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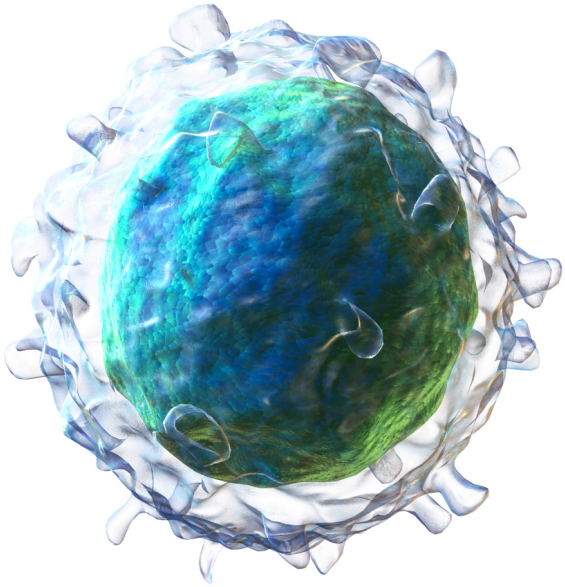

# B cells maturation in infancy and its affect in early allergy

*The appearance of allergy occurs when the immune system fails to develop tolerance to a harmless substance in the environment*

Diploma Thesis in Biological Engineering

EMMA KARLSSON

Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2019

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https://commons.wikimedia.org/wiki/File:Blausen0624LymphocyteBcell(crop).png

Typeset in LaTeX
Gothenburg, Sweden 2019

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# Abstract

The last decades, the presence of allergy has increased in countries belonging to the Western world and over 25 % of Swedish young people are affected of allergic symptoms. One reasonable explanation is the hygiene hypothesis, mentioned by David Strachan 1989, that associate the increase in allergy development with a cleaner lifestyle. Previous studies showed that children who grew up on a farm with animals had a more maturated immune system and therefore developed less allergy. With samples received from the study named Nutritional impact on Immunological maturation during Childhood in relation to the Environment (NICE), I have studied the B cells maturation during early time in life.

By studying subpopulations of B cells, specific surface markers are stained with fluorochromes to be analysed with a flow cytometer. Thus, the development of B cell subpopulations from birth up to 4 months can be charted. Several surface markers can indicate the same subpopulation, i.e. $CD27^{+}$ and $CD24^{hi}CD38^{lo}$ that are expressed by memory B cells. However, the percentage differ from each other and the most liable reason is that the surface markers have different functions in the B cells, and are therefore not only expressed during one phase of the development of B cells.

My results shows that the development of percentage of subpopulations of B cells differ from each other from birth up to 4 months. Correlation tests indicate that $CD27^{+}$ and $CD24^{hi}CD38^{lo}$ could both be used to detect memory B cells. Correlation tests at number of subpopulations had higher correlation coefficient and were strongly significant at birth. Therefore, it would be interesting to continue the analysis of numbers of B cells populations at 48h, 1, 4 and 12 months of life.

In the NICE study, so far 452 children have been examined for allergy and 6.2 % suffered from eczema, 2.4 % of food allergy and 6.2 % of the children had developed asthma at 1 year of age. In this thesis, there was no significant associations between B cell maturity and the development of allergy.

Keywords: NICE cohort, allergy, immune system, B cell, flow cytometry

# Acknowledgements

Jag har under våren spenderat mina dagar på Mikrobiologen på Sahlgrenska Akademin, Göteborgs Universitet. Tack till er som jobbar på sjätte våningen för trevligt bemötande och era hjälpande händer.

Mitt varmaste tack till min handledare Hardis Rabe, som med stort engagemang har hjälpt mig under dessa veckor. Trots en stressig period, har du alltid varit positiv vilket verkligen har inspirerat mig. Tack också för att jag har fått använda dina fina bilder till mitt arbete.

Stort tack till alla inblandade i NICE-studien för jag fick delta och träffa er under konferensen i Umeå. Tack för era tips och inputs.

Tack till mina kursare och för våra luncher tillsammans.

Sist men inte minst vill jag tacka min stöttepelare och sambo Anton som alltid finns vid min sida när jag behöver bolla något eller bara prata.

