Karolinska Institutet

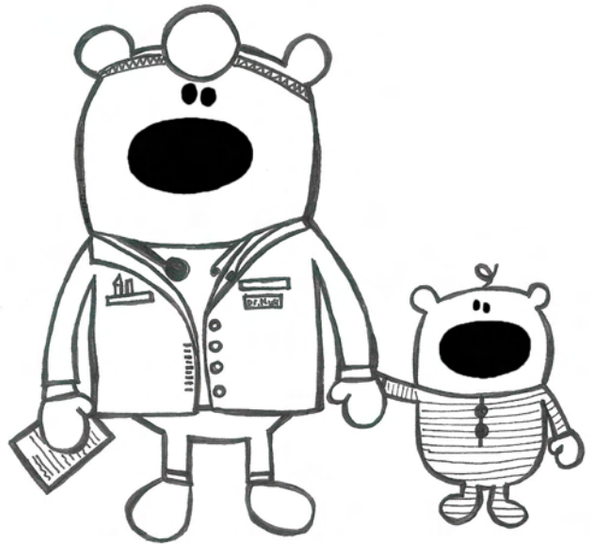
Thesis for doctoral degree (Ph.D.) 2009

Development of screening for primary immunodeficiency

Magdalena Janzi

Thesis for doctoral degree (Ph.D.)
2009

DEVELOPMENT OF SCREENING FOR PRIMARY IMMUNODEFICIENCY



Magdalena Janzi

Karolinska Institutet

From THE DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

DEVELOPMENT OF SCREENING FOR PRIMARY IMMUNODEFICIENCY

Magdalena Janzi

Stockholm 2009

Cover: Drawing by Camilla Åhlén. All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Larserics Digital Print AB.

ISBN 978-91-7409-573-9

The question is not about what you look at, but what you see
- Henry David Thoreau

To all my loves,

ABSTRACT

Primary immunodeficiencies are inherited disorders of the immune system, with an estimated prevalence of 1:500 in the USA. Yet, a majority of the patients are still undiagnosed. Most patients with a diagnosis come in contact with the healthcare due to complications caused by the immunological defects. In many cases, it is crucial that the patients receive treatment before their health is seriously compromised. There is therefore a great need for more efficient ways of identifying the patients. As a lack of serum IgA is included in the phenotype of many of the defects, a population-based screening for IgA deficiency would identify patients with various forms of primary immunodeficiencies. The main benefit of such screening would be the early detection of the disorders followed by appropriate treatment, to decrease the risk of severe infections. We investigated the possibility of a screening for primary immunodeficiencies based on the use of serum microarrays, a high-throughput platform suitable for large-scale analysis of dried blood spot samples collected from newborns.

Serum samples from adult individuals with known levels of serum IgA were used to investigate the potential of serum microarrays in detection of serum IgA. A high level of correlation was observed throughout the material, compared to a conventional method (paper I).

The serum microarrays were then used to establish the prevalence of IgA deficiency in serum samples of Swedish children at the age of four and the IgA was correlated to various health outcomes that are associated to allergy and infections. The prevalence of IgA deficiency was found to be 1:173, as compared to 1:600 in the adult population. IgA deficient children seem to have an increased risk of pseudocroup and parentally reported food hypersensitivity during childhood (paper II).

As the screening should be based on the use of dried blood spots samples, collected within days after birth, the suitability of serum microarrays in analysis of these samples was evaluated in paper III. The levels of complement protein C3 were measured in samples from a C3 deficient patients and controls. The levels of C3 in the control group was significantly higher than in the Swedish C3 deficient patient sample, indicating that serum microarrays are suitable for identification of C3 deficiency using the dried blood spot samples.

The next step in our evaluation was to increase the sensitivity of the serum microarrays/DBSS approach (paper IV). The C2 protein was used, which is less abundant in newborns than C3. None of the tested anti-C2 antibodies was suitable for discrimination between patients and controls using serum microarrays. However, when using ELISA, the levels of C2 in DBSS eluates of C2 deficient patients were significantly lower than in control samples. Thus, the C2 deficiency is present already at birth.

In paper V, serum IgA levels were determined in dried blood spot samples from patients with various forms of primary immunodeficiencies. Only patients born to mothers with selective IgA deficiency lacked IgA at birth. In the remaining patients, the IgA levels were comparable to those of the controls. The results suggest that the serum IgA detected in newborns is to some extent of maternal origin. Thus, a neonatal screening for primary immunodeficiencies based on the determination of IgA levels is not possible due o the presence of maternal IgA in the samples.

LIST OF PUBLICATIONS

I. **Serum microarrays for large scale screening of protein levels.**
Janzi M., Odling J., Pan-Hammarström Q., Sundberg M., Lundeberg J., Uhlén M., Hammarström L., Nilsson P.
Mol Cell Proteomics. 2005, 4(12):1942-7.

II. **Selective IgA deficiency in early life: Association to infections and allergic diseases during childhood.**
Janzi M., Kull I., Sjöberg R., Wan J., Melén E., Bayat N., Östblom E., Pan-Hammarström Q., Nilsson P., Hammarström L.
Clin Immunol. 2009, Jun 19. [Epub ahead of print]

III. **Screening for C3 deficiency in newborns using microarrays.**
Janzi M., Sjöberg R., Wan J., Fischler B., von Döbeln U., Isaac L., Nilsson P., Hammarström L.
PLoS One. 2009, 4(4):e5321.

IV. **Screening for C2 deficiency in newborns.**
Janzi M., von Döbeln U., Jönsson G., Hammarström L.
Manuscript.

V. **Evaluation of lack of IgA as a diagnostic marker for primary immunodeficiency disorders using Guthrie card eluates.**
Janzi M., Pan-Hammarström Q., von Döbeln U., Nordvall L., Fasth A., Hammarström L.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

A-T	Ataxia-telangiectasia
C2	Second component of complement
C3	Third component of complement
CVID	Common variable immunodeficiency
DBSS	Dried blood spot samples
ELISA	Enzyme-linked immunosorbent assay
HIGM	Hyper IgM syndrome
HLA	Human leukocyte antigen
Ig	Immunoglobulin
MHC	Major histocompatibility complex
PBS	Phosphate buffered saline
PIDs	Primary immunodeficiency disorders
SCID	Severe combined immunodeficiency
sIgAD	Selective IgA deficiency
TRECs	T-cell receptor excision circles
XLA	X-linked agammaglobulinemia

1 INTRODUCTION

1.1 PRIMARY IMMUNODEFICIENCY DISORDERS

Primary immunodeficiency disorders (PIDs) comprise a group of inherited defects of the immune system, characterized by an increased risk of infections and in many cases, susceptibility to autoimmunity. PIDs affect components of both the innate and adaptive immunity, including B cells, T cells, complement proteins, macrophages, dendritic cells, NK cells and neutrophils[1]. Since Bruton described the first patient with hypogammagobulinemia in 1952 [2], more than 150 different forms of PIDs have been described. Vetrie et al identified the gene associated with Brutons agammaglobulinemia (X-linked agammaglobulinemia) already in 1993[3], and to date, more than 120 genes have been identified[1]. New forms of PIDs are constantly being described.

Approximately 50% of all known PIDs are associated with B cell defects resulting in inadequate production of antigen-specific antibodies[4]. Example of antibody defects is selective IgA deficiency (sIgAD), common variable immunodeficiency (CVID) and X-linked agammaglobulinemia (XLA), which is the most severe form of antibody defects. 20% of the PIDs are combined B-cell and T-cell immunodeficiencies[5]. The most serious form is the severe combined immunodeficiency (SCID), where survival beyond the first year of life is rare in the patient is left untreated. Approximately 50% of all SCID cases are linked to the X chromosome. 10% of the PIDs result from defects in T-cell maturation or function, for example DiGeorge syndrome, while 18% result from defective phagocytes. Example of the latter is chronic granulomatous disease and the leukocyte adhesion defect[6]. Complement deficiencies occur less frequently than other PIDs and the most common defect is the C2 deficiency.

The estimated prevalence of PIDs in the USA is 1:500[7], thus affecting approximately 600 000 individuals. In Sweden, the true prevalence of PIDs is still unknown, as the few population-based studies that have been reported focus on specific defects. We do however know that the prevalence of sIgAD alone is 1:600[8] in Sweden. Based on these prevalence numbers, this specific defect alone would affect 15 000 individuals. To date, the central registry of patients with known PIDs in Sweden includes approximately 700 individuals, comprising less than 5% of all cases. For other defects, such as the second component of complement (C2) deficiency, the estimated prevalence is approximately 1:20 000 in Western European white populations[9], yielding a total of 450 cases of C2 deficiency in Sweden. 40 cases of C2 deficiency have been identified in Sweden between 1977 and 2002[10], indicating that less than 10% are identified. Most of the PIDs patients come in contact with the health care due to complications caused by the specific defects. The main reason for the late recognition by the health care practitioners is their lack of familiarity with the PIDs. Thus, there is a great need for more reliable ways of identifying the patients, one of which could be to screen the population for the underlying defects of the immune system. The benefit of such screening is the early detection of PID, before serious infections have compromised the patient's health. However, no general, rapid, cost-effective screening method is available today.

To identify a larger portion of PID patients during their first year of life, a screening should preferably be based on the use of a single analytical platform testing one, or a few, common denominators. The method of choice should also be suitable for high-throughput analysis and not require any additional sample collection.

1.2 THE METHOD: SERUM MICROARRAYS

The diagnostic methods used for detection of PIDs today are relatively demanding in terms of labour and time, and are thus not suitable for high-throughput analysis. In contrast, the microarray technology enables fast and cost-effective testing of thousands of samples simultaneously. The development of DNA microarrays enabled a global, large-scale analysis of whole genomes and had an enormous impact on the field of functional genomics. The DNA microarray technique is now relatively mature in terms of available instrumentation for production and analysis, the availability of commercial premade microarrays, robust experimental protocols, and the tools for data analysis. However, DNA microarrays tell us only about the genes themselves and provide little information regarding the functions of the proteins they encode. The protein microarray format was therefore developed to suit global protein analysis where great potential can be envisioned. The possibility of using microarrays for large-scale, parallel measurement is perfectly suited for studies on protein profiles and functions in health and disease.

The principle of highly sensitive "microspots" containing small amounts of capture molecules, accurately detecting low concentrations of analytes were already described 20 years ago[11,12]. The protein microarray applications can generally be divided into functional protein arrays and protein profiling arrays. Functional protein arrays are used to screen large numbers of full-length functional proteins or protein domains for biochemical activity and interaction of proteins with other proteins, lipids, nucleic acids and small molecules. The strength of the functional protein arrays lies in the ability to, in a single experiment, rapidly screen large numbers of protein simultaneously. This enables determination of whole protein interaction networks or determination of all substrates for a specific enzyme[13].
The primary purpose of protein profiling arrays is to simultaneously quantify the levels of specific proteins present in a sample. This approach has been used for antibody specificity profiling[14], detection of circulating antibodies in clinical specimens[15-17], measurements of binding specificity of protein expression libraries[18,19] and protein profiling in cancer tissues[20-24].

As a PID screening would be based on the measurement of one or a few analytes, protein profiling arrays are the preferable choice for such a screening.

The protein profiling arrays can be further divided into two different types; forward phase arrays and reverse phase arrays. In the forward phase format, a bait molecule, most often an antibody, is immobilized on a membrane, thus forming a grid of microspots. As many different bait molecules are printed on a substrate, each microspot will contain one kind of bait molecule. This way, one test sample, containing several analytes of interest, is analyzed per array, using many different bait molecules

simultaneously. The captured proteins are then detected by a second, tagged molecule or by direct labeling. [25]. In contrast, in the reverse phase format, the analytes are immobilized directly on the substrate and each spot represents one test sample containing multiple analytes. Thousands of samples are analyzed simultaneously for a given analyte, using very small amounts of each sample. As reverse phase arrays do not demand labeling of the analyte and do not utilize the sandwich setup using two different antibodies, the risk of experimental variability is decreased[25].

In this work, we have chosen to name our particular approach serum microarrays, to distinct it from other forms of protein microarrays.

1.3 THE SAMPLES: DRIED BLOOD SPOT SAMPLES

Neonatal screening programs for metabolic disorders were introduced in several countries during the 60's. The screening is based on the analysis of dried blood spot samples (DBSS), collected 48 hours after birth, and is now a well-established part of the neonatal healthcare system. The screening of all newborns was introduced in Sweden in 1965, and since 1975 all tested samples are stored in a national biobank. The screening includes phenylketonuria, galactosemia, congenital hypothyroidism, congenital adrenal hyperplasia and biotinidase deficiency; metabolic disorders requiring early treatment to prevent severe injuries. 100 000 children are born in Sweden each year and approximately 50 suffer from one of the five disorders.

There has been a growing interest in the use of the DBSS in large population-based biomarker studies. The study of Tsunami Aftermath and Recovery being the largest to date with 35 000 DBSS collected[26]. In addition, the DBSS have been used in multiplex analysis of inflammatory markers[27] as well as in the previously mentioned SCID screening launched by the Jeffrey Modell Foundation and the State of Wisconsin.

The use of DBSS in a screening for PIDs solves two major issues related to the young age of the participants and the need for early diagnosis. The first issue concerns the problem of collecting new blood samples. An additional blood sampling of the newborns is not ethically defendable and would require tremendous resources in terms of money, personnel and logistics. The use of the already existing DBSS passes round those problems and greatly reduces the overall cost of such a screening. The second issue concerns the need of early diagnosis and subsequent treatment of patients with severe PIDs. Early introduction of adequate treatment is required for prevention of fatal infections. As only a small part of each DBSS is used for the existing screening, the rest of the sample constitutes an invaluable resource of biological material, suitable for use in a high-throughput application.

