenvatting

SUMMARY

Down syndrome is the most common cause of developmental delay in humans. In The Netherlands, each year approximately 250 children with Down syndrome are born. Individuals with Down syndrome suffer from increased incidences of respiratory tract infections, autoimmune disease and haematological malignancies. This triad is reminiscent of immunodeficiencies and has led to the hypothesis of an altered immune system in Down syndrome. The high incidence of respiratory tract infections is an important clinical problem and is suggestive of an immune defect. Therefore, the work described in this thesis was focused on the nature of these respiratory tract infections and dissection of adaptive immunity, especially regarding humoral immunity.

The increased incidence of respiratory tract infections is a well-known clinical problem in children with Down syndrome. However, little is known about the perception of the parents of the nature and frequency of respiratory symptoms such as a runny nose, coughing and shortness of breath. We therefore designed the 'KiDS diary study' (**Chapter 2**). This study is currently running in The Netherlands, and participating parents receive a weekly online questionnaire to report whether their child has been ill and suffers from specific respiratory symptoms. Moreover, we can analyse the impact of these symptoms on daily activities (i.e. school attendance) and medical history of the child and his/her family.

Both parents and doctors empirically report that cognitive and motor development temporarily declines when children with Down syndrome suffer from a respiratory tract infection. In **Chapter 3** we have studied this in a large cohort of 8-year-old children with Down syndrome. We analyzed developmental status, behaviour and Health-Related Quality of Life (HRQoL). Moreover, parents were asked whether their child suffered from recurrent or serious respiratory tract infections. The mean developmental age of 8-year-old children who suffered from recurrent respiratory tract infections was 3 years and 8 months, as compared to 4 years and 1 month in children who did not. Hierarchical regression analysis showed that the presence of parent-reported recurrent respiratory tract infections has a higher impact on developmental age than other morbidities such as congenital heart disease and thyroid disease. Moreover, we showed that behaviour and HRQoL was negatively influenced by the presence of recurrent respiratory tract infections. For future studies, it would be important to determine whether these infections result in decreased practical and social skills in adolescence. If so, this would further stress the importance of research on the prevention as well as treatment of respiratory tract infections in children with Down syndrome.

Since the 1970s, Down syndrome has been the focus of immunological studies. In **Chapter 4**, we present an overview of this work and challenge the paradigm that the alterations of the immune system in Down syndrome are the result of precocious aging. We hypothesize that immunity is intrinsically altered in Down syndrome. Furthermore, we demonstrate that the profound decrease of absolute B cell numbers has received little attention. Therefore, we studied naive B cells, memory B cells and plasma cell differentiation and immunoglobulin production in children with Down syndrome in **Chapters 5 and 6**.

In **Chapter 5**, we showed that children with Down syndrome have a distinct pattern of serum immunoglobulin levels. Total IgG is increased, IgA is normal and IgM is decreased compared to controls. Furthermore IgG1 and IgG3 are increased and IgG2 and IgG4 are decreased. We found that this pattern was not identical to patients without Down syndrome who are suffering from recurrent infections. The high levels of IgG were in line with high frequencies of plasma cells in blood and tonsil (**Chapter 6**). Moreover, naïve B cells from children with Down syndrome readily differentiated into plasma cells upon in vitro stimulation.

As described before, we found decreased numbers of total B cells in the blood in children with Down syndrome as compared to controls. Transitional B cells were normal in number, but naive B cells were decreased despite slightly increased serum BAFF levels. Except for IgM-only memory B cells, all memory subsets were decreased in Down syndrome.

We demonstrated that naive B cells are decreased in Down syndrome. Considering the normal numbers of recent bone marrow emigrants, i.e. transitional B cells, this was most likely not caused by impaired bone marrow output (**Chapter 6**). Furthermore, homeostatic proliferation of naive mature B cells was normal. Thus, we concluded that impaired survival was most likely the cause of reduction in circulating naive B cells. This was confirmed in **Chapter 7**, where we demonstrated that T, B and NK cells of children with Down syndrome showed increased apoptosis compared to controls.

During an antigen response, B cells proliferate and undergo affinity maturation through somatic hypermutation and selection of B cells carrying antibodies with high affinity to antigen. IgM+IgD+ memory ('natural effector') B cells showed a decreased replication history and low levels of somatic hypermutation. Proliferation and somatic hypermutation levels appeared normal in IgA and IgG class-switched memory B cells. Still, IgA memory B cells showed impaired selection for replacement mutations in the antigen-binding region. Thus, IgM and IgA memory was found to be impaired in children with Down syndrome.

In **Chapter 8**, we describe how the newborn screening for Severe Combined Immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA) is affected by Down syndrome. Patients with SCID have decreased T-cells with or without low B-cell numbers. In XLA, patients suffer from agammaglobulinemia as a result of absent B cells. These patients suffer from serious infections that cause high morbidity and in case of SCID, mortality as well. It has been established that early diagnosis improves prognosis significantly. Therefore, a newborn screening assay has been developed based on assessment of T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) in dried blood spots. TRECs and KRECs are stable excision circles that are formed during V(D)J recombination in T and B cells, respectively. Real-time quantitative PCR (RQ-PCR) analysis can be used to determine the number of TRECs and KRECs as a measure for blood T- and B-cell numbers. Because children with Down syndrome have decreased T and B cells caused by impaired survival, we hypothesized that this might result in false-positive screening results. Therefore, we performed the screening assay on neonatal dried blood spots of a large cohort of children with Down syndrome and confirmed lower TREC and KREC numbers in these children. However, the chosen cut-off values determine how many children with Down syndrome would have been designated "positive" by the screening for SCID/XLA.

Thus, in contrast to known immunodeficiencies, children with Down syndrome do not have a defect in plasma cell differentiation and antibody production. Rather, defective B-cell memory seems to underlie the increased frequency of respiratory tract infections. In our studies, we did not find a correlation between the alterations of the B-cell compartment and the clinical features of children with Down syndrome. Future research should therefore include long-term longitudinal follow-up of young children with Down syndrome to determine to what extent the composition and function of naive and memory B cells relates to the infections experienced by these children.

