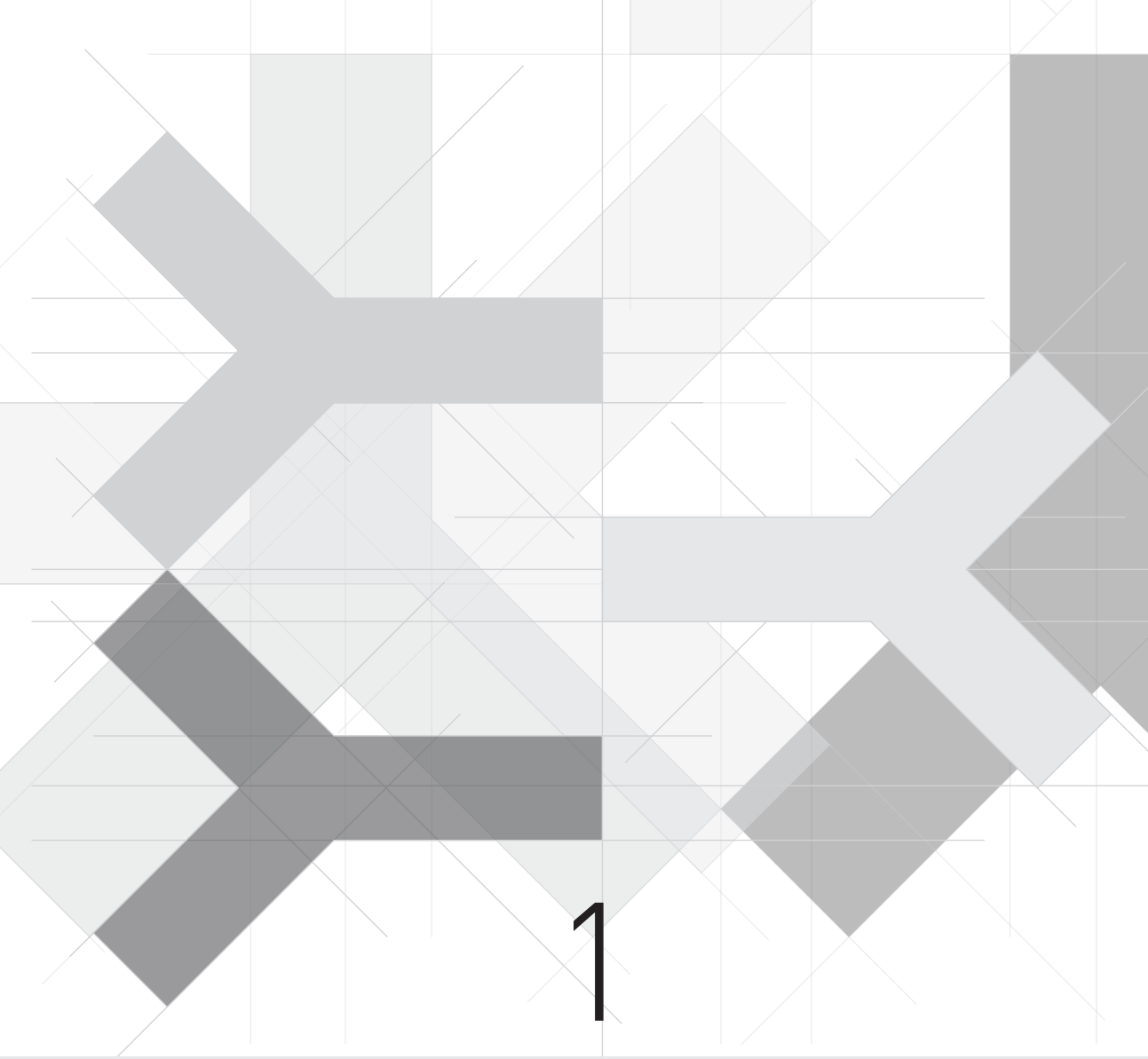

1

GENERAL INTRODUCTION

Down syndrome (DS), named after John Langdon Down, is the most common chromosomal disorder in man. Down syndrome is caused by trisomy of (parts of) chromosome 21.[1] The incidence of Down syndrome in the Netherlands is around 14.6 per 10,000 live births, which results in around 245 live newborns with Down syndrome in the Netherlands each year.[2] Children with DS have distinctive phenotypic facial features such as oblique eye fissure, epicanthus, small ears, flat nasal bridge and a protruding tongue.