1.4 THE COMMON DENOMINATOR: IMMUNOGLOBULIN A

1.4.1 Immunoglobulin A

Immunoglobulin A (IgA) is the second most abundant serum immunoglobulin after IgG and the main immunoglobulin in human secretions - tears, saliva, colostrum,

mucus. Secretory IgA is important for the neutralization of viruses, binding of toxins, agglutination of bacteria, prevention of bacterial binding to epithelial cells, and prevention of entry of food antigens into the circulation. In contrast, the role of serum IgA is less clear. It has been shown that monocytes and granulocytes express receptors binding the Fc portion of IgA and that this binding might activate the phagocytosis of bacteria and fungi[28]. One possible role of serum IgA is suggested to be to remove foreign antigens by the phagocytic system without inducing inflammation[29].

1.4.2 Selective IgA deficiency

sIgAD is the most common primary immunodeficiency in Sweden with an estimated prevalence of 1:600[8]. The prevalence varies between different ethnic groups and ranges from 1:328 in American blood donors[30] to 1:18 550 in Japanese blood donors[31]. The current definition, established by the Pan-American Group for Immunodeficiency and the European Society for Immunodeficiencies, defines sIgAD as serum IgA levels below 0.07 mg/ml with normal IgM and IgG levels in individuals of 4 years of age or older[32] (www.esid.org). As children younger than 4 years of age have a poorly developed IgA secretory system, it is difficult to establish a correct diagnosis.

sIgAD is a mild form of PID and most patients are asymptomatic. Nevertheless, one third of the adult patients suffer from recurrent infections of the upper respiratory tract, gastrointestinal disorders, autoimmune disorders, and possibly allergic diseases[33,34]. The infections are often viral and clinically mild to moderate, while bacterial infections are relatively uncommon[35]. The gastrointestinal diseases include giardiasis, nodular lymphoid hyperplasia, celiac disease, and inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Autoimmune disorders are also frequently seen in sIgAD patients and include rheumatoid arthritis, systemic lupus erythematosus, haemolytic anaemia, thyroiditis, diabetes type I, pernicious anaemia, dermatomyositis, chronic active hepatitis, Addison's disease, Sjögren's syndrome, and idiopathic thrombocytopenic purpura[28,35]. As sIgAD can be transferred between individuals through bone marrow transplantation, the disorder is regarded to be caused by a defect at the stem cell level[36]. Moreover, bone marrow transplantation from a normal donor to an IgA deficient recipient resulted in normal serum IgA levels in the recipient[37].

1.4.3 IgA deficiency as a common denominator in PIDs

Humoral primary immunodeficiencies account for more than 50% of all known forms of PIDs[4] and sIgAD is the most common form of PID in Caucasians. The low or absent serum IgA levels are also included in the phenotype of a vast majority of PIDs and can theoretically be used as a common denominator for neonatal PID screening.
Normal levels of serum IgA can be readily detected today by using conventional routine methods, such as enzyme-linked immunosorbent assay (ELISA) and nephelometry. These methods are however not suitable for the high-throughput design that is required for a national screening. When combining the commercially available and broadly used antibodies for detection of IgA and the reverse phase arrays, a platform may be created that meets the demands of cost- and labour-effectivity required. The IgA-based screening may serve as the first step in an investigation aiming

at identifying patients with specific immune defects that require early treatment. Further investigation would be needed to distinguish these patients from the patients with sIgAD, as the disorder, in most cases, does not require any specific treatment.

The serum microarray/DBSS approach may theoretically also be used for identification of individuals with defects in other parts of the immune system, such as the complement system. Complement deficiencies are often characterized by a lack of specific complement proteins, thus, by using antibodies directed against the proteins of interest, the same platform can be used to identify these individuals at birth.

1.5 THE READ THREAD

1.5.1 Evaluation of the serum microarrays

A basic requirement that needed to be fulfilled before any form of IgA-based screening can be evaluated, was to readily detect serum IgA after immobilizing serum samples directly on a substrate. It was also necessary to asses the specificity and sensitivity of the commercially available anti-IgA antibodies. Therefore, in study I, serum samples from adult individuals with known levels of serum IgA were used to investigate the potential of serum microarrays in detection of serum IgA. The reason for choosing adult serum samples was the relative abundance of serum IgA, thus enabling proper evaluation of the method and the anti-IgA antibodies used. The serum IgA levels in the included samples ranged from <0.05 to 4.9 mg/ml. The suitability of the serum microarrays was evaluated by correlation to the clinically measured serum IgA levels.

1.5.2 Application of serum microarrays to clinical samples

Very few studies have been conducted on the prevalence of IgA deficiency in children and adolescents. IgA levels are very low in newborns and increase steadily throughout the childhood until reaching adult levels during adolescents. In newborns, the serum IgA levels are <0.05 mg/ml, while the normal adult levels range from 0.9 to 4.5 mg/ml (reference values at the Laboratory of Clinical Immunology, Karolinska Hospital, Stockholm). Thus, although the prevalence of sIgAD in adults, primarily blood donors, has been extensively studied[8], the numbers cannot be automatically extrapolated to children and adolescents.
In study II, the serum microarrays were used to establish the prevalence of IgA deficiency in Swedish children at the age of four. The reported prevalence numbers in studies on healthy children[38-50] range from 1:90 in the Netherlands[38] to 1:2095 in Tunisia[50]. The IgA levels from serum microarrays were correlated to IgA levels obtained by sandwich ELISA. The IgA deficiency in Swedish children was also correlated to various health outcomes that are associated to allergy and infections.

1.5.3 Evaluation of serum microarrays for the analysis of C3 levels in dried blood spot samples

As a neonatal screening would require efficient elution of proteins from the DBSS as well as a reliable analysis, the focus of study III was the evaluation of elution

procedures and of the suitability of serum microarrays in analysis of DBSS. For evaluation purposes, a protein suitable for analysis should be as abundant in the blood of newborns as serum IgA is in adults. As the serum IgA levels in newborns are 1000 times lower than in adults, IgA is not a proper protein for evaluation of the method.

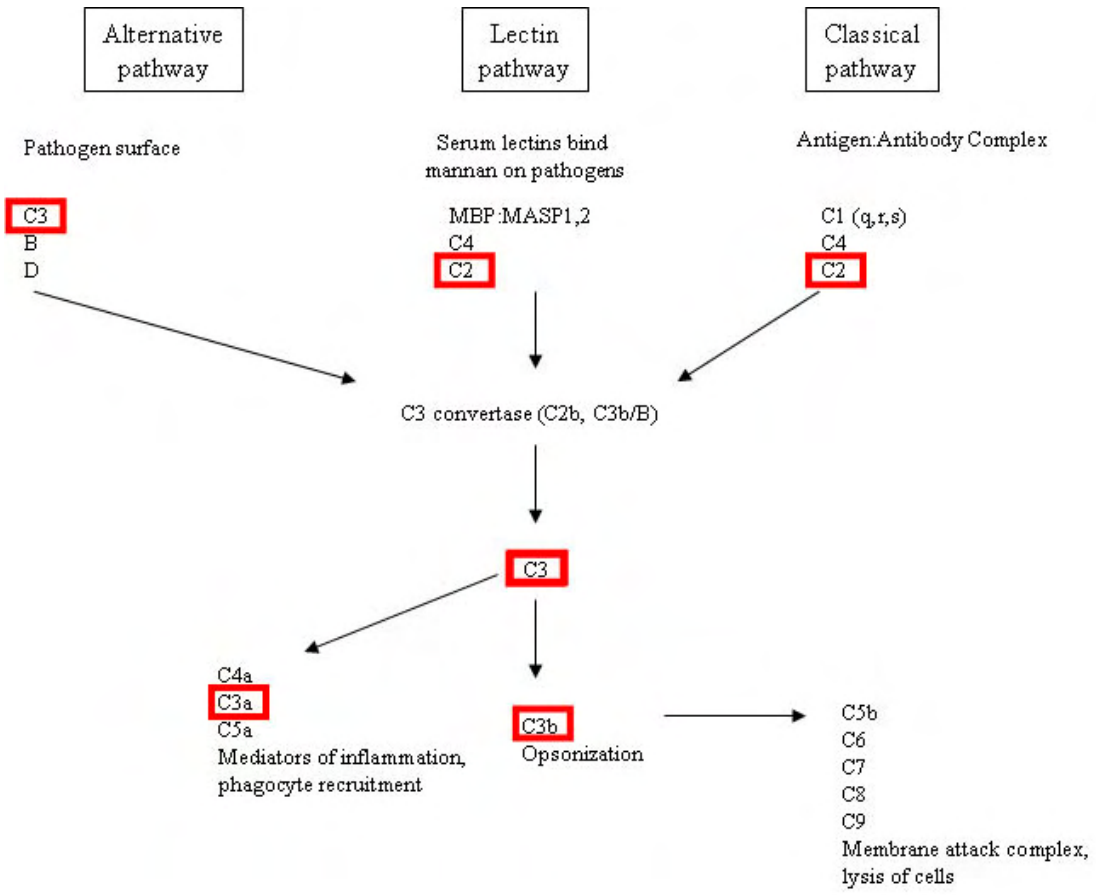


Figure 1. The complement system. The classical pathway is activated by binding of C1q to antibody-antigen complexes which results in the formation of the classical C3 convertase C4b2a. Activation of the lectin pathway by mannan-binding lectin or ficolins results in an identical classical C3 convertase. The alternative pathway is activated on pathogen surfaces that promote spontaneous hydrolysis of native C3, resulting in binding of factor B, generating the alternative pathway C3 convertase C3bBb. Recruitment of C3b molecules leads to formation of C5 convertase and initiation of the lytic pathway. Sequential assembly of C5b to C9 results in the formation of the membrane attack complex.

The third component of complement (C3) is the most abundant protein of the complement system, with a concentration of approximately 1 mg/ml in both adults and newborns[51], making it a suitable protein for the evaluation of the method. C3 plays an important role in the clearing of infections as it is involved in cytolysis, increased vasopermeability, opsonization, clearance of immune complexes and facilitation of B cell proliferation and differentiation. The component C3 plays an important role in all three activation pathways (figure 1) and is required for the formation of C3- and C5-convertases, both essential for the full activation of the complement system. Individuals lacking C3 are therefore highly susceptible to recurrent systemic bacterial infections

and frequently suffer from immune complex-related disorders, such as glomerulonephritis and systemic lupus erythematosus[52]. All patients reported to date have low (<0.07 mg/ml) or undetectable C3 levels. The deficiency is caused by mutations in the *C3* gene, resulting in absence/markedly reduced levels, or dysfunction of the protein[53]. To date, 23 families (31 individuals) have been identified[52,54-56] and ten different, unique mutations, have been found in the *C3* encoding gene[57-64]. Patients lacking C3 often develop life-threatening infections during the first year of life. Current complement analysis in clinical practice is restricted to cases where a patient's health status is brought to attention, often due to severe infections. Thus, an early diagnosis of complement deficiency may decrease the risk of acquiring severe infections during childhood by using prophylactic treatment.

1.5.4 Evaluation of serum microarrays for the analysis of C2 levels in dried blood spot samples

The next step in our evaluation was to increase the sensitivity of the serum microarrays/DBSS approach. For this, another target protein was required in study IV, one that is less abundant in the blood from newborns than C3.

C2 is important for the function of antibody-mediated phagocytosis and bactericidal activity[65], as cleavage of C2 leads to the formation of a C4b2a enzyme complex, the C3 convertase of the classical and the lectin pathway of the complement system[66] (figure 1). The normal concentration of serum C2 level is approximately 26 µg/ml[67] and is reached one month after birth[68]. In cord samples from newborns, the mean serum C2 levels are 87% of those of healthy adults[69]. C2 deficiency is one of the most common inherited complement component deficiencies in the Western world, with an estimated prevalence of 1:20 000 in European descendants[9]. In contrast, no C2 deficient individuals were found among 145 640 Japanese blood donors[70]. The C2 encoding gene is located between the genes coding HLA-B and complement factor B[71]. The most common type I C2 deficiency is characterized by lack of C2 synthesis, usually caused by a homozygous 28-bp deletion in exon six in the C2 gene of the MHC haplotype [HLA-B18, S042, DRB1*15]. Type II C2 deficiency is associated with some uncommon haplotypes and is caused by a selective block of C2 secretion[10,72].

Although many of the C2 deficient patients are asymptomatic, the disorder is often associated with systemic lupus erythematosus and other autoimmune disorders[65]. The incidence of invasive infection has been described to vary between 22-57%[73,74]. A majority of these infections are caused by encapsulated bacteria such as *S. pneumoniae, N. meningitides* and *H. influenzae*[65,75,76]. The treatment usually consists of prophylactic antibiotics treatment or vaccination against encapsulated bacteria and specific treatment of any associated disorders.

1.5.5 Lack of IgA as a diagnostic marker for humoral primary immunodeficiencies

In the last step, the focus of study V was to evaluate the potential of use of DBSS for determination of serum IgA levels in newborns and identification of patients with various forms of humoral primary immunodeficiencies.
Several studies have demonstrated the presence of IgA in the cord blood as well as the presence of B cells in the fetal tissues[77,78] and the cord blood[79-82]. These fetal B cells are regarded to be the source of the fetal IgA found in cord blood[78]. Therefore, the IgA detected in the blood of neonates should theoretically be of fetal origin and thus suitable as a reliable marker for the serum IgA status of the child.