Figuur 1. Aanbidding van Christus (The adoration of the Christ child; circa 1515).
A. Schilderij van Jan Joest van Kalkar (geboren circa 1450-1460, overleden in 1519 in Haarlem). **B.** Meerdere individuen hebben kenmerken van Downsyndroom waaronder een platte neusbrug, opvallende huidplooi ter hoogte van de binnenste ooghoeken (epicanthus plooi) en kleine omhoogstaande neus.
Gedrukt volgens de richtlijn van het "Open Access for Scholarly Content (OASC)" programma van The Metropolitan Museum of Art, New York, Verenigde Staten.

NEDERLANDSE SAMENVATTING

VOOR NIET INGEWIJDEN

Downsyndroom

Jaarlijks worden in Nederland ongeveer 250 kinderen geboren met Downsyndroom. Dit syndroom is daarmee de meest voorkomende oorzaak van ontwikkelingsachterstand bij mensen en wordt veroorzaakt door de aanwezigheid van een extra – derde – chromosoom 21 (trisomie 21). De diagnose wordt meestal tijdens de zwangerschap of vlak na de geboorte gesteld. Pasgeboren kinderen met Downsyndroom hebben een lage spierspanning in het hele lichaam (hypotonie). Daarnaast hebben ze vaak een kenmerkend gelaat met onder andere een platte neusbrug, opvallende huidplooien aan de binnenzijde van het oog (epicanthusplooi), een vergrote tong en kleine oren (Figuur 1). Kinderen met Downsyndroom hebben daarnaast ook vaker last van aangeboren afwijkingen. Zo heeft ongeveer de helft van de kinderen een hartafwijking en hebben ze ook een grotere kans op aangeboren afwijkingen van het maag-darm-stelsel. Deze afwijkingen kunnen zo ernstig zijn dat chirurgisch ingrijpen noodzakelijk is.

In de eerste levensjaren wordt zichtbaar dat kinderen met Downsyndroom zich trager ontwikkelen, bijvoorbeeld omdat ze later beginnen met lopen en praten. De ontwikkeling verschilt per kind en is daarom moeilijk te voorspellen. Kinderen en volwassenen met Downsyndroom kunnen in hun verdere leven te maken krijgen met diverse gezondheidsproblemen. Hiervan zijn luchtweginfecties één van de belangrijkste.

Luchtweginfecties

Kinderen met Downsyndroom hebben in de eerste levensjaren vaker last van luchtweginfecties. De ernst van deze infecties varieert van een eenvoudige verkoudheid tot een ernstige longontsteking, waarvoor opname op een Intensive Care voor kinderen noodzakelijk kan zijn. Er zijn verschillende oorzaken waarom kinderen met Downsyndroom gevoeliger zijn voor het krijgen van luchtweginfecties dan kinderen zonder Downsyndroom. Zo is de aanleg van de luchtwegen anders, maar zijn ook de spieren minder sterk waardoor het voor kinderen met Downsyndroom moeilijker is om diep door te ademen en te hoesten.

Het is niet bekend wat de perceptie van ouders is over hoe vaak kinderen met Downsyndroom last hebben van luchtwegklachten. Daarom hebben wij het onderzoek “KiDS Dagboekstudie” opgezet (**Hoofdstuk 2**). In dit onderzoek ontvangen ouders van kinderen met Downsyndroom wekelijks een vragenlijst

per e-mail. In deze vragenlijst wordt gevraagd of hun kind luchtwegklachten heeft gehad zoals snotteren, hoesten en benauwdheid. Daarnaast krijgen ouders jaarlijks een algemene vragenlijst waarin vragen worden gesteld met betrekking tot de gezinssamenstelling, de dagbesteding en de algemene gezondheid van hun kind. Op dit moment zijn er meer dan 100 ouders die tot medio 2015 meedoen aan dit onderzoek. Wij hopen met dit onderzoek in kaart te brengen hoe vaak er luchtwegklachten zijn en wat het effect daarvan is op het dagelijks functioneren van deze kinderen en hun omgeving (school, werkverzuim ouders).

Ouders en artsen ervaren bij ziekte van een kind met Downsyndroom vaak een terugval in de ontwikkeling. Daarom hebben wij bij een grote groep kinderen met Downsyndroom met een kalenderleeftijd van 8 jaar onderzocht wat de gevolgen zijn van terugkerende luchtweginfecties (**Hoofdstuk 3**). Bij deze kinderen werd de ontwikkeling, het gedrag en de kwaliteit van leven in kaart gebracht. Hiernaast werd aan ouders gevraagd of hun kind vaak of ernstig verkouden was. Op basis van deze vraag werden twee groepen vastgesteld die met elkaar werden vergeleken. In de groep kinderen die veel last heeft van verkoudheden was de gemiddelde ontwikkelingsleeftijd 3 jaar en 8 maanden. Bij kinderen die minder last hadden van deze verkoudheden bedroeg de gemiddelde ontwikkelingsleeftijd 4 jaar en 1 maand. Hieruit blijkt dat de kinderen die veel last hebben van luchtweginfecties 5 maanden verder achterlopen in hun mentale en fysieke ontwikkeling dan kinderen zonder deze klachten. Bovendien liet ons onderzoek zien dat deze kinderen ook vaker last hebben van gedragsproblemen en een lagere kwaliteit van leven ervaren.

Het is belangrijk om te onderzoeken of deze verschillen blijven bestaan als deze kinderen ouder worden. Het zou namelijk kunnen zijn dat deze kinderen door de frequente verkoudheden op latere leeftijd ook minder goed kunnen functioneren. Als dit waar is, is dat een extra reden om meer onderzoek te doen naar luchtweginfecties. Bijvoorbeeld door te kijken of deze infecties voorkomen kunnen worden.