In addition to this, DS subjects show generalized hypotonia, short stature and mental retardation of variable severity. Around fifty percent has a congenital heart defect.[3] Children with DS have multiple ear-nose-throat abnormalities such as stenotic ear canals with smaller or abnormally inserted Eustachian tubes, and midfacial hypoplasia.[4] DS children also have hyperproduction of mucus and lower respiratory tract abnormalities such as laryngo- and tracheomalacia.

Clinical profile of infections, autoimmune disease and malignancies in DS

Nowadays, the majority of Dutch DS children live at home. They are exposed to the same viral and bacterial pathogens and environmental factors as their brothers and sisters. However, children with DS are more frequently ill.[5] They are more prone to respiratory tract infections. This is at least partly explained by a combination of their phenotypical facial features and their anatomical respiratory tract abnormalities together with generalized hypotonia. Complications of respiratory tract infections remain the most important cause of mortality in all age groups in DS.[6-10] DS children also show more haematological malignancies and autoimmune phenomena such as celiac disease, thyroid disease and diabetes mellitus.[11-15] Leukemia (both acute lymphoid and myeloid) and leukemoid reactions show an increased incidence in Down syndrome; estimates of the relative risk are 10-20x higher compared to the non-DS population.[11, 12, 16] Statistics on bacterial sepsis show higher morbidity and mortality figures in DS children.[6, 17] This combination of increased incidence of infections, autoimmune diseases and haematological malignancies has led to the hypothesis of an altered adaptive immune system in Down syndrome.

Several theories have been postulated. During corrective heart surgery it was noted that the DS thymus looked different compared to non-DS thymuses.[18-22] As a result, immunological research in DS focused on T-cell problems for decades. The clinical profile of recurrent infections, autoimmune diseases and malignancies in combination with a higher incidence of Alzheimer-like disease in relatively young DS adults led to the hypothesis of premature ageing or 'immunosenescence' of thymus and T-lymphocytes.[22-24] But the question remains: is that true? This thesis provides more in depth research - both quantitative and qualitative - on the alterations in the adaptive immune system in children with Down syndrome.

The adaptive immune system: basic background

The key players in the adaptive immune response are T- and B-lymphocytes. Both T- and B-lymphocyte precursors are generated from haematopoietic stem cells in the bone marrow. A unique B-cell antigen receptor is created and expressed on the membrane through gene rearrangements without previous antigen-exposure. While B-lymphocytes fully develop in the bone marrow, immature T-cell-precursors migrate to the thymus. Within the thymus, T-cell-precursors can only survive when their T-cell receptors can interact with self major histocompatibility complexes (MHC) expressed on cell membranes. Thymocytes binding to MHC-class I differentiate into cytotoxic-T-lymphocytes (Tc), thymocytes binding to MHC-class II differentiate into helper-T-lymphocytes (Th).

Naive Th-, Tc- and B-lymphocytes migrate to the secondary lymphoid organs (e.g. spleen, lymph nodes, gut- and mucosa-associated lymphoid tissue respectively GALT and MALT), and proliferate and differentiate into multiple different effector and memory subpopulations after antigen exposure.

B-lymphocytes react directly to antigen exposure by producing immunoglobulins (Igs). Extracellular pathogens such as bacteria are the main target of these Igs (humoral response).

Tc interact with antigen presented on MHC-class I molecules, which almost all human cells express, and can act directly as "killing machines". Tc are specifically suited for strong cellular immune responses against tumour cells and intracellular pathogens such as viruses.

Th can only interact with antigen-presenting cells (APCs) expressing MHC-class II molecules. Examples of APCs are B-lymphocytes, dendritic cells and phagocytes.