2 AIM

2.1 GENERAL AIM

The aim of the investigations has been to develop a high-throughput method for detection of PIDs in newborns using DBSS.

2.2 SPECIFIC AIMS

1. Evaluation of serum microarrays as a suitable high-throughput platform for determination of serum protein levels in thousands of samples simultaneously.

2. Evaluation of serum microarrays as a suitable high-throughput platform for identification of individuals with IgA deficiency using serum samples, determination of prevalence of IgA deficiency in Swedish children and association of IgA deficiency in Swedish children to allergy- and infection-related health outcomes.

3. Evaluation of serum microarrays as a suitable platform for identification of patients with PIDs involving high abundance serum proteins using DBSS.

4. Evaluation of serum microarrays as a suitable platform for identification of patients with PIDs involving low abundance serum proteins using DBSS.

5. Evaluation of DBSS as suitable source of biological material for determination of serum IgA levels in newborns and identification of patients with primary immunodeficiency disorders.

3 PATIENTS AND METHODS

Details concerning all the different methods used can be found in the papers, I-V.

3.1 PATIENTS

In study I, 2009 serum samples with known IgA concentrations (determined using nephelometry at the department of Clinical Immunology, Karolinska Hospital, Huddinge, Sweden), were included. The samples had been referred from patients with a history of increased susceptibility to infections. 1191 samples had normal IgA levels (0.7–3.65 mg/ml), 153 had increased IgA levels, and 665 had low IgA levels including 99 IgA-deficient samples (<0.05 mg/ml).

For study II, serum samples from 2 423 4-year-old children were included. The children participated in a prospective study (the Children, Allergy, Milieu, Stockholm, Epidemiological survey [BAMSE]) in a predefined area of Stockholm. In addition, serum samples collected at the 8-year follow-up from the individuals lacking IgA at 4 years of age were tested using sandwich ELISA (n = 10).

In study III, serum samples from an adult Swedish C3 deficient patient[83] and twelve individuals with normal C3 levels were collected at the department of Clinical Chemistry, KUS Huddinge, Sweden. A serum sample from an adult Brazilian C3 deficient patient[84] was collected at Laboratory of Complement, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil and lyophilized. Before use, this C3 deficient serum was reconstituted in 100 µl of 1x phosphate buffered saline (PBS). In addition, neonatal DBSS from the Swedish C3 deficient patient (collected in 1985), 13 controls collected 1983–1998 and 256 freshly collected controls (2 dots, each 3 mm in diameter, corresponding to approximately 3 µl plasma in total) were obtained from the Center for Inherited Metabolic Diseases, Karolinska University Hospital Huddinge, Sweden.

In study IV, ten serum samples from C2 deficient patients and four serum samples from partially C2 deficient patients (heterozygous for a 28-bp deletion in exon six in the C2 gene) were collected at the Institute of Laboratory Medicine, Section of Microbiology, Immunology, University Hospital of Lund, Sweden. 21 serum samples from C2 sufficient controls were collected at the Division of Clinical Immunology, Karolinska Institutet, KUS Huddinge, Sweden. Four original DBSS from C2 deficient individuals (collected 1979-1988) and freshly collected DBSS (2 dots) from 100 controls were obtained from the Center for Inherited Metabolic Diseases, Karolinska University Hospital Huddinge, Sweden.

In study V, 115 DBSS were obtained from the Center for Inherited Metabolic Diseases, Karolinska University Hospital, Stockholm, Sweden (2 dots). The samples were divided into three groups depending on origin: Individuals with sufficient IgA levels when tested later in life (adult serum IgA levels of 0.5 mg/ml or more) (group I, n=51), patients with PIDs impairing humoral immunity, born to healthy mothers or mothers with sIgAD (group II, n=44), and individuals with normal adult serum IgA levels, born to IgA deficient mothers (group III, n=20). Group II includes 26 patients with sIgAD, 11 with severe combined immunodeficiency (RAG-1 deficient n=3, X-linked SCID (gamma chain gene mutation) n=3, Jak3 deficient n=1, 4 unknown), 3 with the hyper

IgM syndrome (HIGM) (CD40L mutations), and 4 with ataxia-telangiectasia (A-T). All PID patients in group II lack serum IgA at present or at time of preparation for hematopoietic stem cell transplantation (4-29 years of age).

3.2 GENERAL OVERVIEW OF THE SERUM MICROARRAYS USED FOR SERUM AND DBSS ANALYSIS

The serum microarrays were used in paper I-IV. Although with different target proteins and changes in procedure, depending on the aim of each study, the principle for the serum microarrays is the same (figure 2).

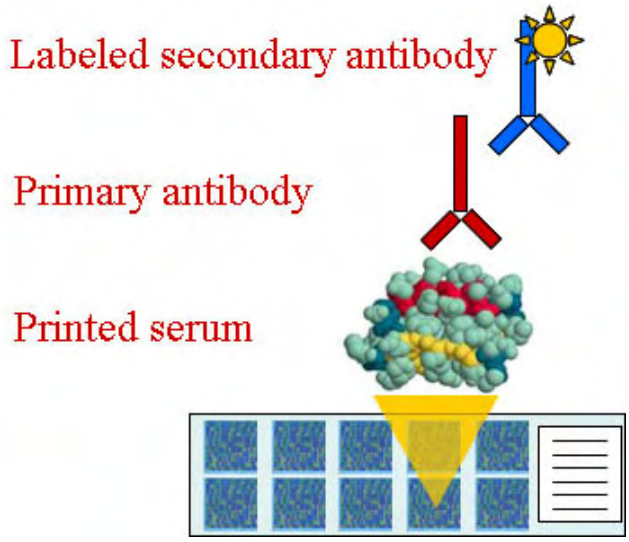


Figure 2. Schematics of serum microarrays (reverse phase protein microarrays). The bound serum is probed with antibodies for detection of antigens present in the sample. Detection is accomplished by a secondary antibody.

The first step of the procedure is the immobilization of the serum samples on a substrate, in our case, epoxy slides. The printing generates microspots, each microspot representing one serum sample. Usually, each serum sample is printed in replicates on each slide, thus being represented by several microspots. The serum samples are printed onto the slides using a printing robot (figure 3).

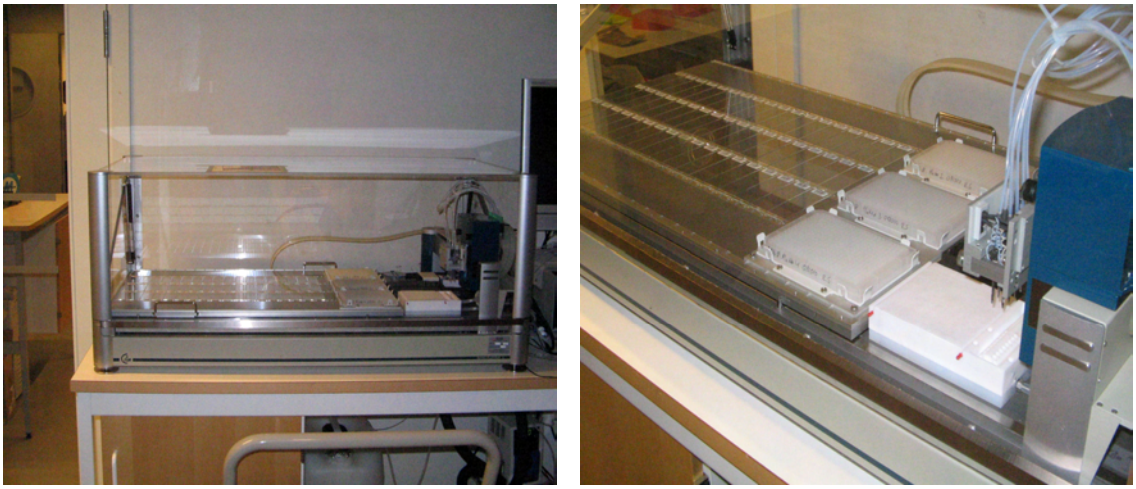


Figure 3. Printing robot Nano-plotter 2.0, Gesim, used for printing of all samples included in paper II-IV. The robot deposits approximately 0.4 nl/drop.

One slide can contain approximately 10 000 microspots, all of which are analyzed simultaneously. In the next step, the analyte of interest needs to be targeted using antibodies. The antibodies are added to the whole slide at the same time, and will bind to all microspots containing the analyte of interest. A consequence of this is that microspots lacking the analyte will not bind any of the primary antibodies. Subsequently, the slides are incubated with a secondary antibody that is directed against the primary antibody. The secondary antibody will thus bind only to microspots where the analyte of interest is present. As the secondary antibody is fluorescently labeled, a signal will appear in the microspots that contain the analyte (figure 4). The slides are then scanned for the intensity of the fluorescent emission from the bound labeled secondary antibodies (figure 5). The created files can then be used in an image analysis software to obtain the signal intensities for each microspot present on the slide.

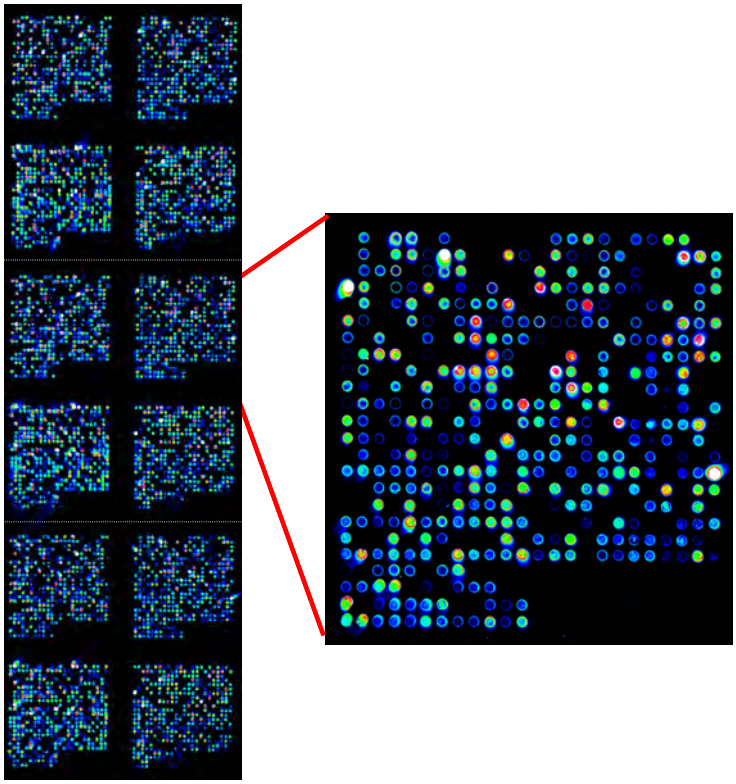


Figure 4. Visualization of the fluorescence intensities of the serum array with 3 x 2000 spotted serum samples. The enlarged area corresponds to ~500 patient samples.

3.3 OPTIMIZATION OF SERUM MICROARRAYS FOR ANALYSIS OF IGA LEVELS IN SERUM SAMPLES

As reverse phase protein microarrays had not been previously used for analysis of serum IgA levels by direct printing of serum, study I started with an optimization of the printing procedure. The system was initially optimized using three different serum samples with known IgA concentrations of 0.07, 0.20, and 1.04 mg/m respectively. The three samples were diluted in 10-fold steps 1:10-1:1,000,000 in 1x PBS and arrayed onto 25 x 75-mm SuperEpoxy slides (ArrayIt, Telechem) using an eight-pin and ring GMS 427 arrayer (Affymetrix). Twelve identical blocks were printed on each slide with each block containing all three samples in all dilutions. By using a 16-well adhesive silicone mask (Schleicher & Schuell BioSciences), the primary antibody (rabbit anti-

human IgA; DAKO; 4.6 mg/ml) was added to the slide in a dilution series (1:5,000, 1:10,000, 1:20,000, 1:40,000, 1:60,000, and 1:100,000). The labeled secondary antibody (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes; 2 mg/ml) was diluted in the same manner and added to the slides (one concentration per slide). The slides were scanned in a G2565BA array scanner (Agilent).

Figure 5. The array scanner G2565BA, Agilent, used for scanning all the samples included in paper I-IV.

3.4 EVALUATION OF ELUTION PROCEDURE FROM ORIGINAL DBSS

In study V, an evaluation of the elution procedures was performed using freshly collected blood, containing 1.5 mg/ml IgA. The blood was used to create several DBSS that were then eluted under various conditions (temperature and time of elution). Serum IgA levels were then determined by sandwich ELISA (as described below). The ratio between the IgA levels obtained after elution and the actual level of 1.5 mg/ml was then calculated. All DBSS in study II-V were subsequently eluted for one week at 4°C.

3.5 ELUTION OF PROTEINS FROM ORIGINAL DBSS

Elution of proteins from DBSS was performed in studies III-V.

Each DBSS is approximately 3 mm and contains approximately 1.5 µl plasma. One DBSS spot is approximately one fifth of one whole DBSS sample. Each whole DBSS sample contains approximately 15 µl of whole blood, which corresponds to approximately 7-8 µl of plasma. Therefore, as one fifth of the total plasma volume in the whole DBSS is approximately 1.5 µl, this corresponds to one DBSS spot with 3mm in diameter.
In study III and IV, the two DBSS spots per individual, containing approximately 3 µl of plasma in total, were soaked one week at 4°C in PBS to a final dilution of 1:50.