Het afweersysteem bij Downsyndroom

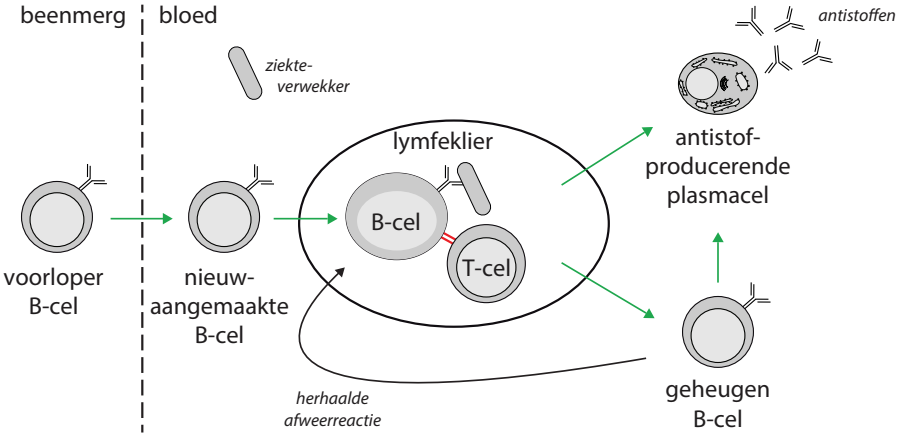
Naast luchtweginfecties hebben kinderen met Downsyndroom een grotere kans op het ontwikkelen van bloedkanker (leukemie) en auto-immuunziekten. Bij deze laatste groep ziekten richt het afweersysteem zich op onderdelen van het eigen lichaam. De auto-immuun ziekten die bij Downsyndroom vaker voorkomen zijn suikerziekte (diabetes mellitus type 1), glutenintolerantie (coeliakie) en te langzaam werkende schildklier (hypothyreoïdie).

Een hogere frequentie van luchtweginfecties, leukemie en auto-immuunziekten wordt ook vaker gezien bij patiënten met een afweerstoornis. Dit is de reden dat onderzoek wordt gedaan naar het afweersysteem bij Downsyndroom. In **Hoofdstuk 4** wordt een overzicht gegeven van het onderzoek dat in het verleden is verricht. Hieruit blijkt dat diverse onderdelen van het afweersysteem bij Downsyndroom verminderd functioneren. Daarnaast concluderen wij dat er relatief weinig aandacht is besteed aan een specifiek type afweercel: de B-cel. Deze witte bloedcellen verzorgen de aanmaak van antistoffen en hebben hierdoor een belangrijke functie in het afweersysteem. Daarom werden de B-cellen bij kinderen met Downsyndroom bestudeerd in dit proefschrift.

Het afweersysteem

Het menselijk lichaam wordt door het afweersysteem beschermd tegen ziekteverwekkers zoals bacteriën en virussen. Om deze taak uit te kunnen voeren bestaat het afweersysteem uit verschillende soorten afweercellen en afweerstoffen die allen een specifieke functie hebben. Zodra een ziekteverwekker het lichaam binnendringt zullen onderdelen van het aangeboren afweersysteem reageren. Deze eerste reactie is snel, al binnen enkele uren, maar deze afweercellen reageren op veel verschillende ziekteverwekkers en kunnen zich niet exclusief richten tegen de betreffende bacterie of virus. T- en B-cellen kunnen zich wel specifiek richten tegen een bacterie of virus, maar het duurt 5 tot 7 dagen voordat hun afweerreactie op gang komt. Een dergelijke afweerreactie is effectiever omdat de antistoffen die geproduceerd worden door B-cellen specifiek gericht zijn tegen de betrokken ziekteverwekker.

B-cellen worden aangemaakt in het beenmerg (voorloper B-cel) waar ervoor gezorgd wordt dat iedere B-cel uniek is door veranderingen in het DNA te maken (Figuur 2). Iedere B-cel kan hierdoor één specifieke eigenschap van ziekteverwekkers herkennen. Er zijn dus veel verschillende B-cellen nodig om eigenschappen van alle soorten virussen en bacteriën te herkennen. Nieuw-aangemaakte B-cellen komen in het bloed als zij volledig gevormd zijn en een eigenschap van ziekteverwekkers kunnen herkennen. Zodra ze in contact komen met de specifieke eigenschap vindt een afweerreactie plaats in een lymfeklier. Omdat iedere B-cel een andere eigenschap kan herkennen, zullen meer B-cellen reageren bij dezelfde ziekteverwekker. Tijdens deze reactie wordt het DNA van deze B-cellen iets aangepast. Dit doen ze met de hulp van T-cellen. Het doel is dat ze hierdoor de ziekteverwekker nog beter kunnen herkennen. De B-cellen die de ziekteverwekker het beste herkennen zullen geselecteerd worden om uit te rijpen tot een geheugen B-cel en antistof-producerende plasmacel. De geheugen B-cellen



Figuur 2. Afweerreactie van B-cellen.
B-cellen worden gemaakt in het beenmerg (voorloper B-cel) en komen in het bloed zodra ze volledig werkzaam zijn (nieuw-aangemaakte B-cel). Zodra een B-cel een ziekteverwekker herkent, vindt een afweerreactie plaats in een lymfeklier. Deze reactie wordt versterkt met hulp van T-cellen. Hierna worden ze geheugen B-cellen en antistof-producerende plasmacellen. De geheugen B-cellen kunnen sneller reageren als ze opnieuw dezelfde ziekteverwekker herkennen. De plasmacellen kunnen antistoffen maken. Deze antistoffen kunnen aan de specifieke ziekteverwekker hechten waardoor de andere onderdelen van het afweersysteem de ziekteverwekker kunnen herkennen en onschadelijk maken.

wachten in het bloed totdat ze opnieuw dezelfde ziekteverwekker herkennen. Geheugencellen zijn geprogrammeerd om sneller te reageren. De antistoffen die door plasmacellen worden aangemaakt kunnen zich via de bloed- en lymfebanen over het hele lichaam verspreiden. Omdat ze aan de specifieke ziekteverwekker hechten kunnen de andere onderdelen van het afweersysteem de ziekteverwekker beter herkennen en onschadelijk maken.

B-cellen bij Downsyndroom

Er zijn verschillende ziektes bekend waarbij de B-cellen niet goed werken. Patiënten met deze ziekten hebben – net zoals kinderen en volwassenen met Downsyndroom – last van herhaalde luchtweginfecties, auto-immuunziekten en vormen van kanker. In **Hoofdstuk 5** hebben wij daarom gekeken naar de samenstelling van de verschillende B-cellen bij Downsyndroom. Hierin laten wij zien dat het totaal aantal B-cellen bij jonge en oudere kinderen verlaagd is. Daarnaast hebben wij gekeken of de samenstelling van B-cellen anders is bij kinderen met Downsyndroom die vaak luchtweginfecties hebben. Dat konden wij niet aantonen.