Th are responsible for coordination and communication with both innate and adaptive immune cells; in that sense they serve as immunoregulators. Th can help both humoral and cellular immune responses.

Lymphocyte distribution in the human body

Flow cytometric analysis using specific cell surface (cluster of differentiation, CD) markers can differentiate between the various naive and memory B- and T-lymphocyte subpopulations in peripheral blood. However, peripheral blood lymphocytes only represent around 2% of the total number of lymphocytes in the human body.[25] Lymphocytes continuously circulate through the body and migrate to their preferred sites situated in primary and secondary lymphoid organs. The majority of lymphocytes actually reside in the secondary lymphoid organs, especially in the lymph nodes (estimated 40% of total lymphocytes). So, by analysing peripheral blood, only a minor fraction of the lymphocyte population can be visualized (Figure 1). Therefore, conclusions based on peripheral blood analysis should be drawn with caution.

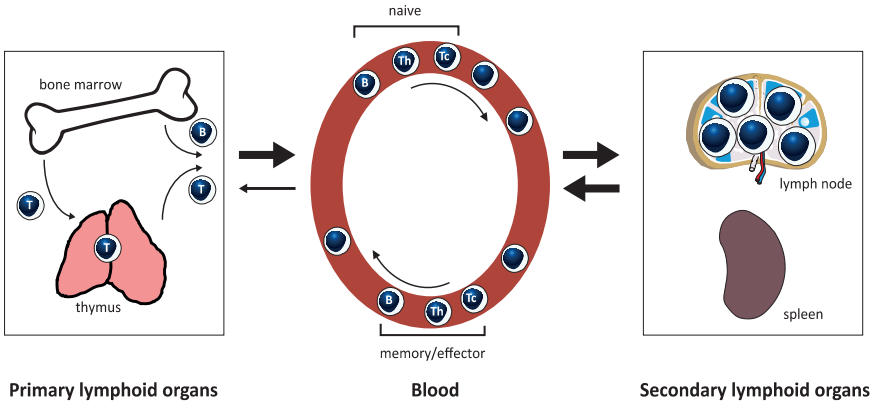

Figure 1 Analysing lymphocytes in peripheral blood represents around 2% of the total lymphocyte population, as the majority of lymphocytes reside in primary (bone marrow, thymus) and secondary lymphoid organs (such as lymph node and spleen).

Figure 1 is adapted from *Werkboek Immunologie* G.T. Rijkers et al.

Also, the distribution of lymphocytes can vary between individuals and groups.[26, 27] For instance in Down syndrome, an increased genetic expression of lymphocyte homing receptors and cell adhesion molecules is found in thymic epithelium and thymocytes, potentially leading to an inefficient release of T-lymphocytes into the peripheral blood and thereby alteration of the distribution of T-lymphocytes in the body. Moreover, the results of one specific blood analysis do not necessarily reflect a permanent difference, as many factors – e.g. recently encountered antigens – can strongly influence the amount of lymphocytes circulating in the blood.[27] Exposure to pathogens can also leave a personalised fingerprint, which shape is dependent on factors such as age at time of contact, season, environment, race, and sequence of encounters. Especially viruses trigger cytotoxic-T-lymphocytes to differentiate and proliferate to specific subpopulations. For example, after primary cytomegalovirus (CMV) contact, a persistent increase of CMV-specific terminally differentiated Tc-lymphocytes ($CD3^+CD8^+CD45RA^+CD27^-$) is seen; CMV leaves a typical fingerprint in the Tc population.[28] The individual set-point is defined by the degree of immunocompetence during the primary CMV-contact: immunocompromised people produce higher numbers of CMV-specific terminally differentiated Tc-lymphocytes in an attempt to keep CMV latent, but they are still more prone to CMV reactivation and severe infection-related morbidity.[29-31] The number of terminally differentiated Tc in combination with the clinical picture after primary CMV infection can therefore be

used as an indicator for an assessment of the immune status of the host during the encounter with CMV.