3.6 PREPARATION OF SIMULATED DBSS FROM A BRAZILIAN C3 DEFICIENT SERUM SAMPLE AND CONTROLS

In study III, simulated DBSS were prepared from the Brazilian serum sample, as no fresh blood or original DBSS were available. Twenty five µl of the blood cells from the adult Swedish C3D patient were added to 25 µl of the Brazilian serum sample. A volume of 25 µl (corresponding to ~ 13 µl of plasma) of the mixed sample was then transferred onto filter paper. The filter paper was diluted 1:50 in PBS with 0.5% Tween20 and soaked for one week at 4°C. The Swedish C3D serum sample and 0.9% NaCl, mixed with 25 µl of blood cells from the Swedish C3D patient, were used as negative controls and 9 serum samples with normal levels of C3 as positive controls.

3.7 DETERMINATION OF IGA, C3 AND IGG LEVELS IN SERUM SAMPLES AND DBSS BY SERUM MICROARRAYS

Serum microarrays were used for protein determination in studies I-IV.
Determination of IgA levels in serum samples using serum microarrays was performed in study I and II. Serum samples were diluted 1:10, the primary antibodies (rabbit anti-human IgA; DAKO) was diluted 1:100 000 to a final concentration of 46 ng/ml, and the labeled secondary antibodies (Alexa Fluor 555 goat anti-rabbit IgG; Molecular Probes) was diluted 1:60 000 to a final concentration of 33 ng/ml.
In study III, serum microarrays were used for determination of C3 levels in serum samples. After evaluation of signal intensities, the samples were diluted 1:100, the primary antibodies (rabbit anti-human C3c; DAKO, Denmark) were diluted 1:100 000 to a final concentration of 115 ng/ml, followed by the labeled secondary antibodies (Alexa Fluor 555 conjugated goat anti-rabbit IgG; Molecular Probes, USA) diluted 1:60 000 to a final concentration of 33 ng/ml. The samples were printed using GMS 427 arrayer (Affymetrix) in study I and Nano-plotter 2.0 (Gesim) in study II-IV.
The DBSS in study III were, similarily to the serum samples, diluted to 1:100 prior to serum microarray analysis. The primary antibodies (rabbit anti-human C3c; DAKO, Denmark) were diluted 1:500 to a final concentration of 2.3 µg/ml. The labeled secondary antibodies (Alexa Fluor 555 conjugated goat anti-rabbit IgG; Molecular Probes, USA) were diluted 1:2 000 to a final concentration of 1 µg/ml.
In some DBSS in study III, serum IgG levels were determined using serum microarrays. The primary antibodies (rabbit anti-human IgG; DAKO, Denmark) were diluted 1:100 000 to a final concentration of 83 ng/ml and the labeled secondary antibodies (Alexa Fluor 555 conjugated goat anti-rabbit IgG; Molecular Probes, USA) were diluted 1:60 000 to a final concentration of 33 ng/ml.
In study IV, six different anti-human C2 antibodies were evaluated for determination of C2 levels using microarrays: polyclonal rabbit anti-human C2 (4.9 mg/ml; a gift from professor L. Truedsson, Institute of Laboratory Medicine, Section of Microbiology, Immunology, University Hospital of Lund), a polyclonal sheep anti-human C2 (10 mg/ml; The Binding Site, UK), a polyclonal goat anti-human C2 ($\geq$ 20 mg/ml; Quidel, USA), a monoclonal mouse anti-human C2 HYB 050-04 (1 mg/ml; Antibodyshop, Denmark), a monoclonal mouse anti-human C2 HYB 050-05 (1 mg/ml; Antibodyshop, Denmark), a monoclonal mouse anti-human C2 HYB 050-08 (1 mg/ml; Antibodyshop, Denmark).

All six antibodies were tested using a dilution of 1:5000. Subsequently, secondary antibodies were added at a final concentration of 400 ng/ml (1:5000) (one per slide): Alexa Fluor 555 conjugated goat anti-rabbit IgG antibodies (Molecular Probes, USA), Alexa Fluor 555 conjugated goat anti-sheep IgG antibodies (Molecular Probes, USA), Alexa Fluor 555 conjugated goat anti-mouse IgG antibodies (Molecular Probes, USA), Alexa Fluor 555 conjugated rabbit anti-goat IgG antibodies (Molecular Probes, USA).

3.8 DETERMINATION OF IGA, IGG, C3, AND C2 LEVELS IN SERUM SAMPLES AND DBSS BY ELISA

ELISA was used for determination of protein levels in study II-V.
Serum IgA levels were determined by sandwich ELISA in study II, using rabbit anti-human IgA antibodies (DAKO, Denmark) at a final concentration of 1.2 mmg/ml and alkaline phosphatase-conjugated rabbit anti-human serum IgA antibodies (Jackson ImmunoResearch Laboratories, USA) at a final concentration of 0.6 mmg/ml. All samples were titrated against a six-fold serially diluted standard. p-Nitrophenyl phosphate (Sigma-Aldrich, USA) was added and the absorbance (405 nm) was read on a Vmax microplate reader (Molecular Devices, USA). A mean concentration was obtained for each sample using Deltasoft JV 1.8 (Biometallics Inc, USA).
In study II, the IgG levels of some individuals were determined by sandwich ELISA. Polyclonal rabbit anti-human IgG antibodies (DAKO, Denmark) and alkaline phosphatase-conjugated polyclonal rabbit anti-human IgG antibodies (DAKO, Denmark) were added at a final concentration of 0.6 mmg/ml and 1.1 mmg/ml respectively. The same protocol as for determination of serum IgA was followed.
In study III, levels of C3 were determined in serum samples using sandwich ELISA. Polyclonal rabbit anti-human C3c antibodies (DAKO, Denmark) at a final concentration of 3.8 µg/ml were used as primary antibodies and horseradish peroxidase-conjugated polyclonal goat anti-human C3c antibodies (Nordic Immunological Laboratories, the Netherlands) at a final concentration of 3.3µg/ml were used as secondary antibodies. The absorbance was read at 450 nm on a Vmax microplate reader (Molecular Devices, USA). The DBSS used in study III were tested for C3 levels using the same procedure as the serum samples. The same dilutions of samples and antibodies were applied.
In study IV, six different anti-human C2 antibodies were evaluated for determination of C2 levels using ELISA: polyclonal rabbit anti-human C2 (4.9 mg/ml; a gift from professor L. Truedsson, Institute of Laboratory Medicine, Section of Microbiology, Immunology, University Hospital of Lund), a polyclonal sheep anti-human C2 (10 mg/ml; The Binding Site, UK), a polyclonal goat anti-human C2 (≥ 20 mg/ml; Quidel, USA), a monoclonal mouse anti-human C2 HYB 050-04 (1 mg/ml; Antibodyshop, Denmark), a monoclonal mouse anti-human C2 HYB 050-05 (1 mg/ml; Antibodyshop, Denmark), a monoclonal mouse anti-human C2 HYB 050-08 (1 mg/ml; Antibodyshop, Denmark). All antibodies were tested on C2 sufficient serum samples and a C2 deficient negative control. All antibodies were tested as primary antibody (1:3 000) and as secondary antibodies (1:1 000). Subsequently, alkaline phosphatase-conjugated antibodies diluted 1:1000, directed against the respective secondary antibodies: alkaline phosphatase-conjugated donkey anti-sheep

IgG antibodies (Binding Site, UK), alkaline phosphatase-conjugated sheep anti-rabbit IgG antibodies (Sigma-Aldrich, USA), alkaline phosphatase-conjugated rabbit anti-mouse IgG (DakoCytomation, Denmark) or alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma-Aldrich, USA) were added. p-Nitrophenyl phosphate (Sigma-Aldrich, USA) was added and the absorbance was read on a Vmax microplate reader (Molecular Devices, USA).

After the evaluation in study IV, the C2 levels were determined in serum samples and DBSS by ELISA using polyclonal rabbit anti-human C2 antibodies (a gift from professor L. Truedsson, Institute of Laboratory Medicine, Section of Microbiology, Immunology, University Hospital of Lund, Sweden) added at a concentration of 1.6 mmg/ml (1:3000), sheep anti-human C2 antibodies (The Binding Site, UK) added at a concentration of 1 mmg/ml (1:1000), followed by alkaline phosphatase-conjugated donkey anti-sheep IgG antibodies (Binding Site, UK) diluted 1:1000.

In study V, IgA levels were determined in DBSS using the same primary and secondary antibodies as described for study II. Samples with non-detectable IgA levels were considered deficient. Subsequently, total serum IgG levels were determined in individuals with undetectable IgA levels at birth using sandwich ELISA. The same protocol as for study II was followed.

For study II-V, a mean concentration was obtained for each sample using Deltasoft JV 1.8 (Biometallics Inc, USA).

3.9 TOTAL PROTEIN CONTENT IN DBSS

In study III, an assessment of the protein composition was made in DBSS showing low levels of C3 and controls, using a SDS-polyacrylamide gradient gel (NuPAGE 4–12% Bis-Tris pre cast gel, Invitrogen, USA). MultiMark rainbow molecular weight marker (Invitrogen, USA) was used as a standard and the gel was stained with Coomassie Brilliant Blue.

3.10 DEFINITION OF ALLERGY- AND INFECTIONS-ASSOCIATED HEALTH OUTCOMES IN CHILDREN

In study II, allergy- and infection-associated health outcomes were correlated to IgA deficiency in young children. Data on parental allergy and various exposures were collected using a questionnaire when the children were 2 months old in average. At 1, 2, 4 and 8 years of age, the questionnaires focused on the children's allergic symptoms and possible confounding factors. Data on environmental factors including maternal age, maternal smoking habits, breast-feeding and parental allergies were collected from parent-answered questionnaires when the children were on average 2 months old. Questions regarding symptoms related to allergic diseases and infections in their children were answered by the parents at years 1, 2, 4 and 8. Pseudocroup, pneumonia, bronchitis and respiratory syncytial virus infection were parent reported and defined as doctor's diagnosis during the 12 months prior to answering the questionnaire. Parentally

reported pseudocroup is defined as a sudden spasm of the larynx that occurs in children and is marked by difficult breathing with prolonged noisy inspiration, normally caused by viral infection. *Parentally reported asthma* at years 1 and 2 was defined as at least 3 episodes of wheezing in combination with treatment with inhaled steroids or signs of hyperactivity[85]. At 4 and 8 years of age the definition included at least 4 episodes of wheezing in the last 12 months or at least 1 episode of wheezing combined with inhaled steroids treatment[86]. *Parentally reported eczema* was defined as dry skin and itchy rash continuously for at least 2 weeks in the previous 12 months with a typical distribution (face, outer limbs, folds of elbows/wrists, behind the knees, or fronts of ankles) and/or doctor's diagnosis[87]. *Parentally reported wheeze* was defined as at least 1 episode of wheezing in the last 12 months. *Sensitization to inhalant allergens* was defined as positive Phadiatop® (IgE-antibody levels ≥ 0.35 kU_A/l) against cat, dog, horse, birch, timothy, mugwort, *Dermatophagoides pteronyssinus*, or *Cladosporium* species. *Sensitization to food allergens* was defined as positive fx5® (IgE-antibody levels ≥ 0.3 kU_A/l) against cow's milk, hen's egg, codfish, soybean, peanut or wheat. *Parentally reported food hypersensitivity* was defined as parentally reported symptoms (wheeze, itchy eyes and/or running nose, facial oedema, urticaria, eczema or vomiting/diarrhoea) related to ingestion of a certain food[88].

3.11 DATA ANALYSIS OF SERUM MICROARRAYS

All slides were scanned by the G2565BA array scanner (Agilent, USA) and the image analysis in study I-IV was performed using GenePixPro 5.1 (Axon Instruments) with non-circular feature alignment. In study I, the median intensities were used to scale the array intensities *versus* the IgA concentrations previously measured by nephelometry. A scaling factor was calculated individually for each replicated set of samples as the ratio between the median of the array intensities with the median of the concentrations. In study II, the median intensity of each spot was averaged based on replicates and the correlation between the microarrays and ELISA was calculated using R console (www.r-project.org).

3.12 STATISTICAL ANALYSES

In study II, the χ^2 test was used for determination of differences in the distribution of selected characteristics and association with selected health outcomes among children with respect to their serum IgA levels, $p < 0.05$ was regarded as significant. The analysis were performed for children with IgA deficiency (<0.07 mg/ml), partial IgA deficiency (0.07-0.49 mg/ml) and normal IgA levels ($\geq$0.5 mg/ml). The statistical analyses were performed using Stata 9.0 (College Station, TX, USA).
In study III and IV, the calculations were performed by Microsoft Excel. In study V, the statistical analyses were performed using XLSTAT.

4 RESULTS

4.1 PAPER I

There is a great need for comprehensive proteomic analysis of large patient cohorts of plasma and serum samples to identify biomarkers of human diseases. In this paper, we describe a new antibody-based proteomic approach involving a reverse array format where serum samples are spotted on a microarray. This enables all samples to be screened for their content of a certain serum protein in a single experiment using target-recognizing antibodies and fluorescently labeled secondary antibodies. The procedure is illustrated with the analysis of the IgA levels in 2009 spotted serum samples, and the data are compared with clinical routine measurements. We could show that the present limit of detection in spotted complex serum is below 1 µg/ml, well below the IgA levels defining sIgAD. For the clinical samples, the median coefficient of viariation was 15% and only 10% for the samples with IgA concentrations above the deficiency range, indicating a satisfying robustness comparable to other methods. A high level of correlation was observed throughout the material.