In het bloed kan gemeten worden hoeveel antistoffen er zijn. Hierbij wordt een onderscheid gemaakt tussen verschillende soorten antistoffen: IgG, IgA en IgM. Bij kinderen met Downsyndroom zagen we dat de bloedwaarden van IgG hoog waren, van IgA normaal en van IgM verlaagd. Daarnaast kan IgG onderverdeeld worden in

IgG1, IgG2, IgG3 en IgG4. Bij Downsyndroom zijn IgG1 en IgG3 verhoogd en IgG2 en IgG4 verlaagd. De verschillen in de antistofwaarden bleken specifiek te zijn voor Downsyndroom en hingen niet samen met het toegenomen aantal infecties. Toch zijn de specifieke antistoffen die gemaakt worden na vaccinaties vrijwel normaal bij Downsyndroom.

In **Hoofdstuk 6** wordt dieper ingegaan op de afweerreactie van B-cellen. In het beschreven onderzoek werd uitgebreider gekeken naar de aantallen van de verschillende B-cellen in het bloed. Hierbij zagen we dat de aantallen nieuw-aangemaakte B-cellen en geheugen B-cellen verlaagd zijn. Daarentegen waren plasmacellen verhoogd in aantal.

Een belangrijk deel van de afweerreactie van B-cellen vindt plaats in lymfeklieren en keelamandelen. Daarom hebben wij keelamandelen van kinderen met Downsyndroom onderzocht met een microscoop; wij zagen een normale opbouw. Daarnaast werd onderzocht hoe B-cellen van kinderen met Downsyndroom uitrijpen tot plasmacellen. Hiervoor werden B-cellen in het laboratorium gestimuleerd met stoffen die eigenschappen hebben van ziekteverwekkers én stoffen die normaal door het lichaam aangemaakt worden om een afweerreactie te stimuleren. Dit onderzoek liet zien dat de uitrijping van B-cellen naar plasmacellen bij Downsyndroom normaal is.

In het bloed werden de geheugen B-cellen verder onderzocht. Zoals besproken passen B-cellen hun eigenschappen aan zodat hun reactie tegen ziekteverwekkers verbetert. Ons onderzoek heeft laten zien dat de specifieke geheugen B-cellen die met name belangrijk zijn voor afweerreacties in het bloed en slijmvliezen van darmen en luchtwegen dit minder goed kunnen. Het is mogelijk dat kinderen met Downsyndroom om deze reden vaker ziek zijn.

Zoals werd besproken, zijn de nieuw-aangemaakte B-cellen verlaagd bij Downsyndroom. Dit kan komen door verminderde aanmaak van B-cellen in het beenmerg, onvoldoende celdeling of toegenomen celdood van B-cellen. In **Hoofdstuk 6** beschrijven wij dat de aanmaak vanuit het beenmerg en de celdeling van deze nieuw-aangemaakte B-cellen normaal is. Het leek dus waarschijnlijk dat B-cellen sneller doodgaan waardoor het aantal B-cellen afneemt. Dit konden wij bevestigen in **Hoofdstuk 7**.

De onderzoeken die wij beschreven hebben in **Hoofdstukken 5, 6 en 7** laten duidelijke afwijkingen zien in de uitrijping van B-cellen bij Downsyndroom. Echter,

het was niet mogelijk om vast te stellen of deze afwijkingen ook daadwerkelijk leiden tot meer luchtweginfecties.

Toepassing van onderzoeksresultaten

Afweerstoornissen zijn ziekten waarbij het afweersysteem niet goed functioneert. Een aantal van deze ziekten wordt veroorzaakt doordat er geen T-cellen en/of B-cellen aanwezig zijn. Deze kinderen worden ernstig ziek in het eerste levensjaar. Het is daarom belangrijk dat de diagnose op zo vroeg mogelijk leeftijd wordt gesteld. In een aantal landen is daarom de hielprikscreening bij pasgeborenen aangepast om deze ziektes vroegtijdig op te sporen. Bij deze screening wordt op de vijfde dag na de geboorte bloed afgenomen bij het kind en onderzocht op specifieke bloedwaarden (TRECs en KRECs) die een maat zijn voor het aantal T- en B-cellen in het bloed.

Omdat kinderen met Downsyndroom ook lagere aantallen T- en B-cellen in hun bloed hebben, wilden wij vaststellen wat de invloed hiervan is op de hielprikscreening voor deze ernstige afweerstoornissen (**Hoofdstuk 8**). Wij hebben daarom hielprikkaartjes van kinderen met Downsyndroom onderzocht. Dit liet zien dat het zeker mogelijk is dat deze screening onbedoeld afwijkend is bij kinderen met Downsyndroom door een verlaging van hun T- en B-cellen.

Conclusie

Dit proefschrift laat zien dat luchtweginfecties een grote invloed hebben op kinderen met Downsyndroom. Het is daarom belangrijk om vast te stellen of deze gevolgen ook op volwassen leeftijd blijven bestaan. Dit is een belangrijke reden om meer onderzoek te verrichten naar mogelijkheden om deze infecties te voorkomen en te behandelen. Daarom hebben wij uitgebreid onderzoek uitgevoerd naar B-cellen en antistoffen. B-cellen bij Downsyndroom laten inderdaad duidelijke afwijkingen zien, wat kan bijdragen aan de verhoogde gevoeligheid voor het krijgen van luchtweginfecties. Om vast te stellen hoe groot deze bijdrage is, is het nodig om jonge kinderen met Downsyndroom langdurig te volgen. Alleen dan kan worden vastgesteld hoe de afwijkingen in het afweersysteem precies samenhangen met het aantal luchtweginfecties.

Addendum

DANKWOORD

Nu de afronding van het proefschrift nadert is het de hoogste tijd om mijn dankwoord te schrijven. Terugkijkend op de afgelopen acht jaar zijn er veel mensen geweest die een bijdrage hebben geleverd aan de totstandkoming van “mijn boekje”. Graag wil ik dan ook mijn dank uitspreken naar hen.