Vaccination types as models for T-cell (in)dependent antigen response

The adaptive immune system uses different pathways to respond to antigens: T-cell dependent (TD) and T-cell independent (TI), respectively. Different types of vaccination are used in this thesis as a model to study these different immune response pathways. All models have their limitations. A vaccine is not a surrogate for experiencing an infection for various reasons. The route is different: vaccinations are administrated intramuscularly in most cases, whereas pathogens invade across barriers (e.g. respiratory tract, skin) resulting in differences in antigen-presentation and immune response.

Also, the composition is different: vaccinations are made of attenuated live viruses, inactivated or killed organisms, inactivated toxins or segments of the pathogens. They can contain extra elements, such as a carrier protein (conjugate) or adjuvants to boost the immune response and push the immune response in a certain direction: e.g. a TI or TD antigen response. In reality, most pathogens share properties of TD and TI antigens.[32-34]

Finally, comparison is difficult: different methods and vaccination schemes co-exist side-by-side, but universal antibody cut-off levels are being used despite of the differences. The majority of the documented protective antibody cut-off levels are based on studies performed in healthy males (e.g. soldiers), but they are applied to children and elderly as well. Post-vaccination protection for an individual should be based not only on the quantitative antibody level, but also on the antibody quality measured by avidity and opsonisation capacity and most importantly the lack of disease occurrence. However, for most vaccines, combined quantitative and qualitative studies and reference values specifically for children are lacking.

T-cell dependent antigen response

The T-cell dependent antigen response is primarily determined by the combination of T-lymphocyte function, T-B interaction and B-lymphocyte function (Figure 2). An adequate T-cell dependent antigen response will ultimately lead to activated class-switched B-lymphocytes producing immunoglobulins with high affinity, and will also result in the formation of memory T- and B-lymphocytes.

Peptide antigen has to be processed and presented by an antigen-presenting cell (APC). Antigen-loaded APCs can then migrate to secondary lymphoid organs (e.g. lymph nodes, spleen) and present a specific antigen on their MHC-class II complex to the T-cell receptor (TCR) of helper-T lymphocytes (Th). If the MHC-class II-peptide complex is recognized by TCR of Th, an immunological synapse will be formed in the interface between APC and (B- or T-)lymphocyte. The formation consists of different

clusters of rings: in the centre, the B-cell or T-cell receptor which is bound to a MHC-antigen complex presented by APC, with surrounding clusters consisting of different adhesion molecules. For example in the peripheral supramolecular activation cluster (pSMAC), lymphocyte function-associated antigen 1 (LFA-1) on Th binds to intercellular adhesion molecule 1 (ICAM-1) on APC. The immunological synapse coordinates cell-signalling, activation and differentiation. The Th becomes activated and moves towards B-cell areas in secondary lymphoid organs.

B-lymphocyte activation is initiated following recognition of a specific antigen by the B-cell receptor (BCR). Activated B-lymphocytes can produce IgM instantly. The activated B-lymphocytes move towards the activated Th-lymphocytes in the secondary lymphoid organs and can present processed peptide-antigen on their MHC-II molecules to TCR of the abovementioned activated Th-lymphocytes.

With help from Th, B-lymphocytes can undergo class-switch recombination and somatic hypermutation. Apart from finding the right Th-B match, co-stimulatory molecules creating an immunological synapse between B-Th are necessary as well.

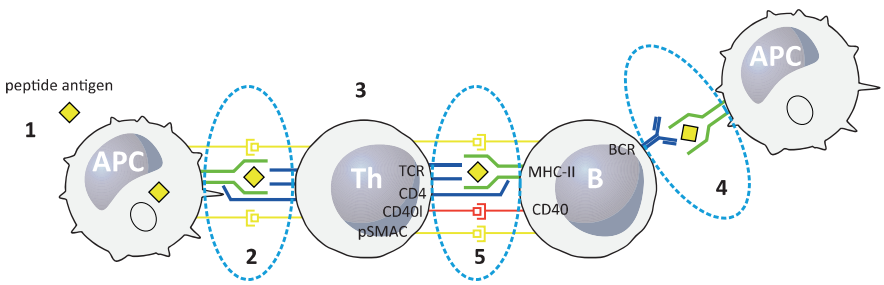

Figure 2 Stepwise approach of T-cell dependent antigen response.