4.2 PAPER II

Serum microarrays enable simultaneous analysis of thousands of samples and are suitable for high-throughput analysis. This platform generally generates semi-quantitative data but is not perfectly suited for absolute measurements. However, it is highly applicable to initial screening procedures for relative measurements of medium and highly abundant proteins in complex samples. The two methods were used simultaneously to investigate whether the IgAD samples identified by traditional ELISA were also identified by the serum microarrays. 14 children with IgAD were identified using ELISA, giving a prevalence one in 173. The fluorescent signals in the IgAD group when using serum microarrays (mean signal strength of 1600) were significantly lower ($p < 0.01$) than in the remaining sample set. Serum microarrays are therefore suitable for identification of IgA deficient patients. For association of IgA deficiency in Swedish children with allergy- and infection-related health outcomes, we found a significantly increased risk of parentally reported pseudocroup at year 1 and pseudocroup at 1 and/or 2 years of age compared to non-deficient children. For allergic diseases, IgAD children were found to have a significantly increased risk of parentally reported food hypersensitivity at 4 years of age. At 8 years of age there was a tendency to increased risk for food hypersensitivity ($p = 0.05$). The health outcomes did not differ significantly between children with partial IgA deficiency (0.07–0.49 mg/ml) and those with normal serum IgA levels.

4.3 PAPER III

DBSS from newborns are widely used in neonatal screening for selected metabolic diseases and diagnostic possibilities for additional disorders are continuously being evaluated. In this study, we investigated whether patients with C3 deficiency can be identified by the analysis of DBSS eluates using reverse phase serum microarrays. No C3 was detected in the DBSS eluate from the Swedish patient using serum microarrays.

In the control group, C3 was readily detected in all samples, with a correlation between the two methods of 0.49. The mean C3 level of the 13 stored controls was significantly lower than the mean level of the freshly collected controls. The levels of C3 in the control group was significantly higher than in the Swedish C3 deficient patient sample, indicating that serum mciroarrays are suitable for identification of C3 deficiency using DBSS eluates. Similar to the original DBSS of the Swedish patient, no C3 was detected in the Brazilian DBSS or negative controls, while all positive controls had detectable levels of C3, using serum microarrays. Six control DBSS showed consistently low intensities on serum microarrays but normal C3 levels on ELISA. A number of low molecular weight bands were noted in these samples, suggesting degradation of serum proteins. No such bands were seen in the eluates from the C3 deficient patient or the normal-intensity controls, suggesting that C3 is one of the degraded proteins in these six low-intensity samples.

4.4 PAPER IV

In this study, we investigated if DBSS collected at birth can be used for identification of subjects with C2D. C2 is found at much lower levels in newborns than C3. The criss-cross evaluation of six different anti-C2 antibodies for sandwich ELISA showed that the polyclonal rabbit anti-human C2 antibodies were the only antibodies suitable for coating. The polyclonal sheep anti-human C2 and polyclonal goat anti-human C2 were suitable as secondary antibodies. The evaluation of antibodies for serum microarrays showed that none of the tested antibodies are suitable for analysis; due to lack of distinction between patient and control samples.

Patient serum samples and controls were tested for C2 levels using sandwich ELISA. No C2 was detected in the sera of the deficient patients. In contrast, detectable levels were seen in all control samples and samples from partially deficient patients. The DBSS from C2 deficient patients and controls were also tested using sandwich ELISA. The levels of C2 in DBSS eluates of C2 deficient patients were significantly lower than in control samples.

4.5 PAPER V

Primary immunodeficiency disorders are caused by inherited defects of the immune system. There is a great need for a rapid and cost-effective neonatal screening to diagnose affected children early in life, thus improving the clinical outcome of the most severe forms of primary immunodeficiency disorders. Serum IgA were readily detected in all DBSS eluates in control group. In 16 of 44 PID patients, no IgA was detected at birth. In the remaining 28, the IgA levels were comparable to those of the controls. All 16 PID patients lacking IgA at birth are diagnosed with sIgAD and are born to sIgAD mothers. Ten sIgAD patients had detectable serum IgA levels at birth and all ten were born to IgA sufficient mothers. Among 20 children with normal IgA levels at follow up (1-16 years), born to sIgAD mothers, 10 lacked serum IgA at birth.

5 DISCUSSION AND FUTURE PERSPECTIVES

5.1 THE POTENTIAL OF SERUM MICROARRAYS IN LARGE SCALE SCREENING OF PROTEIN LEVELS

Today, a majority of PID patients are not diagnosed until brought to attention due to recurrent and/or severe infections. A majority of the individuals would benefit from prophylactic treatment with antibiotics and/or immunoglobulins or in more severe cases, hematopoietic stem cell transplantation. Children with more severe forms of PIDs, such as SCID and HIGM, risk a fatal outcome if left undiagnosed or diagnosed late, as many of them acquire severe, life-threatening infections during the first year(s) of life. One of the reasons why a majority of individuals are left undiagnosed is the lack of high-throughput methods for analysis. The potential of a reverse phase protein microarray system with spotted serum samples is huge, as there is an urgent need for sensitive and robust methods for large scale screening of protein levels in serum. During this project, we investigated the possibility of using serum microarrays for detection and semi-quantification of specific serum proteins associated with certain forms of PIDs.

We believe that an effective screening that identifies a majority of PID patients should involve the use of a single analytical platform. Therefore, a common denominator in a majority of individuals with PID needs to be identified. As IgA deficiency is included not only in the phenotype of most antibody deficiencies, but also in a majority of other forms of severe primary immunodeficiencies, determination of serum IgA levels could potentially serve as a basis for such a screening. It should, however, be noted that in some forms of SCID such as ADA deficiency, RAG1/2 defects and complete DiGeorge syndrome, a substantial part of the patients do not exhibit IgA deficiency during the first year of life. Therefore, these patients will not be identified using the proposed approach. It should also be emphasized that although the screening will result in identification of patients with selective IgA deficiency, these patients are not included in the target group for this screening as selective IgA deficiency is a mild form of PID. Thus the screening would constitute a first step in the evaluation of the patients, followed by in-depth analysis to identify the specific defect.

In paper I, we could show that serum IgA can be readily detected in 6 000 microspots on one single array slide and the capacity for simultaneous analysis of a very large number of serum samples in an array format can be extended beyond those 6000 microspots to at least double the amount. This means that an even further optimized system has the potential to allow large scale screening of huge clinical sample collections.

One of the limiting parameters in serum microarrays is the theoretical sensitivity, originating from the small number of molecules transferred to the arrays during printing. Only 0.4 nl of a complex sample is transferred to a slide per microspot. Thus since only a fraction of the proteins present are available for detection, it is inherent in the technology that low-abundant proteins will be difficult to detect without proper signal amplification. With the present setup, the limit of detection for C3 in serum is

approximately 500 ng/ml, and 1 µg/ml for serum IgA. The sensitivity of the arrays could be increased by using undiluted or concentrated samples or with higher concentrations of primary and secondary antibodies. Yet other approaches may include the use of sera depleted of albumin, IgG, and other highly abundant serum proteins, thus the sensitivity would potentially increase at least one magnitude. The main issue would then be to develop an automated and robust sample preparation procedure. Alternative detection systems such as those based on thin film planar waveguides[89] and various signal amplification procedures would also increase the sensitivity of the serum microarrays. The printing of the microspots results in immobilization of the serum samples. When immobilizing complex samples, a relevant concern is always that of all proteins being kept constant compared to in solution. One approach is then to use epoxide, which can bind proteins covalently in several different ways, utilizing both primary amines and hydroxyl- and thiol-based attachment. This general binding will reduce the risk of bias when binding proteins. Although further tests are needed to determine the proportion between bound and applied proteins, we found no indications in our studies that it would pose a problem in our analyses. Also if the immobilization efficiency is equal for all the samples, it will possible to handle it by proper data normalization.

The overall median coefficient of variation of the serum microarrays for detection of serum IgA in serum samples was 15% and only 10% for the samples above the deficiency range. This indicates a satisfying robustness comparable to other methods as the average coefficient of variation for nephelometry is 5-10% and antibody-based immunoassays in general have an average coefficient of variation of 10–15%. Nephelometry is a routine fluid phase immunoprecipitation technique currently used for quantification of serum proteins. The samples with the highest deviation between the concentrations determined by nephelometry and the array results have in general either a very low concentration or a significantly higher variation between replicates. There are no obvious reasons for the high variation and thereby high deviation for some samples. Further investigations are needed to clarify whether the compositions of those samples deviate from the average. To determine concentrations in subsequent studies where the concentrations are unknown, there will be a need to establish proper standard curves and also to introduce a quality filtering based on variance.

Compared to nephelometry and the radial immunodiffusion method used for low level analysis, two techniques that are not well suited for large scale screening due to the cost and work-load, the serum microarrays offer several major improvements. Only a few microliters, depending on spotting equipment, of serum is adequate for hundreds of microarrays, and each slide can then be used to screen thousands of samples for the content of a specific serum protein. Furthermore the reagent cost is drastically reduced because of the very large amount of samples analyzed simultaneously.

In study II, the serum microarrays were used to successfully identify 14 individuals lacking serum IgA. The findings were confirmed by sandwich ELISA. Therefore, it would be theoretically possible to screen thousands of samples and subsequently quantify serum IgA levels in individuals with the lowest intensities obtained on serum microarrays, using ELISA.

5.2 THE USE OF DBSS IN NEONATAL PID SCREENING

The DBSS are normally collected within 72 hours after birth and can be stored for many years. In study III, IV and V, we used such stored DBSS for determination of C3, C2 and IgA levels. One concern when using old samples is that the long-time storage might result in a degradation of the proteins. The patient DBSS used for study III had been stored for 21 years and for study V for 2 to 28 years prior to analysis. Therefore, to exclude that the lack of the target protein in the patient samples is due to degradation, we included control DBSS that had been stored for an equal amount of time. When analyzing the long-time stored DBSS controls in study III, we could detect C3 in all samples, thus indicating that the lack of C3 in the patient DBSS is not a result of protein degradation. The undetectable C3 levels in the freshly collected serum sample from the C3 deficient patient also support this notion as well as the absence of low molecular weight bands (indicating degradation) when running the SDS-PAGE.
In study IV, we could show that the levels of C2 are significantly lower in patient samples than control samples when using sandwich ELISA. This indicates that the DBSS might be suitable for identification of patients with C2 deficiency already at birth. So far, we have, however, been unsuccessful in finding an anti-C2 antibody that enables us to discriminate between patient samples and controls when using serum microarrays. Only a handful of commercial anti-human C2 antibodies are available to date. Three of the tested antibodies, all polyclonal, were suitable for sandwich ELISA using the current protocol. In contrast, mone of the monoclonal antibodies showed distinction between patients and controls, which might be due to low amount of serum C2 in the samples combined with the antibodies binding one single epitope. Furthermore, none of the tested antibodies could differentiate between patient and control samples using the serum microarrays. It is not clear why some antibodies fail in the microarray settings but it may depend on production method, storage buffers, storage procedures or other factors.

In study III, higher concentrations of primary and secondary antibodies were required for the serum microarray analysis of the DBSS than for the analysis of the serum samples. As the composition of the DBSS eluates differs from that of the serum samples in terms of total protein content and viscosity, these parameters may be important for the piezo-electric printing procedure and might thus influence the amount of the sample that is actually transferred to the slide.

A fundamental question that needs to be addressed before an IgA based assay can be tested in newborns, is the origin of the serum IgA found in the DBSS. Studies have shown the presence of B cells in fetal tissues[77,78] as well as in the cord blood[79-82], that have been proposed to be the source of serum IgA found in the cord blood[78]. If the serum IgA found in the cord blood would be only of fetal origin, a lack of detectable IgA at birth would reflect the inability of the child to produce IgA. This would then enable us to identify children with defective IgA production.

In study V, we found that 16 out of 44 PID patients lacked IgA at birth. All 16 are today diagnosed with selective IgAD, thus all of the patients with severe forms of PIDs had detectable serum IgA levels at birth. We found that only patients born to sIgAD

mothers lacked IgA at birth, while patients born to mothers with normal IgA levels had detectable amounts of IgA at birth. The lack of IgA in the fetal samples does not seem to be caused by maternal anti-IgA antibodies, as no such association was seen in our samples. Based on our findings, we propose that there is a hitherto not fully appreciated transport/diffusion of IgA from mother to child during pregnancy. This notion is supported by several previous studies suggesting a possible transfer of IgA across the placenta[90-93]. Malek and colleagues[91] investigated the transport of various proteins across human placenta by comparing the concentrations in maternal and fetal blood. A correlation was seen between fetal and maternal levels of serum IgA (0.66), suggesting that diffusional transfer does take place. Gurevich and colleagues[92] reported that precursor endocrine cells of 4-7 weeks old embryos were weakly positive for IgA but IgA-secreting lymphocytes did not appear until after 10-11 weeks of pregnancy, again suggesting a transplacental transfer. Similarly, Ben-Hur and colleagues[93] found that the components of the placental barrier were positive for IgA in 3.5-8 weeks old embryos, but lymphocytes were first seen after 9-11 weeks. These findings suggest that maternal IgA might be transferred to the embryo during the early prenatal period. However, the Am2 allotype has been shown to be present in the cord blood of some children even though the mother lacks this antigen, thus indicating a fetal origin of at least some of the neonatal IgA[90].

A matter that needs to be discussed in the current setting is also the specificity of the rabbit anti-human IgA antibodies that are used for detection of IgA in the DBSS eluates. We believe that the perfect overlap of lack of IgA in PID babies and maternal sIgAD validates the specificity of the anti-human IgA antibodies used. If the antibodies would have bound unspecifically to any maternal or fetal anti-rabbit antibodies of isotypes other than IgA, we would expect a more random distribution of our findings.