Mijn bijzondere dank gaat uit naar alle kinderen en hun ouders die belangeloos hebben meegewerkt aan de verschillende onderzoeken die onderdeel uitmaken van dit proefschrift. Ik hoop dat dit onderzoek in de toekomst een bijdrage levert aan betere zorg voor kinderen met het syndroom van Down.

Bij deze wil ik mijn promotoren, co-promotor en overige leden van de promotiecommissie bedanken.

Prof. dr. E. de Vries, beste Esther, zonder jou zou dit proefschrift er nooit zijn geweest. Tijdens mijn co-schap kindergeneeskunde in het Jeroen Bosch Ziekenhuis heb je me enthousiast gemaakt voor immunologisch onderzoek bij het syndroom van Down. Dit is de start geweest van een vruchtbare samenwerking. Door je focus op klinische vraagstellingen zijn veel originele onderzoeksideeën ontstaan welke we samen hebben kunnen verwezenlijken. Ik heb bewondering voor je doorzettingsvermogen en je nooit aflatende kritische blik. Deze eigenschappen zijn niet alleen waardevol geweest voor de inhoud van dit proefschrift, maar hebben ook zeker bijgedragen aan je benoeming tot hoogleraar. Ik wens je veel succes toe bij de invulling van deze leerstoel en de oratie die je binnenkort zal uitspreken. Dankjewel!

Prof. dr. J.J.M. van Dongen, beste Jacques, heel erg bedankt voor onze samenwerking. Je betrokkenheid bij mij en mijn onderzoek heb ik zeer gewaardeerd. Je opbouwende reacties en kritische vragen hebben een duidelijke invloed gehad op het eindresultaat.

Dr. M.C. van Zelm, beste Menno, ik ben trots dat jij mijn co-promotor bent. Ik ben je dankbaar voor je optimisme en kritische (biologische) vragen waarmee je mij hebt weten te stimuleren om verder te gaan. Had ik maar een fractie van jouw schrijftalent... Je hebt je rol als co-promotor glansrijk vervuld, dankjewel!

Prof. dr. H. Hooijkaas, prof. dr. A.J. van der Heijden en prof. dr. R. de Groot, ik wil jullie danken voor de beoordeling van het manuscript en dat jullie zitting willen nemen in de promotiecommissie. Beste Ronald, dankjewel voor onze goede gesprekken en steun in de afgelopen jaren.

Prof. dr. H. Moll en dr. L. Bont, hartelijk dank dat jullie plaats willen nemen in de Grote Commissie.

Een groot deel van het onderzoek heeft plaatsgevonden in het Jeroen Bosch Ziekenhuis. Nadat ik als ANIOS op de afdeling kindergeneeskunde heb gewerkt voelde het iedere keer weer als thuiskomen als ik patiënten mocht includeren op de polikliniek en dagbehandeling. Dankjewel!

Beste Eugenie, op het laboratorium Klinische chemie en hematologie heb je me inhoudelijk en praktisch vaak geholpen. In het begin heb je me wegwijs gemaakt in de flowcytometrie en me betrokken bij het B-cel project. Later hebben we samen het apoptose-onderzoek uitgewerkt en heb je me vaak geholpen met praktische problemen. Het blijft voor mij een raadsel hoe je zoveel details van de verschillende onderzoeken kan onthouden! Ik wens je heel veel succes met je huidige projecten en ik hoop dat dit op den duur zal leiden tot je eigen promotie.

Ik ben alle coauteurs dankbaar die ieder met hun eigen expertise en kritische input hebben bijgedragen aan de diverse publicaties.

Prof. dr. Roeland van Hout, dankjewel voor je methodologische bijdrage aan het onderzoeksprotocol van de KiDS dagboekstudie.

Ko en Helma, dankjewel dat jullie mij wilde betrekken bij het grote Down-onderzoek van TNO. Beste Helma, je geduld om mij steeds weer uit te leggen hoe de ingewikkelde statistiek in elkaar zat is bewonderingswaardig. Ik wens je veel succes toe met je verdere opleiding tot kinderarts. Dr. Minne Fekkes en dr. Elise Dusseldorp, dankjewel voor jullie bijdrage aan ons artikel.

Beste Benjamin, dankjewel voor al het werk dat je heb verzet op het laboratorium in het kader van het Down-project. Beste Mirjam en Gertjan, dankjewel voor de fijne samenwerking! De toevoeging van "jullie" CVID-patiënten heeft het artikel een extra dimensie gegeven. Dr. Carel van Noesel, dank voor de beoordeling van de tonsillen. Dr. Louis Boon, dank voor de antilichamen. Martijn Alles, Noortje Puts en dr. Ronald Admiraal, dank voor jullie hulp bij het verzamelen van de tonsillen.

Dr. Jacqueline Leuvenink, bedankt voor je bijdrage aan het apoptose-onderzoek.

Dear Stephan, thank you very much for introducing me to the neonatal screening assay for severe immunodeficiencies. It was a pleasure to work with you in the laboratory in Leipzig. Prof. dr. Lennart Hammarström and dr. Ulrika von Döbeln, thank you very much for your contribution during our project. Dr. Vinus Bok, Paul van Zwieten, Anne-Marie van Wermeskerken, Adam Bolz,† Felix Dikken, Wil Rijnvos en Ed van Leer, dank dat jullie zoveel ouders benaderd hebben voor ons onderzoek. Het RIVM ben ik dankbaar voor hun medewerking bij het hielprikonderzoek.

In het afgelopen jaar heb ik de mogelijkheid gehad mijn onderzoek af te ronden op de afdeling Immunologie van het Erasmus MC. Benjamin, Britt, Diana, Halima,

Jorn, Katharina, Magda en Marieke, door jullie heb ik mij vanaf het begin welkom gevoeld op de afdeling en binnen de BCD-groep. Ingrid, bedankt voor je hulp op het lab.

Vanuit de staf Kindergeneeskunde/Kinder-IC van het Radboudumc Amalia Kinderziekenhuis heb ik veel steun gekregen om mijn opleiding en onderzoek met elkaar te kunnen combineren. Dit draagt zeker bij aan de fantastische opleiding Kindergeneeskunde van het Radboudumc!