1. Peptide antigen uptake and processing by antigen-presenting cell (APC).
2. Immunological synapse between APC-Th: antigen presentation on MHC-II by APC to TCR on helper-T-lymphocyte (Th). Antigen-recognition by TCR on Th. Cellular binding between Th and APC through costimulating peripheral supramolecular adhesion molecules (pSMAC) such as LFA-1 and ICAM-1, creating an immunological synapse.
3. Activation of Th and Th movement towards B-cell areas, generating cytokines activating both Th and APC.
4. Antigen-recognition of B-lymphocyte by BCR, antigen presentation by APC possible. Antigen-processing and presentation on MHC-II by B-cell. Movement of activated B-cell in lymph node towards Th.
5. Immunological synapse between B-Th: antigen-presentation on MHC-II of B-cell to TCR on Th. Antigen-recognition by TCR on Th. Cellular binding between Th and B-cell through costimulating peripheral supramolecular adhesion molecules (pSMAC) such as LFA-1 and ICAM-1, creating an immunological synapse. CD40-CD40L connection necessary for class switch recombination (CSR) of activated B-lymphocytes towards the production of IgG, IgA or IgE.

For instance, B-lymphocytes can only class-switch from the production of IgM to the production of IgG, IgA and IgE after interaction between CD40 molecules on B-lymphocytes with CD40L molecules on activated Th. Through this class-switch recombination (CSR) process, B-lymphocytes can adapt their Ig effector functions while maintaining antigen specificity.

Repeated exposure to T-lymphocyte dependent antigens activates selected clones of memory B-lymphocytes to undergo somatic hypermutation (SHM), leading to increased antigen specificity with higher affinity immunoglobulins. These processes also result in the production of memory B-lymphocytes inducing long lasting protection and faster immune responses when antigen is repeatedly encountered.[33, 34] Examples of TD antigens used in this thesis are tetanus toxoid and influenza A/H1N1 vaccinations.

T-cell independent antigen response

Bacteria can use camouflage techniques to prevent Th help, for instance by using a coat of polysaccharides. B-lymphocytes can mount an immune response against these polysaccharides without T-cell help, because these molecular structures are repetitive and therefore do not need peptides or antigen-presentation on MHC to activate the B-lymphocyte. This is called a T-cell independent (TI) type 2 immune response (Figure 3a). Polysaccharides can extensively crosslink B-cell antigen receptors and deliver a prolonged and persistent signal to the B-lymphocyte.[32] TI immune response is essential for rapid antibody production and early protection especially to blood-borne pathogens. However, without Th help, no B-lymphocyte class switch recombination or somatic hypermutation will take place and no memory B-lymphocytes will be produced. The TI immune response can be used to investigate the maturation and quality of B-lymphocytes. An example of a specific TI type 2 antigen used in this thesis is 23-valent pneumococcal polysaccharide vaccine (PPV23).

The immature adaptive immune system in infants under 2 years of age is unable to induce an adequate TI response in response to polysaccharides. To overcome this problem, new types of vaccines have been developed. By conjugating a peptide to the polysaccharide antigen, an adequate TD immune response can be induced, even in young children (Figure 3b). Examples of this type of immune response used in this thesis are the heptavalent pneumococcal conjugate vaccine (PCV7) and meningococcal serotype C (MenC) vaccine.

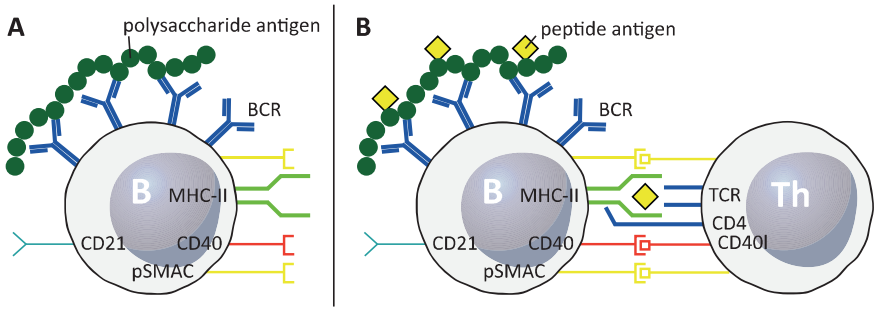

Figure 3 Stepwise approach of T-cell independent antigen response (polysaccharide PS) and PS+conjugate.