There is a possibility that the serum IgA detected in the present study might be due to microbreaks in the maternofetal barrier, leading to low levels of IgA contamination. However, 10 out of 20 IgA sufficient children born to sIgAD mothers (group III) had detectable serum IgA levels at birth, suggesting that both the mother and the fetus contribute to the total serum IgA detected in neonatal blood. We thus conclude that a screening for PIDs, based on serum IgA levels in neonatal blood is not possible, due to a transplacental transfer of maternal IgA.

5.3 THE NEED OF HIGH-THROUGHPUT DIAGNOSTIC TOOLS FOR PID - EXAMPLES OF NEW APPROACHES

As there is a great need for a high-throughput screening for PIDs, new approaches are currently being developed. In a recent study by Ingvarsson et al, 2007, sandwich antibody microarrays were used for multiplex analysis of eight complement proteins (including C3) in unfractioned serum and plasma[94], showing promising results for protein profiling in complex samples. However, the inherent format of the sandwich antibody arrays rules out the simultaneous screening of large numbers of samples that is required for population screening.

The most promising approach to date is a novel neonatal screening for SCID[6,95,96] that was presented in 2005. The suggested technique is based on quantitation of T-cell receptor excision circles (TRECs) that are generated during the maturation of T cells[97]. The known gene mutations result in a combined cellular and humoral immunodeficiency and most SCID patients have no, or very few, T cells. Therefore, only trace amounts of TRECs can be found in their blood. Recently, a study presented further optimization of the method, by enhancing the yield of DNA extraction and increasing the amplification efficiency, as well as showing a substantially lower rate of false positives[98]. In 2004, SCID was found to meet the criteria for inclusion in newborn screening. In January 2007, a pilot study aiming at developing a neonatal screening test for SCID was launched as collaboration between the Jeffrey Modell Foundation and the Wisconsin State Laboratory of Hygiene and Children's Hospital of Wisconsin and in January 2008, SCID was added to the neonatal screening program in the state of Wisconsin. It is anticipated that several additional states will join the pilot screening program. Criteria include infants who are asymptomatic at birth, serious medical consequences without treatment, availability of confirmatory tests and effective treatment, and improved outcomes with early intervention.The limitation of this approach is its dependence on lack of TRECs, thus focusing on a small part of patients with PIDs.

5.4 PREVALENCE OF SIGAD IN SWEDISH CHILDREN AND ITS ASSOCIATION TO ALLERGIC DISEASES AND INFECTIONS

In study II, we identified 14 sIgAD subjects among 2423 Swedish children, giving a prevalence of 1:173. As the prevalence of sIgAD in the adult Swedish population is approximately 1:600[8], it might suggest a dynamic process with the deficiency being more common among children due to their yet not fully mature IgA synthesis system, but an actual increase in prevalence cannot be ruled out. A majority of the previous studies have reported prevalence numbers ranging from 1:90 to 1:509, thus showing the same tendency to a higher prevalence in children than in adults. However, because of the differences in the definition of sIgAD, such as age of the subjects and the cut-off for serum IgA levels, it is difficult to compare the results from the different studies. In a majority of the paediatric studies, the age range of the children is broad and often no information is available regarding its distribution. This is of importance as the prevalence numbers will probably differ between very young children and adolescents.
In study II, we have a very homogenous study population in respect to age, as the samples were collected within a range of a few months prior to or after their 4th birthday.

We found that the IgA deficiency in our cohort of Swedish children (study II) is significantly associated to pseudocroup and food hypersensitivity. As the significant association to pseudocroup is seen during early childhood, we believe that it strengthens the assumed infectious aetiology in these children. Alternatively, the IgA deficient children may be more prone to developing pseudocroup as a result of respiratory infections without a general increase in susceptibility. Pseudocroup is caused by viral infections (mainly parainfluenzae) while e.g. pneumonia and otitis (health outcomes included in study II, showing no significant association) are mainly

caused by bacterial infections. As reported previously, the infections in IgA deficient individuals are often viral and clinically mild to moderate, while bacterial infections are relatively uncommon[35]. The association to pseudocroup early in life, and lack thereof later on, might be due to the set-in of compensatory mechanisms. Age-related compensatory mechanisms have previously been reported in patients with other forms of primary immunodeficiency disorders, such as C3 deficiency[58,99] where individuals prone to infections during childhood became symptom-free adults. As the association to pseudocroup at 1 and/or 2 years of age is significant, it strengthens the finding of an increased risk in early childhood. The combined analysis at 1 and/or 2 years of age also increased the number of affected children and correspondingly the power.

As for the association to food hypersensitivity, symptoms from the GI tract are a common feature of symptomatic IgAD patients. This made us suspect, when deciding on which health outcomes to focus on, that allergic manifestations of the GI tract would be predominant. Other allergy-related variables were chosen on the basis of previously reported associations. We believe that the significantly increased risk of food hypersensitivity seen in our cohort might indicate an association between IgA deficiency in childhood and allergic manifestation in the gastro-intestinal tract. Furthermore, although not significant, there is a trend of increased risk for food hypersensitivity at all ages, thus strengthening the assumption of a real association. As we did not find any association between sIgAD and increased levels of specific IgE, it suggests that the increased risk for food hypersensitivity in sIgAD children is not IgE-mediated. It is important to point out that the lack of association to other infection- or allergy-related health outcomes does not necessarily exclude an association, due to the small number of deficient children. The study nevertheless yielded significant results, suggesting that the associations are of clinical relevance. As food hypersensitivity in this study is parentally reported might be a limitation as bias may be introduced into the results. However, similar definitions have been used in other population based studies[88,100-102], and there is a statistically significant difference in prevalence of parentally reported food hypersensitivity between children with, and without sIgAD.

The association of allergic diseases and sIgAD is still under discussion. Some studies have reported an increased frequency of allergic diseases in IgA deficient patients[103,104] while other show no increased frequencies of allergic diseases[105-109] compared to the general population[110]. However, when discussing allergic diseases and sIgAD, it should be noted that allergies are clinically heterogeneous and may have different underlying mechanisms depending on factors such as heredity and age[111]. It is thus possible that transient sIgAD, IgA deficiency that disappears with age, may predispose children to allergies during certain periods of time, as suggested by several studies[112,113]. Many studies have shown that a fair proportion of the paediatric IgAD cases are transient[42,114-121]. A study on normalization of IgA levels in deficient children showed that 6 out of 18 subjects with total IgAD normalized their IgA levels with time[119]. In contrast, Plebani et al., reported that out of 40 children with total IgAD, none had reached normal serum IgA levels at follow-up 1.5 to 9 years later[120] nor had any of the 36 children with total IgAD that were followed up 5 years later by de Laat et al[116].Among the ten IgA deicient children with serum samples available at 8 years of age in study II, we found one subject with a normalized IgA level, while four remained partially deficient.

There are some issues regarding a large scale screening that do require consideration. One of them is the fact that a screening would result in identification of individuals with partial IgA deficiency. However, when comparing children with partial IgAD to those with normal IgA levels, we noted no significant differences, suggesting that the impaired health outcomes identified in this study are limited to children with a total lack of serum IgA. Based on these results, we propose that partial IgA deficiency seems to be clinically irrelevant, as the present findings suggest no significant difference in clinical findings when compared to children with normal levels of serum IgA.

5.5 FUTURE PERSPECTIVES

The idea of a neonatal IgA screening was born based on two assumptions, that IgA deficiency is included in the phenotype of various PIDs and that the IgA detected DBSS eluates is of fetal origin. As we could show in study V, the latter assumption was not correct and much of the detected IgA is maternal. Thus, an IgA screening will not give us any useful information on the IgA status of the child.

However, we believe that the microarray technology has great potential in the field of PID-diagnostics and new approaches should be evaluated. We are currently investigating the possibility of a multi-analyte antibody suspension bead array system[122] to pin-point specific defects in PID patients. In a first trial we will use 71 antibodies targeting 63 unique proteins that are involved/potentially involved in PIDs. The tests will be performed on serum samples from patients with various forms of PIDs as well as controls. Preliminary results indicate a potential to explore such diseases as CVID, HIGM, leukocyte adhesion defect, SCID and A-T. By using the antibody suspension bead arrays, multiple samples can be analyzed simultaneously and each sample is tested for a variety of proteins. Before large-scale screenings are possible however, the sensitivity issues need to be further addressed. Moreover, new antibodies towards potential targets are continuously being generated and implemented. In a future assay system, multiple serum samples will be analyzed on up to 100 proteins in parallel, enabling screening for most PIDs simultaneously.

While the serum microarrays are utilizing a two-dimensional arrangement of the samples on epoxy slides, the antibody suspension bead arrays utilize spectrally distinguishable beads to form an array consisting of a set of currently up to 100 bead signatures. In suspension, the beads with different color codes and different immobilized capturing molecules are mixed. Thus, each color coded bead is coupled to one antibody targeting one of the proteins of interest. The serum samples are then biotin-labeled and incubated with the mix of color coded beads. For detection purposes, R-Phycoerythrin-labeled streptavidin is added and a signal intensity value is obtained for each antibody in the mixture. The current set-up utilizes the 96-well-plate-platform, where one serum sample is added per well and enables the analysis of more than 200 serum samples within less than two days[122].

6 CONCLUSIONS

The aim of this thesis has been to develop a high-throughput method for detection of PIDs in newborns using DBSS. The conclusions drawn from the individual papers in this thesis are:

1. The microarray format has a potential to become an important tool for serum screening. If the serum microarrays are designed to contain sera from patients with certain diseases as well as normal sera and are combined with a program for high throughput generation of antibodies, great possibilities arise for large scale biomarker discovery strategies (paper I).

2. The serum microarrays have been shown to be suitable for identification of IgA deficient individuals using clinical samples. We also show that the prevalence of IgAD in Swedish children at 4 years of age is higher than in adults and the IgA deficient children seem to have an increased risk of pseudocroup early in life and may be at a higher risk of having concurrent allergic manifestations (paper II).

3. The serum microarrays were successfully used to distinct patients with C3 deficiency from C3 sufficient controls using dried blood spot samples. The findings suggest that patients with deficiencies of specific high abundance serum proteins can be identified by analysis of DBSS using reverse phase protein microarrays (paper III).

4. Six different anti-human C2 antibodies were tested for C2-binding in sandwich ELISA and serum microarrays. Three polyclonal antibodies were suitable for detection of C2 proteins using sandwich ELISA and a clear distinction was noted between patients and controls. None of the tested antibodies was suitable for identification of C2 deficiency using serum microarrays. The findings suggest that C2 deficiency can be detected already at birth using dried blood spot samples but the anti-C2 antibodies available to date are not suitable for identification of C2 deficient patients using serum microarrays. Therefore, additional anti-human C2 antibodies need to be tested for their applicability in a high-throughput serum microarray setting (paper IV).

5. In our cohort, the mother is the major contributor of IgA in the DBSS eluates. We thus conclude that a screening for PIDs, based on serum IgA levels in neonatal blood is not possible, due to a transplacental transfer of maternal IgA (paper V).

7 POPULAR SCIENCE SUMMARY

The immune system is made up of special cells, proteins, tissues, and organs, defends people against germs and microorganisms every day. In most cases, the immune system manages to keep us healthy and prevents infections. Sometimes, however, problems with the immune system can lead to illness and infection. Immunodeficiencies occur when a part of the immune system is not working properly. Sometimes a person is born with an immunodeficiency — these are called primary immunodeficiencies, although the symptoms may not show up until later in life. There are different types of primary immunodeficiencies and they affect different parts of the immune system. IgA deficiency is the most common immunodeficiency disorder. IgA is an immunoglobulin that is found primarily in the saliva and other body fluids that help guard the entrances to the body. IgA deficiency is a disorder in which the body does not produce enough of the antibody IgA. People with IgA deficiency tend to have allergies or get more colds and other respiratory infections, but the condition is usually not severe. One of the most severe forms of primary immunodeficiency is called Severe combined immunodeficiency (SCID), which is also known as the "bubble boy disease" after a Texas boy with SCID who lived in a germ-free plastic bubble. SCID is a serious immune system disorder which makes it almost impossible to fight infections.
Some forms of primary immunodeficiencies are very rare, while others are more common. In the USA more than 600 000 individuals are affected by some form of primary immunodeficiency. Despite the large number of affected individuals, a majority of them are still undiagnosed. Most of the patients come in contact with the health care first after acquiring complications because of their defective immune system. In some cases, these complications seriously compromise the patient's health and might be life-threatening. Because of this, there is a huge need for more effective ways to find individuals with primary immunodeficiencies before they acquire these complications, as an early diagnosis would enable appropriate treatment.

In many forms of primary immunodeficiencies, the patients lack IgA. That means, that by measuring the levels of IgA, you can theoretically find out which individuals have some kind of primary immunodeficiency. By measuring the IgA levels in a whole population very early on, we could find many of the individuals that have a primary immunodeficiency but have not acquired any complications yet. This would have to be done on newborns, as some of the defects cause severe injury very early in life. Today, all newborns in Sweden are tested for a handful of very severe disorders. This is done by collecting blood from the newborns and drying it on a filter paper that is later used for the analysis. We believe that these so called dried blood spot samples (DBSS) can also be used for determination of IgA levels in the newborns. Furthermore, as a screening of this sort would be done on ten-thousands of children every year, we need a method to determine the IgA that is very efficient, fast and inexpensive. A method of our choice is called serum microarrays and in this thesis, we have evaluated the possibility to use serum microarrays in determination of IgA levels using DBSS.