Beste Jos, het vertrouwen dat jij hebt in jouw AIOS is groot en voor iedereen merkbaar. Door jouw steun heb ik de mogelijkheid gehad om een deel van mijn opleidingstijd te kunnen besteden aan de laatste loodjes van het onderzoek. Ook gaf je mij de mogelijkheid om met de opleiding tot klinisch farmacoloog te starten. Dank hiervoor!

Beste Janiëlle, ik ben erg blij dat jij mijn mentor wilde zijn! Onze eerste ontmoeting en onze gesprekken hebben me heel erg geholpen om een betere dokter te kunnen worden. Veel succes als plaatsvervangend opleider, een rol die – wat mij betreft – op jouw lijf geschreven is.

Dit is de plek waar ik de (oud) arts-assistenten kindergeneeskunde wil bedanken voor hun grote bijdrage aan mijn fantastische opleidingstijd en de mogelijkheid om successen en tegenslagen in het onderzoek ("het werkstuk") te kunnen delen. Mede door jullie ben ik (vrijwel) iedere dag met plezier naar mijn werk gegaan!

Alex, Bart, Bas, Gijs, Ilonka, Loes, Lydia, Marc, Martine, Mieke, Miel, Remco, Roel, Sharon, Susan, Willemien, jullie vriendschap betekent heel veel voor me. Ook al spreken ik jullie soms tijden niet, ik weet dat we er voor elkaar zijn. Hanneke, dankjewel voor alle fijne gesprekken die wij gehad hebben.

Lieve Maaike, jouw rol in dit proefschrift is bijna niet te beschrijven. Door een toevallig lot zijn wij allebei betrokken geraakt bij het Down onderzoek en dat heeft niet alleen een gouden samenwerking opgeleverd maar bovenal een waardevolle vriendschap. Het voelt goed om onze successen én tegenslagen met elkaar te kunnen delen. Het was een grote eer je bij te mogen staan tijdens jouw promotie en ik ben dan ook erg blij dat jij mijn paranimf bent.

Lieve Karin, we hebben een heel groot deel van onze opleidingen met elkaar gedeeld (met hier en daar een zwangerschapsverlof aan jouw kant). Ik heb je leren kennen als een duizendpoot die haar leven met passie leidt en voor iedereen klaar staat. Onze vriendschap is heel waardevol voor mij en daarom doet het mij heel

goed dat jij ook naast mij staat tijdens de promotie. Heel veel succes met alle nieuwe projecten die op je pad gaan komen en ik hoop dat we snel die koffie in Central Park kunnen drinken.

Pap en mam, je hebt geen keuze waar je wieg staat maar ik mag van geluk spreken dat ik bij jullie ben opgegroeid. Jullie zijn er altijd voor me geweest en hebben me gesteund in het bereiken van alle doelen.

Peter, we zijn op sommige vlakken heel verschillend maar soms ook zó hetzelfde. Ik ben je dankbaar dat je er altijd voor mij bent en wens je alle geluk samen met Willemijn. Chris en Ria, dankjewel dat ik deel mag uitmaken van jullie gezin.

Last, maar zeker not least: Jeroen. Je steunt me bij alles wat ik onderneem, ook al neemt dit menig weekend in beslag en gaan we hierdoor naar Toronto verhuizen. Dankjewel, wat ben ik blij met jou in mijn leven!

CURRICULUM VITAE

Ruud Verstegen werd op 12 april 1983 geboren in dorpje Zeeland (Noord-Brabant). Op de boerderij van zijn ouders groeide hij op, samen met zijn broer Peter. Ruud behaalde in 2001 zijn VWO diploma aan het Udens College waarna hij Geneeskunde ging studeren aan de Universiteit Leiden. Deze studie vervolgde hij aan de Radboud Universiteit Nijmegen waar hij in 2007 het artsexamen behaalde.

Het werd Ruud tijdens zijn studie snel duidelijk dat hij kinderarts wilde worden. Hij heeft daarom na zijn studie op de Kinderafdeling van het Queen Elizabeth Central Hospital in Blantyre, Malawi gewerkt. Hierna werkte hij als arts-assistent niet in opleiding (ANIOS) in het Jeroen Bosch Ziekenhuis ('s-Hertogenbosch) en werd hij aangenomen voor de opleiding tot kinderarts welke hij in januari 2009 startte in het Canisius Wilhelmina Ziekenhuis te Nijmegen (opleider dr. B. Semmekrot). In het Radboudumc te Nijmegen (opleider dr. J.M.T Draaisma) heeft Ruud de opleiding vervolgd vanaf juli 2010 en afgerond in september 2014.

Naast zijn opleiding tot kinderarts is Ruud in januari 2013 gestart met de opleiding tot klinisch farmacoloog welke hij in het voorjaar van 2015 zal afronden (opleiders dr. C. Kramers, prof. dr. D. Burger en dr. M. te Loo).

Het onderzoek dat beschreven wordt in dit proefschrift werd gestart tijdens het senior coschap kindergeneeskunde in het Jeroen Bosch Ziekenhuis. Het vervolg van het onderzoek werd uitgevoerd naast zijn klinische werkzaamheden. Hierbij diende het Jeroen Bosch Ziekenhuis als thuisbasis en werden deelonderzoeken uitgevoerd op de afdeling Immunologie van het Erasmus MC (Rotterdam), bij TNO Kwaliteit van Leven (Leiden) en bij het ImmunoDeficiencyCenter Leipzig (Leipzig, Duitsland) in samenwerking met de afdeling Klinische immunologie van het Karolinska Institutet (Stockholm, Zweden).

Ruud heeft het geluk zijn leven te mogen delen met zijn partner Jeroen. Zij wonen samen in de havenstad Rotterdam en zullen in juli 2015 verhuizen naar Toronto (Canada) waar Ruud de opleiding tot Kinderreumatoloog zal volgen in The Hospital for Sick Children (SickKids).