A. PS: Extensive polysaccharide crosslinking on BCRs: B-cell activation signal 1. Extra activation through C3d recognition by CD21 on B-cell. Extra stimulation through cytokines produced by macrophages/T-lymphocytes possible. TI antigen response: primarily IgM.
B. PS with conjugate: Th help possible through conjugate (peptide) uptake and processing by B-lymphocyte. Peptide antigen-presentation on MHC-II of B-cell to TCR on Th. Costimulating peripheral supramolecular adhesion molecules (pSMAC) creating immunological synapse, connection CD40-CD40L necessary for class switch recombination (CSR) of B-lymphocytes. Th activation: effector and memory cells, cytokine production. TD antigen response: primarily IgG.

THESIS OUTLINE

This thesis gives more insight in the adaptive immune system of children with Down syndrome. In **PART 1** both T- and B-lymphocytes subpopulations and immunoglobulin (IgG, IgA, IgM) levels in different age groups in DS children are analyzed. In **Chapter 2,** an overview of past literature on the DS adaptive immune system is given. **Chapter 3** investigates the current hypothesis of immunosenescence by comparing the literature on immunological alterations and clinical profiles in normal ageing, progeria syndromes and DS. In **Chapters 4 and 5** both T- and B-lymphocyte subpopulations and immunoglobulin (IgG, IgA, IgM) levels in different age groups in DS children are analyzed and compared with healthy age-matched control children. Terminally differentiated cytotoxic-T-lymphocyte counts in relation to cytomegalovirus are compared with non-DS subjects with different immunocompetence status. The clinical picture of infections, autoimmune disease, allergy and malignancies is correlated with these quantitative results. In **PART 2** the quality of B- and T-lymphocyte responses to different types of antigen is investigated through vaccination studies as a model for TD and TI immune responses. In **Chapters 6-9** five different vaccinations

are used. Tetanus toxoid (chapter 6), influenza A/H1N1 (chapter 7) are examples of protein antigens eliciting TD responses. Meningococcal C (MenC; chapter 8) and heptavalent pneumococcal conjugate (PCV7; chapter 9) vaccinations are examples of protein-conjugated polysaccharide antigens eliciting TD responses. 23-Valent pneumococcal polysaccharide vaccine (PPV23; chapter 9) is an example of a polysaccharide antigen eliciting a TI response. A comparison is made with data from the literature on ageing, on DiGeorge syndrome (DGS), thymectomy and HIV (all mainly T-lymphocyte deficiencies), and on common variable immunodeficiency disorders (CVID) and specific polysaccharide antibody deficiency (SPAD) (mainly B-lymphocyte immunodeficiencies). **PART 3** contains the summary and discussion.

AIM OF THE THESIS

In this thesis the following questions are addressed:

1. What alterations - both qualitative and quantitative - can be found in T- and B-lymphocytes in DS?
2. Do these alterations fit the current hypothesis of early immunosenescence?
3. Do children with DS have an intrinsic primary immunodeficiency (PID)?
4. Do the immunological alterations in combination with the clinical profile fit a specific known PID pattern or do children with Down syndrome have their own profile?