In paper I, we used serum microarrays to determine the levels of IgA in serum from adults. The IgA levels had been tested previously using a routine method and then we compared those results to the ones we obtained with serum arrays. The results were

very similar using the two methods and it was concluded that serum microarrays can be used for detection of IgA. This was important to check as no one had ever used serum microarrays in the way we used it in this study.
In paper II, we used the serum microarrays to try to find IgA deficiencies in a set of more than 2 000 Swedish children, to get more information on how common the IgA deficiency is in young people. We also wanted to investigate if the children with IgA deficiency have more infections and allergies than normal. The results showed that serum microarrays can be used to identify individuals with IgA deficiency and that the disorder is more common in children than in adults. Also, these children did have an increased risk of certain infections and of food-related allergies during childhood.

In paper I and II, the tests were performed using serum samples that had been collected when the individuals were a couple of years old or adult. The reason is that the amounts of IgA are increasing with age and there is a lot of IgA in these serum samples. However, as mentioned, a screening for primary immunodeficiencies has to be done very early in life and therefore we want to use DBSS. Serum samples are quite sticky while DBSS are very "watery", as they are eluted using the PBS buffer. This may be important for the serum microarrays, and we wanted to investigate if this difference does affect the results. To do this, we needed to measure a protein that is very abundant in DBSS. As the levels of IgA are extremely low in newborns, we chose to test the DBSS for a different protein, namely C3. In paper III, the levels of C3 were measured in patients that we know lack C3 as well as in individuals with normal levels of C3. The results showed that serum microarrays detected C3 in all DBSS from individuals with normal C3 levels but they did not detect any C3 in the patient samples. This shows that serum microarrays can be used for detection of proteins in DBSS.

In paper IV, we wanted to see if serum microarrays can detect proteins that are not as common in newborns as C3. We chose a protein called C2 and tested different ways of detecting it using serum microarrays. The results showed that the approaches currently to detect this specific protein using serum microarrays are not working properly, as we could not tell which of the samples that belonged to patients lacking C2 and which belonged to individuals with normal C2 levels. Still, we showed that C2 deficiency is present already at birth.

In paper V, we focused on the determination of IgA levels in DBSS. We wanted to see if the very low levels of IgA in DBSS can be detected using routine methods and if patients with various forms of primary immunodeficiencies, all lacking IgA as adults, lacked IgA as newborns. We found that only some patients lacked IgA at birth, while the rest had the same levels of IgA as healthy individuals. The only patients lacking IgA at birth were those born to mothers that lacked IgA. This led us to the conclusion that some of the IgA in the DBSS of the newborn children is actually produced by the mother and not the child. Therefore, it indicates that a screening for primary immunodeficiencies based on levels of IgA in the child is not possible, as we not only measure the IgA of the child but also that of the mother.

8 ACKNOWLEDGEMENTS

So then, this is the end of this journey. Along the way, I have had the pleasure to meet some amazing, interesting people whom I would like to thank for helping me in one way or another.

First and foremost, I would like to thank all the participants who took part in my research.

My supervisor, **Lennart Hammarström,**
I am truly grateful for everything you have taught me during these six years. From the first encounter, when you showed me "my" working place after talking to me for less than one hour, you have inspired me to follow my heart. You have taught me what it means to be passionate about ones work.

My co-supervisor, **Pan Qiang-Hammarström,**
For all the help with my manuscripts, your advices have been invaluable. I have great respect for your knowledge and your instinctive feel for research. I hope you will continue your important work in science, because science needs strong women like you.

My co-supervisor, **Sven Arne Silfverdal,**
Thank you for your good advice and supervision.

Collaborators:

Peter Nilsson, for your expertise and tons and tons of help on paper I-IV. Without you, this thesis would have never happened.

Ronald Sjöberg, Maja Neiman, Jinghong Wan and **Mårten Sundberg,** for all the hours of planning and printing and analyzing the samples in paper I-IV. **Maja**, a big thank you for helping me with the thesis! Good luck guys!

Inger Kull, Erik Melén and **Magnus Wickman** for the great support, enthusiasm and knowledge that made it possible to complete the wonderful BAMSE-paper. It taught a lot about a whole new field. Also, a BIG thank you for all the encouragement when things were falling apart, it helped a lot.

Eva Hallner and **Stina Gustavsson,** for helping me with the BAMSE-samples, TWICE! It is always a pleasure to work with you and hear more about golf, sailing, grandchildren and trips to France!

Ulrika von Döbeln, for the expertise within the field of neonatal screening.

Lennart Truedsson and **Göran Jönsson,** for the expertise within the complement field, great collaboration and for answering all my questions…hundreds of them.

Present and former members of the Hammarström-group:

Anna, for all those great chats about science, work, love, future. I have greatly enjoyed your company and really appreciate all you taught me about the life during and beyond the PhD-studies.

Neha, for all the good times laughing and talking about life. Thank you for your help with preparation of the thesis, everything you do is prepared with such care.

Naradja, thank you for your warm heart and beautiful laughter. You are such an amazing woman, I learned a lot from you about fighting for what you believe in. I wish you a life full of beautiful moments and I will keep my eyes open for your first record!

Harold, Bea, Kasper, Ashwin, thank you all for giggling and laughing your way through the day, your enthusiasm makes wonders!

Sara, for bringing some good memories from Linköping. Enjoy your wonderful kids. Wish you all the best in the future.

Charlotte, Kerstin, Rene and **Ingegerd,** for your smiles and enthusiasm, you are all amazing women! Thank you all for the help in the lab, what would I do without you!

Ryan, for all the great tips on house renovation and all the great chats on life in general.

My colleagues **Gavad, Du, Hai, Lily, Yoko, Cheryl, Nina, Sonal, Gokce, Alberto,** for your nice company and smiles.

I would also very much like to thank all the personnel at **Clinical Immunology (F79)**, for always greeting me with a smile and being very, very helpful, even when I showed up for the 10th time in one day, confused, with lists and samples and no clue!

I am grateful for all the support provided by the Post-Graduate School of Health Care Sciences, especially **Jan Ekstrand** and **Ingeborg van der Ploeg** and all the wonderful fellow PhD students.

My dear friends:

Sait, always been there, always will be, thank you for adding the crazy-dimension to my life.

Narges, you will be a great scientist! It was a pleasure working with you and it is a thrill to have you as a friend! Love your laughter, the way we "fikar" us through depressions, your fighting-spirit! We are going to create great things together! ☺

There are so many people that truly made a difference, but as this section is already getting out of hand, I want you to give you a group hug: **Evi, Marcus, Magdalena, Gracia,** thank you for helping me to find my path.

To my cool, wonderful and loving family:

My mom, the coolest woman alive, nothing is too hard for you.
My dad, my rock, you are always there.
My brother **Akram,** I always look up to you, and his lovely girlfriend **Camilla,** you are so strong, you can do anything! Thank you for the beautiful cover!!
My brother **Adam** and my sister **Suzanne,** my bundles of joy, you have taught me a lot about dreams.

Tons of love and kisses to my new family: **Olga, Achour, Gabriel** and **Ingrid,** for welcoming me with open arms.

Ronny – the love of my life, thank you for your unconditional love, your support through the years has been my life-line. You have been there in my darkest moments, always lifting me up, giving me hope and strength. I am thrilled that you joined me on this journey and I can't wait till the next destination! Love you endlessly.

9 REFERENCES

1. Geha, R.S. et al. Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. *J. Allergy Clin. Immunol* **120**, 776-794(2007).
2. Bruton, O.C. Agammaglobulinemia. *Pediatrics* **9**, 722-728(1952).
3. Vetrie, D. et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* **361**, 226-233(1993).
4. Woroniecka, M. & Ballow, M. Office evaluation of children with recurrent infection. *Pediatr. Clin. North Am* **47**, 1211-1224(2000).
5. Noroski, L.M. & Shearer, W.T. Screening for primary immunodeficiencies in the clinical immunology laboratory. *Clin. Immunol. Immunopathol* **86**, 237-245(1998).
6. Chan, K. & Puck, J.M. Development of population-based newborn screening for severe combined immunodeficiency. *J. Allergy Clin. Immunol* **115**, 391-398(2005).
7. Primary immunodeficiency diseases. Report of an IUIS Scientific Committee. International Union of Immunological Societies. *Clin. Exp. Immunol* **118 Suppl 1**, 1-28(1999).
8. Hammarström, L. & Smith, C. Genetic approach to common variable immunodeficiency and IgA deficiency. *Primary immunodeficiency diseases: A molecular and genetic approach.* 313-325(2007).
9. Pickering, M.C. et al. Systemic lupus erythematosus, complement deficiency, and apoptosis. *Adv. Immunol* **76**, 227-324(2000).
10. Jönsson, G. et al. Hereditary C2 deficiency in Sweden: frequent occurrence of invasive infection, atherosclerosis, and rheumatic disease. *Medicine (Baltimore)* **84**, 23-34(2005).
11. Ekins, R.P. Multi-analyte immunoassay. *J Pharm Biomed Anal* **7**, 155-168(1989).
12. Ekins, R. & Chu, F. Multianalyte microspot immunoassay. The microanalytical 'compact disk' of the future. *Ann. Biol. Clin. (Paris)* **50**, 337-353(1992).
13. Hall, D.A., Ptacek, J. & Snyder, M. Protein Microarray Technology. *Mech Ageing Dev.* **128**, 161–167(2007).
14. Schweitzer, B., Predki, P. & Snyder, M. Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics* **3**, 2190-2199(2003).
15. Robinson, W.H. et al. Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med* **8**, 295-301(2002).
16. Lueking, A. et al. A nonredundant human protein chip for antibody screening and serum profiling. *Mol. Cell Proteomics* **2**, 1342-1349(2003).
17. de Wildt, R.M. et al. Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol* **18**, 989-994(2000).
18. Büssow, K. et al. A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. *Nucleic Acids Res* **26**, 5007-5008(1998).
19. Miller, J.C. et al. Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* **3**, 56-63(2003).
20. Paweletz, C.P. et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* **20**, 1981-1989(2001).

21. Wulfkuhle, J.D. et al. Signal pathway profiling of ovarian cancer from human tissue specimens using reverse-phase protein microarrays. *Proteomics* **3**, 2085-2090(2003).
22. Grubb, R.L. et al. Signal pathway profiling of prostate cancer using reverse phase protein arrays. *Proteomics* **3**, 2142-2146(2003).
23. Nishizuka, S. et al. Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. *Cancer Res* **63**, 5243-5250(2003).
24. Nishizuka, S. et al. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. *Proc. Natl. Acad. Sci. U.S.A* **100**, 14229-14234(2003).
25. Espina, V. et al. Protein microarrays: molecular profiling technologies for clinical specimens. *Proteomics* **3**, 2091-2100(2003).
26. McDade, T.W., Williams, S. & Snodgrass, J.J. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography* **44**, 899-925(2007).
27. Skogstrand, K. et al. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. *Clin. Chem* **51**, 1854-1866(2005).
28. Cunningham-Rundles, C. Physiology of IgA and IgA deficiency. *J. Clin. Immunol* **21**, 303-309(2001).
29. Conley, M.E. & Delacroix, D.L. Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense? *Ann. Intern. Med* **106**, 892-899(1987).
30. Clark, J.A. et al. Selective IgA deficiency in blood donors. *Am. J. Clin. Pathol* **80**, 210-213(1983).
31. Kanoh, T. et al. Selective IgA deficiency in Japanese blood donors: frequency and statistical analysis. *Vox Sang* **50**, 81-86(1986).
32. Conley, M.E., Notarangelo, L.D. & Etzioni, A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin. Immunol* **93**, 190-197(1999).
33. Ballow, M. Primary immunodeficiency disorders: antibody deficiency. *J. Allergy Clin. Immunol* **109**, 581-591(2002).
34. Pan-Hammarström, Q. & Hammarström, L. Antibody deficiency diseases. *Eur. J. Immunol* **38**, 327-333(2008).
35. Klemola, T. Deficiency of immunoglobulin A. *Ann. Clin. Res* **19**, 248-257(1987).
36. Hammarström, L. et al. Transfer of IgA deficiency to a bone-marrow-grafted patient with aplastic anaemia. *Lancet* **1**, 778-781(1985).
37. Kurobane, I. et al. Incidental correction of severe IgA deficiency by displacement bone marrow transplantation. *Bone Marrow Transplant* **7**, 494-495(1991).
38. Stoop, J.W. et al. Serum immunoglobulin levels in healthy children and adults. *Clin. Exp. Immunol* **4**, 101-112(1969).
39. Berg, T. & Johansson, S.G. Immunoglobulin levels during childhood, with special regard to IgE. *Acta Paediatr Scand* **58**, 513-524(1969).
40. Collins-Williams, C. et al. Incidence of isolated deficiency of IgA in the serum of Canadian children. *Ann Allergy* **30**, 11-23(1972).
41. Cassidy, J.T. & Nordby, G.L. Human serum immunoglobulin concentrations: prevalence of immunoglobulin deficiencies. *J. Allergy Clin. Immunol* **55**, 35-48(1975).
42. Chandra, R.K. et al. Clinical immunological studies of children with selective IgA deficiency. *Afr. J. Clin. Exp. Immunol.* **3**, 282-292(1982).