PhD PORTFOLIO

Name PhD student:	Ruud Verstegen
Departments:	Paediatrics, Jeroen Bosch Hospital, 's-Hertogenbosch Immunology, Erasmus MC, Rotterdam
Research school:	Molecular Medicine
PhD period:	January 2007 – January 2015
Promotors:	prof. dr. J.J.M. van Dongen prof. dr. E. de Vries
Copromotor:	dr. M.C. van Zelm

PhD TRAINING

In depth courses at the Erasmus MC

2014	Introductory course on Molecular Medicine
2014	Advanced course on Immunology
2014	Introductory course on Regulations and Organization for Clinical Investigators (BROK: Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers)
2014	Introductory and advanced course on flowcytometry

Seminars and Workshops

2009 and 2013	Young Investigators Day of the Paediatric Association Of The Netherlands (NVK), Veldhoven, The Netherlands
2010	Evidenced Based Medicine, Radboud University Medical Center
2013	Time Management Training, Radboud University Medical Center
2013	Symposium "Immunobiology of primary antibody deficiencies", Erasmus MC
2014	6th Research Master Class of the European Society for Paediatric Infectious Diseases (ESPID), Dublin, Ireland
2014	Leiden FutureLab advanced course on Trial Design and Clinical Trial Application

National conferences: oral presentations

November 2007	Annual meeting of the Paediatric Association Of The Netherlands (NVK), Veldhoven, The Netherlands *Decreased expression of CD21 on B-lymphocytes in Down syndrome: association with decreased pneumococcal antibody response?*

November 2008 Annual meeting of the Paediatric Association Of The Netherlands (NVK), Veldhoven, The Netherlands
Decreased percentage of CD23+ B-lymphocytes in Down syndrome: association with decreased antibody production?

November 2012 Annual meeting of the Paediatric Association Of The Netherlands (NVK), Veldhoven, The Netherlands
The impact of recurrent respiratory tract infections on development, behaviour and health related quality of life in 8-year-old children with Down syndrome

December 2012 Dutch Working Group for Immunodeficiencies (WID), Utrecht, The Netherlands
Peripheral development of B-lymphocytes in Down syndrome

November 2013 Annual meeting of the Paediatric Association Of The Netherlands (NVK), Veldhoven, The Netherlands
B-lymphocyte development in Down syndrome

November 2013 Annual meeting of the Paediatric Association Of The Netherlands (NVK), Veldhoven, The Netherlands
Newborn screening for Severe Combined Immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA): false-positive results caused by Down syndrome

National conferences: poster presentation

March 2014 18th Molecular Medicine Day, Rotterdam, The Netherlands
Defective B-cell memory in patients with Down syndrome

International conferences: oral presentations

May 2014 32nd annual meeting of the European Society for Paediatric Infectious Diseases (ESPID), Dublin, Ireland
Defective B-cell memory in patients with Down syndrome

International conferences: poster presentations

October 2008 13th biennial meeting of the European Society for Immunodeficiencies (ESID), 's-Hertogenbosch, The Netherlands
Decreased CD21+ and CD23+ B-lymphocytes in Down syndrome children: association with decreased antibody production?

June 2011 29th annual meeting of the European Society for Paediatric Infectious Diseases (ESPID), The Hague, The Netherlands
Impact of respiratory tract infections in children with Down syndrome

September 2011	Annual meeting of the European Society for Social Paediatrics and Child Health (ESSOP), Maastricht, The Netherlands *Prevention of recurrent respiratory tract infections in Down syndrome: what could potentially be gained?*
October 2011	52nd annual meeting of the European Society for Paediatric Research (ESPR), Newcastle, United Kingdom *Impact of respiratory tract infections on developmental skills in children with Down syndrome*
October 2012	15th biennial meeting of the European Society for Immunodeficiencies (ESID), Florence, Italy *Patients with Down syndrome have defects in blood B-cell numbers, replication history and somatic hypermutation that resemble a distinct subset of common variable immunodeficiency*
June 2013	31th annual meeting of the European Society for Paediatric Infectious Diseases (ESPID), Milan, Italy *Incidence of respiratory symptoms in children with Down syndrome: feasibility of a web-based parent-reported prospective study*

Committees

2010-2012	Research committee, Radboud University Medical Center – Amalia Children's Hospital

Memberships

2007-present	Member of the Paediatric Association Of The Netherlands (NVK)
2009-present	Junior member of the European Society for Immunodeficiencies (ESID)
2010-present	Member of the Dutch Society for Paediatric Rheumatology (NVKR)
2010-present	Member of the Working Group for Paediatric Infectious Diseases and Immunology (SPII) of the Paediatric Association Of The Netherlands (NVK)

Teaching

2009-present	Supervision of medical students and interns, Radboud University Medical Center – Amalia Children's Hospital
2009-present	13 lectures on infectious, autoimmune and autoinflammatory diseases as well as immunodeficiencies, Radboud University Medical Center – Amalia Children's Hospital

LIST OF PUBLICATIONS

- M.A.A. Kusters, R.H.J. Verstegen, E.F.A. Gemen, E. de Vries. Intrinsic defect of the immune system in children with Down syndrome; a review. Clin Exp Immunol 2009; 156:189-193
- M.A.A. Kusters, E.F.A. Gemen, R.H.J. Verstegen, P.C. Wever, E. de Vries. Down syndrome T-lymphocyte subpopulation counts seem to favour intrinsic defect over early senescence. Pediatr Res. 2010; 67(5):557-62.
- R.H.J. Verstegen, M.A.A. Kusters, E.F.A. Gemen, E. de Vries. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help? Pediatr Res. 2010; 67(5):563-9.
- R.H.J. Verstegen, M. Theodore, H. van de Klerk, E. Morava. Lymphatic edema in congenital disorders of glycosylation. JIMD Rep. 2012; 4:113-6.
- M.A.A. Kusters, R.H.J. Verstegen, E. de Vries. Down syndrome: is it really characterized by precocious immunesenescence? Aging Dis. 2011; 2(6):538-45.
- K.M. Brouwer, W.F. Daamen, D. Reijnen, R.H.J. Verstegen, G. Lammers, T.G. Hafmans, R.G. Wismans, T.H. van Kuppevelt, R.M. Wijnen. Repair of surgically created diaphragmatic defect in rats with use of a de novo constructed porous collagen scaffold. J Tissue Eng Regen Med. 2013; 7(7):552-61.
- R.H.J. Verstegen, H.B.M. van Gameren-Oosterom, M. Fekkes, E. Dusseldorp, E. de Vries, J.P. van Wouwe. Significant impact of recurrent respiratory tract infections in children with Down syndrome. Child Care Health Dev. 2013; 39(6):801-9.
- E.F.A. Gemen, R.H.J. Verstegen, J. Leuvenink, E. de Vries. Increased circulating apoptotic lymphocytes in children with Down syndrome. Pediatr Blood Cancer 2012; 59(7):1310-2.
- R.H.J. Verstegen, S. Borte, L.A. Bok, P.H.T. van Zwieten, U. von Döbeln, L. Hammarström, E. de Vries. Impact of Down syndrome on the performance of neonatal screening assays for severe primary immunodeficiency diseases. J Allergy Clin Immunol. 2014; 133(4):1208-11.
- R.H.J. Verstegen, R.W.N.M. van Hout, E. de Vries. Epidemiology of respiratory symptoms in children with Down syndrome: a nationwide prospective web-based parent-reported study. BMC Pediatrics 2014, 14:103.
- R.H.J. Verstegen, G.J. Driessen, S.J.W. Bartol, C.J.M. van Noesel, L. Boon, M. van der Burg, J.J.M. van Dongen, E. de Vries, M.C. van Zelm. Defective B-cell memory in patients with Down syndrome. J Allergy Clin Immunol. 2014;134(6):1346-53.