REFERENCES

1. Hassold TJ, Jacobs PA. Trisomy in man. Annu Rev Genet 1984;18:69-97.
2. 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.
3. Tolksdorf M, Wiedemann HR. Clinical aspects of Down's syndrome from infancy to adult life. Hum Genet Suppl 1981;2:3-31.
4. Ramia M, Musharrafieh U, Khaddage W, Sabri A. Revisiting Down syndrome from the ENT perspective: review of literature and recommendations. Eur Arch Otorhinolaryngol 2013.
5. Broers CJ, Gemke RJ, Weijerman ME, Kuik DJ, van Hoogstraten IM, van Furth AM. Frequency of lower respiratory tract infections in relation to adaptive immunity in children with Down syndrome compared to their healthy siblings. Acta Paediatr 2012;101:862-7.
6. Garrison MM, Jeffries H, Christakis DA. Risk of death for children with down syndrome and sepsis. J Pediatr 2005;147:748-52.
7. 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.
8. Oster J, Mikkelsen M, Nielsen A. Mortality and life-table in Down's syndrome. Acta Paediatr Scand 1975;64:322-6.
9. 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.
10. 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.
11. 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.
12. Fabia J, Drolette M. Malformations and leukemia in children with Down's syndrome. Pediatrics 1970;45: 60-70.
13. Sanchez-Albisua I, Storm W, Wascher I, Stern M. How frequent is coeliac disease in Down syndrome? Eur J Pediatr 2002;161:683-4.
14. 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.
15. Anwar AJ, Walker JD, Frier BM. Type 1 diabetes mellitus and Down's syndrome: prevalence, management and diabetic complications. Diabet Med 1998;15:160-3.
16. Fraumeni JF, Jr., Manning MD, Mitus WJ. Acute childhood leukemia: epidemiologic study by cell type of 1,263 cases at the Children's Cancer Research Foundation in Boston, 1947-65. J Natl Cancer Inst 1971;46:461-70.
17. Hill DA, Gridley G, Cnattingius S, et al. Mortality and cancer incidence among individuals with Down syndrome. Arch Intern Med 2003;163:705-11.
18. Benda CE, Strassmann GS. The thymus in mongolism. J Ment Defic Res 1965;9:109-17.
19. Dybdahl H, Henriques UV. Thymic epithelial abnormalities in patients with congenital heart disease and Down's syndrome. APMIS 1993;101:73-4.
20. Larocca LM, Lauriola L, Ranelletti FO, et al. Morphological and immunohistochemical study of Down syndrome thymus. Am J Med Genet Suppl 1990;7:225-30.
21. Levin S, Schlesinger M, Handzel Z, et al. Thymic deficiency in Down's syndrome. Pediatrics 1979;63:80-7.
22. Lott IT. Down's syndrome, aging, and Alzheimer's disease: a clinical review. Ann N Y Acad Sci 1982;396:15-27.
23. Cuadrado E, Barrena MJ. Immune dysfunction in Down's syndrome: primary immune deficiency or early senescence of the immune system? Clin Immunol Immunopathol 1996;78:209-14.
24. Seger R, Buchinger G, Stroder J. On the influence of age on immunity in Down's syndrome. Eur J Pediatr 1977;124:77-87.
25. Westermann J, Pabst R. Distribution of lymphocyte subsets and natural killer cells in the human body. Clin Investig 1992;70:539-44.
26. Westermann J, Pabst R. Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? Immunol Today 1990;11:406-10.

27. Bonilla FA, Oettgen HC. Normal ranges for lymphocyte subsets in children. J Pediatr 1997;130:347-9.
28. Appay V, Dunbar PR, Callan M, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 2002;8:379-85.
29. Kuijpers TW, Vossen MT, Gent MR, et al. Frequencies of circulating cytolytic, CD45RA+CD27-, CD8+ T lymphocytes depend on infection with CMV. J Immunol 2003;170:4342-8.
30. Almanzar G, Schwaiger S, Jenewein B, et al. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. J Virol 2005;79:3675-83.
31. Bekker V, Bronke C, Scherpbier HJ, et al. Cytomegalovirus rather than HIV triggers the outgrowth of effector CD8+CD45RA+. AIDS 2005;19:1025-34.
32. Defrance T, Taillardet M, Genestier L. T cell-independent B cell memory. Curr Opin Immunol 2011;23:330-6.
33. Good-Jacobson KL, Tarlinton DM. Multiple routes to B-cell memory. Int Immunol 2012;24:403-8.
34. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. Nat Rev Immunol 2009;9:15-27.