43. Oyeyinka, G.O. et al. Range of normal serum immunoglobulin (IgG, IgA and IgM) values in Nigerians. *Afr J Med Med Sci* **13**, 169-176(1984).
44. Ezeoke, A.C. Selective IgA deficiency (SIgAD) in Eastern Nigeria. *Afr J Med Med Sci* **17**, 17-21(1988).
45. Geller-Bernstein, C. et al. IgA, IgG, IgM, and IgE levels in normal, healthy, non-atopic Israeli children. *Ann Allergy* **61**, 296-299(1988).
46. Pereira, L.F. et al. Prevalence of selective IgA deficiency in Spain: more than we thought. *Blood* **90**, 893(1997).
47. Cataldo, F. et al. Celiac disease and selective immunoglobulin A deficiency. *J. Pediatr* **131**, 306-308(1997).
48. Pratesi, R. et al. Prevalence of coeliac disease: unexplained age-related variation in the same population. *Scand. J. Gastroenterol* **38**, 747-750(2003).
49. Ertekin, V. et al. Prevalence of celiac disease in Turkish children. *J. Clin. Gastroenterol* **39**, 689-691(2005).
50. Ben Hariz, M. et al. Prevalence of celiac disease in Tunisia: mass-screening study in schoolchildren. *Eur J Gastroenterol Hepatol* **19**, 687-694(2007).
51. Ritchie, R.F. et al. Reference distributions for complement proteins C3 and C4: a practical, simple and clinically relevant approach in a large cohort. *J. Clin. Lab. Anal* **18**, 1-8(2004).
52. S Reis, E., Falcão, D.A. & Isaac, L. Clinical aspects and molecular basis of primary deficiencies of complement component C3 and its regulatory proteins factor I and factor H. *Scand. J. Immunol* **63**, 155-168(2006).
53. Singer, L., Colten, H.R. & Wetsel, R.A. Complement C3 deficiency: human, animal, and experimental models. *Pathobiology* **62**, 14-28(1994).
54. Fujioka, H. et al. A case of C3 deficiency with a novel homozygous two-base deletion in the C3 gene. *Am. J. Med. Genet. A* **138**, 399-400(2005).
55. Kida, M. et al. The first confirmed case with C3 deficiency caused by compound heterozygous mutations in the C3 gene; a new aspect of pathogenesis for C3 deficiency. *Blood Cells Mol. Dis* **40**, 410-413(2008).
56. Ghannam, A. et al. Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells. *J. Immunol* **181**, 5158-5166(2008).
57. Botto, M. et al. Molecular basis of hereditary C3 deficiency. *J. Clin. Invest* **86**, 1158-1163(1990).
58. Botto, M. et al. Homozygous hereditary C3 deficiency due to a partial gene deletion. *Proc. Natl. Acad. Sci. U.S.A* **89**, 4957-4961(1992).
59. Singer, L. et al. Inherited human complement C3 deficiency. An amino acid substitution in the beta-chain (ASP549 to ASN) impairs C3 secretion. *J. Biol. Chem* **269**, 28494-28499(1994).
60. Huang, J.L. & Lin, C.Y. A hereditary C3 deficiency due to aberrant splicing of exon 10. *Clin. Immunol. Immunopathol* **73**, 267-273(1994).
61. Matsuyama, W. et al. Identification of a novel mutation (Tyr1081Ter) in sisters with hereditary component C3 deficiency and SLE-like symptoms. *Hum. Mutat* **17**, 79(2001).
62. Da Silva Reis, E. et al. Homozygous hereditary C3 deficiency due to a premature stop codon. *J. Clin. Immunol* **22**, 321-330(2002).
63. Reis, E.S., Nudelman, V. & Isaac, L. Nonsense-codon-mediated decay in human hereditary complement C3 deficiency. *Immunogenetics* **55**, 667-673(2004).
64. Tsukamoto, H. et al. Molecular analysis of a novel hereditary C3 deficiency with systemic lupus erythematosus. *Biochem. Biophys. Res. Commun* **330**, 298-304(2005).

65. Alper, C.A. et al. Immunoglobulin deficiencies and susceptibility to infection among homozygotes and heterozygotes for C2 deficiency. *J. Clin. Immunol* **23**, 297-305(2003).
66. Walport, M.J. Complement. First of two parts. *N. Engl. J. Med* **344**, 1058-1066(2001).
67. Cooper, N. Laboratory investigations of complement proteins and complement receptors. *Baillieres Clin Immunol Allergy* **2**, 263-293(1988).
68. Glovsky, M.M., Ward, P.A. & Johnson, K.J. Complement determinations in human disease. *Ann. Allergy Asthma Immunol* **93**, 513-522; quiz 523-525, 605(2004).
69. Johnson, U., Truedsson, L. & Gustavii, B. Complement components in 100 newborns and their mothers determined by electroimmunoassay. *Acta Pathol Microbiol Immunol Scand C* **91**, 147-150(1983).
70. Inai, S. et al. Inherited deficiencies of the late-acting complement components other than C9 found among healthy blood donors. *Int. Arch. Allergy Appl. Immunol* **90**, 274-279(1989).
71. Carroll, M.C. et al. A molecular map of the human major histocompatibility complex class III region linking complement genes C4, C2 and factor B. *Nature* **307**, 237-241(1984).
72. Johnson, C.A. et al. Type I human complement C2 deficiency. A 28-base pair gene deletion causes skipping of exon 6 during RNA splicing. *J. Biol. Chem* **267**, 9347-9353(1992).
73. Figueroa, J.E. & Densen, P. Infectious diseases associated with complement deficiencies. *Clin. Microbiol. Rev* **4**, 359-395(1991).
74. Jönsson, G. et al. Hereditary C2 deficiency in Sweden: frequent occurrence of invasive infection, atherosclerosis, and rheumatic disease. *Medicine (Baltimore)* **84**, 23-34(2005).
75. Bittleman, D.B. et al. Recurrent infections, pericarditis and renal disease in a patient with total C2 deficiency and decreased NK cell function consistent with acute rheumatic fever and systemic lupus erythematosus. *Ann. Rheum. Dis* **53**, 280-281(1994).
76. Densen, P. Complement deficiencies and meningococcal disease. *Clin. Exp. Immunol* **86 Suppl 1**, 57-62(1991).
77. Ben-Hur, H. et al. Immunoglobulin A in the epithelium of the respiratory tract and intrahepatic bile ducts of fetuses and newborns with pneumonia and sepsis. *Hum Antibodies* **8**, 119-123(1997).
78. Gurevich, P. et al. An immunohistochemical study of the secretory immune system in human fetal endocrine glands and their precursors. *Early Pregnancy* **5**, 191-200(2001).
79. Andersson, U., Bird, G. & Britton, S. Cellular mechanisms of restricted immunoglobulin formation in the human neonate. *Eur. J. Immunol* **10**, 888-894(1980).
80. Gathings, W.E., Lawton, A.R. & Cooper, M.D. Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. *Eur. J. Immunol* **7**, 804-810(1977).
81. Gudmundsson, K.O. et al. Immunoglobulin-secreting cells in cord blood: effects of Epstein-Barr virus and interleukin-4. *Scand. J. Immunol* **50**, 21-24(1999).
82. Miyawaki, T. et al. Ig isotypes produced by EBV-transformed B cells as a function of age and tissue distribution. *J. Immunol* **140**, 3887-3892(1988).
83. Lannergård, A. et al. Immune function in Swedish élite orienteers. *Scand J Med Sci Sports* **11**, 274-279(2001).
84. Ulbrich, A.G. et al. Hereditary human complement C3 deficiency owing to reduced levels of C3 mRNA. *Scand. J. Immunol* **53**, 622-626(2001).

85. Kull, I. et al. Breast feeding and allergic diseases in infants-a prospective birth cohort study. *Arch. Dis. Child* **87**, 478-481(2002).
86. Kull, I. et al. Breast-feeding reduces the risk of asthma during the first 4 years of life. *J. Allergy Clin. Immunol* **114**, 755-760(2004).
87. Böhme, M. et al. Atopic dermatitis and concomitant disease patterns in children up to two years of age. *Acta Derm. Venereol* **82**, 98-103(2002).
88. Ostblom, E. et al. Phenotypes of food hypersensitivity and development of allergic diseases during the first 8 years of life. *Clin. Exp. Allergy* **38**, 1325-1332(2008).
89. Pawlak, M. et al. Zeptosens' protein microarrays: a novel high performance microarray platform for low abundance protein analysis. *Proteomics* **2**, 383-393(2002).
90. Cederqvist, L.L. & Litwin, S.D. Production of alpha 1 and alpha 2 immunoglobulin heavy chains during fetal life. *J. Immunol* **112**, 1605-1608(1974).
91. Malek, A., Sager, R. & Schneider, H. Transport of proteins across the human placenta. *Am. J. Reprod. Immunol* **40**, 347-351(1998).
92. Gurevich, P. et al. An immunohistochemical study of the secretory immune system in human fetal endocrine glands and their precursors. *Early Pregnancy* **5**, 191-200(2001).
93. Ben-Hur, H. et al. Transport of maternal immunoglobulins through the human placental barrier in normal pregnancy and during inflammation. *Int. J. Mol. Med* **16**, 401-407(2005).
94. Ingvarsson, J. et al. Design of recombinant antibody microarrays for serum protein profiling: targeting of complement proteins. *J. Proteome Res* **6**, 3527-3536(2007).
95. Puck, J.M. Population-based newborn screening for severe combined immunodeficiency: steps toward implementation. *J. Allergy Clin. Immunol* **120**, 760-768(2007).
96. Puck, J.M. Severe combined immunodeficiency: new advances in diagnosis and treatment. *Immunol. Res* **38**, 64-67(2007).
97. Hazenberg, M.D. et al. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J. Mol. Med* **79**, 631-640(2001).
98. Baker, M.W. et al. Development of a routine newborn screening protocol for severe combined immunodeficiency. *Journal of Allergy and Clinical Immunology* **In Press, Corrected Proof**,
99. Sano, Y. et al. Hereditary deficiency of the third component of complement in two sisters with systemic lupus erythematosus-like symptoms. *Arthritis Rheum* **24**, 1255-1260(1981).
100. Ostblom, E. et al. Reported symptoms of food hypersensitivity and sensitization to common foods in 4-year-old children. *Acta Paediatr* **97**, 85-90(2008).
101. Ostblom, E. et al. Patterns of quantitative food-specific IgE-antibodies and reported food hypersensitivity in 4-year-old children. *Allergy* **63**, 418-424(2008).
102. Ostblom, E. et al. The impact of food hypersensitivity reported in 9-year-old children by their parents on health-related quality of life. *Allergy* **63**, 211-218(2008).
103. Aghamohammadi, A. et al. IgA deficiency: correlation between clinical and immunological phenotypes. *J. Clin. Immunol* **29**, 130-136(2009).
104. Buckley, R.H. & Dees, S.C. Correlation of milk precipitins with IgA deficiency. *N. Engl. J. Med* **281**, 465-469(1969).
105. Ammann, A.J. & Hong, R. Selective IgA deficiency: presentation of 30 cases and a review of the literature. *Medicine (Baltimore)* **50**, 223-236(1971).
106. Burgio, G.R. et al. Selective IgA deficiency: clinical and immunological evaluation of 50 pediatric patients. *Eur. J. Pediatr* **133**, 101-106(1980).

107. Grundbacher, F.J. & Massie, F.S. Levels of immunoglobulin G, M, A, and E at various ages in allergic and nonallergic black and white individuals. *J. Allergy Clin. Immunol* **75**, 651-658(1985).
108. Plebani, A. et al. Comparison of the frequency of atopic diseases in children with severe and partial IgA deficiency. *Int. Arch. Allergy Appl. Immunol* **82**, 485-486(1987).
109. Savilahti, E., Pelkonen, P. & Visakorpi, J.K. IgA deficiency in children. A clinical study with special reference to intestinal findings. *Arch. Dis. Child* **46**, 665-670(1971).
110. Gupta, R. et al. Burden of allergic disease in the UK: secondary analyses of national databases. *Clin. Exp. Allergy* **34**, 520-526(2004).
111. Martinez, F.D. et al. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N. Engl. J. Med* **332**, 133-138(1995).
112. Soothill, J.F. et al. Predisposing factors and the development of reaginic allergy in infancy. *Clin. Allergy* **6**, 305-319(1976).
113. Taylor, B. et al. Transient IgA deficiency and pathogenesis of infantile atopy. *Lancet* **2**, 111-113(1973).
114. Chandra, R.K. Serum levels and synthesis of IgG subclasses in small-for-gestation low birth weight infants and in patients with selective IgA deficiency. *Monogr Allergy* **20**, 90-99(1986).
115. Blum, P.M., Hong, R. & Stiehm, E.R. Spontaneous recovery of selective IgA deficiency. Additional case reports and a review. *Clin Pediatr (Phila)* **21**, 77-80(1982).
116. de Laat, P.C., Weemaes, C.M. & Bakkeren, J.A. Immunoglobulin levels during follow-up of children with selective IgA deficiency. *Scand. J. Immunol* **35**, 719-725(1992).
117. Joller, P.W., Buehler, A.K. & Hitzig, W.H. Transitory and persistent IgA deficiency. Reevaluation of 19 pediatric patients once found to be deficient in serum IGA. *J Clin Lab Immunol* **6**, 97-101(1981).
118. Kutukculer, N. et al. Increases in serum immunoglobulins to age-related normal levels in children with IgA and/or IgG subclass deficiency. *Pediatr Allergy Immunol* **18**, 167-173(2007).
119. Ostergaard, P.A. Clinical and immunological features of transient IgA deficiency in children. *Clin. Exp. Immunol* **40**, 561-565(1980).
120. Plebani, A. et al. Clinical heterogeneity and reversibility of selective immunoglobulin A deficiency in 80 children. *Lancet* **1**, 829-831(1986).
121. Roberton, D.M. et al. IgG subclass concentrations in absolute, partial and transient IgA deficiency in childhood. *Pediatr. Infect. Dis. J* **9**, S41-45(1990).
122. Schwenk, J.M. et al. Antibody suspension bead arrays within serum proteomics. *J. Proteome Res* **7**, 3168-3179(2008).