A

LIST OF ABBREVIATIONS

ACTB	β-actin
AID	Activation-induced cytidine deaminase
ALL	Acute lymphoblastic leukaemia
AMC	Age-matched controls
AML	Acute myeloid leukaemia
AT	Ataxia Telangectasia
AV	Annexin-V
BAFF	B cell–activating factor
BAFFR	BAFF receptor
BCR	B-cell receptor
β-hCG	β human chorionic gonadotrophin
BSA	Bovine serum albumin
CBCL	Child Behavior Checklist
CD	Cluster of differentiation
CDR	Complementarity-determining region
CHD	Congenital heart disease
CSR	Class switch recombination
CVID	Common Variable Immunodeficiency Disorders
DGS	DiGeorge syndrome
DS	Down syndrome
EDTA	Ethylenediaminetetraacetic acid
ENT	Ear, nose and throat
ESID	European Society for Immunodeficiencies
EUROCAT	European Surveillance of Congenital Anomalies
FITC	Fluorescein isothiocyanate
FR	Framework region
GALT	Gut-associated lymphoid tissue
HLA	Human leukocyte antigen
HRQoL	Health Related Quality of Life
ICOS	Inducible T-cell costimulator
Ig	Immunoglobulin
IgκREHMA	Igκ restriction enzyme hot-spot mutation assay
IL	Interleukin
KREC	Kappa-deleting recombination excision circle
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
MLL	Mixed lineage leukaemia (MLL)
MSCA	McCarthy Scales of Children's Abilities
NBS	Nijmegen Breakage Syndrome
NK	Natural killer
PAPP-A	Pregnancy-associated plasma protein A
PBS	Phosphate buffered saline
PD-1	Programmed cell-death protein 1
PE	Phycoerythrin
PE-Cy5	PE-cyanine 5
PI	Propidium iodide
PID	Primary immunodeficiency
RPMI	Rosewell Park Memorial Institute
RQ-PCR	Realtime quantitative polymerase chain reaction
RRTI	Recurrent respiratory tract infections
SCID	Severe Combined Immunodeficiency
SHM	Somatic hypermutation
SPAD	Specific antibody deficiency
TACI	Transmembrane activator and CAML interactor
TACQOL	TNO-AZL Children's Quality of Life
Tc	Cytotoxic T cell
TCR	T-cell receptor
TFH	Follicular helper T cells
Th	Helper T cell
TLR	Toll-like receptor
TMD	Transient myeloproliferative disorder
TPO	Thyroid peroxidase
TREC	T-cell receptor excision circle
XLA	X-linked agammaglobulinemia

AFFILIATIONS OF CO-AUTHORS

S.J.W. Bartol	Department of Immunology, Erasmus MC, Rotterdam
L.A. Bok	Department of Paediatrics, Máxima Medical Center, Veldhoven
L. Boon	Bioceros B.V., Utrecht
S. Borte	Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden; Translational Centre for Regenerative Medicine, University of Leipzig, Leipzig, Germany; ImmunoDeficiencyCenter Leipzig at Hospital St Georg gGmbH Leipzig, Jeffrey Modell Diagnostic and Research Center for Primary Immunodeficiencies, Leipzig, Germany
M. van der Burg	Department of Immunology, Erasmus MC, Rotterdam
U. von Döbeln	Division of Metabolic Diseases, Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Solna, Stockholm, Sweden
J.J.M. van Dongen	Department of Immunology, Erasmus MC, Rotterdam
G.J. Driessen	Department of Paediatric Infectious Disease and Immunology, Erasmus MC, Rotterdam
E. Dusseldorp	Department of Child Health, Netherlands Organisation for Applied Scientific Research TNO, Leiden
M. Fekkes	Department of Child Health, Netherlands Organisation for Applied Scientific Research TNO, Leiden
H.B.M. van Gameren-Oosterom	Department of Child Health, Netherlands Organisation for Applied Scientific Research TNO, Leiden; currently Department of Paediatrics, Leiden University Medical Center, Leiden
E.F.A. Gemen	Laboratory of Clinical Chemistry and Haematology, Jeroen Bosch Hospital, 's-Hertogenbosch
L. Hammarström	Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden
R.W.N.M. van Hout	Department of Linguistics, Radboud University Nijmegen, Nijmegen
M.A.A. Kusters	Department of Paediatrics, Jeroen Bosch Hospital, 's-Hertogenbosch; currently Department of Paediatrics, Radboud University Medical Center - Amalia Children's Hospital, Nijmegen
J. Leuvenink	Laboratory of Clinical Chemistry and Haematology, Jeroen Bosch Hospital, 's-Hertogenbosch
C.J.M. van Noesel	Department of Pathology, Academic Medical Center, Amsterdam
E. de Vries	Department of Paediatrics, Jeroen Bosch Hospital, 's-Hertogenbosch; Tilburg University, Tilburg
J.P. van Wouwe	Department of Child Health, Netherlands Organisation for Applied Scientific Research TNO, Leiden
M.C. van Zelm	Department of Immunology, Erasmus MC, Rotterdam
P.H.Th. van Zwieten	Department of Paediatrics, Juliana Children's Hospital, The Hague