Emma Karlsson, Gothenburg, May 2019

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# Glossary

| | |
|---|---|
| ANTIBODY | Or Immunoglobulin (Ig). Protein by plasma cells |
| ANTIGEN | Substance that can be recognized by lymphocytes that is foreign for the body |
| ATOPY | The formation of IgE antibodies against environmental proteins |
| $CD4^+$ | Surface marker for helper T cells |
| $CD5^+$ | Surface marker for immature transitional B cells |
| $CD8^+$ | Surface marker for cytotoxic T cells |
| $CD20^+$ | Surface marker for all B cells |
| $CD24^{hi}CD38^{hi}$ | Surface marker for immature transitional B cells |
| $CD24^{lo}CD38^{lo}$ | Surface marker for mature naive B cells |
| $CD24^{hi}CD38^{lo}$ | Surface marker for memory B cells |
| $CD27^+$ | Surface marker for memory B cells |
| CLASS-SWITCHED | Induce B cells to start producing IgE, IgA, IgG |
| ENDEMIC DISEASE | A disease that strikes a certain population or geographic area |
| COMPLEMENT SYSTEM | Complement to the immune system |
| CYTOKINES | Proteins with chemical signal |
| DNA POLYMERASE | Enzyme in the DNA synthesis |
| ENDOTHELIAL LAYER | Cells that cover the inside of the blood vessel |
| EPITHELIAL LAYER | A tissue type consisting of cells |
| $IgD^{hi}IgM^{lo}$ | Surface marker for mature naive $IgD^+$ B cells |
| $IgD^{lo}IgM^{hi}$ | Surface marker for memory $IgM^+$ B cells |
| INTERLEUKIN | Or cytokine. Protein with a chemical signal |
| LYMPHATIC SYSTEM | Consisting of lymphatic fluid, part of immune system |
| LYSED | Cell lysis, death |
| MACROPHAGES | Cells that are part of the innate immune system and acts as phagocytes |
| MHC CLASS I AND II | Molecule complexes that are needed to present peptide from an antigen to T cells |
| MONOCLONAL AB | Identical antibodies (ab) |
| MONONUCLEAR CELLS | Cells in the blood that has a specific non-segmented nucleus, i.e. monocytes and lymphocytes |
| TNF | Tumor necrosis factor. A cytokine that causes inflammation |

# 1

# Introduction

## 1.1 Background

The presence of allergy has increased considerably the last decades and it is the most frequent disease in economical developed countries and those with an Western lifestyle [1, 2, 3]. Allergy is an endemic disease and over a quarter of Swedish young people is affected of allergic symptoms [4]. Development of an allergic disease is a result caused by a failure to develop immune tolerance against a harmless allergen [2]. The atopic or allergic march is a allergic progress several children undergoes during childhood. It usually starts with atopic eczema and/or food allergy, followed with asthma. The presence of allergy can either remain or disappear after the atopic march [5, 6].

The year of 1989, David Strachan presented the hygiene hypothesis. The theory is built on the increase of allergy development might associate with a cleaner lifestyle and thus lack of microbe exposure [7, 8]. A previous study, called the farmflora birth-cohort study, showed that children who grew up on a farm developed less allergy compared to non-farmers children. The farmers children had a more maturated immune system, i.e. they had a low proportion of immature B cells, but higher of memory B cells compared to non-farmers children. [2, 9, 10]. Their results indicate the importance to study the development of the immune system, especially with affection from the environment, during early time of life.

Samples for this project are received from the cohort study named Nutritional impact on Immunological maturation during Childhood in relation to the Environment (NICE). 655 families are followed from gestational week 28 and their infants up to 4 years of age. During this period, comprehensive biological samples are collected and questionnaires and examinations are performed. Allergies that currently has been diagnosed in the NICE study are atopic eczema, food allergy and asthma. The recruitment to the study began 2015 and the study is expected to end 2022 [11].

## 1.2 Aim

The overall aim of this thesis is to study the B cell maturation during the first 4 months of life and if the B cell maturation might be correlated with allergy development at 12 months of age.

A subsequent aim of the thesis is to evaluate whether different surface markers combinations, used to identify the same B cells subpopulations, show the same maturation patterns over the first 4 months of life.

## 1.3 Delimitation

The adaptive immune system consists of, beyond B cells, T cells which not will be included in this thesis.

Analysis of number of B cells with flow cytometry was done only at birth.

# 2
# Theory

## 2.1 Immune system

The immune system is divided into two parts; the innate and the adaptive immune system. The innate immune system acts unspecific and immediately against a wide range of invaders and their common patterns called pathogen-associated molecular patterns (PAMPs). The adaptive immune system contains lymphocytes with the ability to react with a specific immune response toward antigens, i.e. any substance that can be recognized by the adaptive immune response. Despite the division, the two parts collaborates when an infection occurs [12].

The immune system have four main roles. The first one is *Immunological recognition*, to detect the infectious substance. The second role is *Immune effector functions* with purpose to attack the harmful pathogen and, if possible, eliminate it. *The immune regulatory* is the third and an important role of the immune system. If the regulation of immune responses fails, the consequences could lead to allergy or autoimmune diseases, such as diabetes and inflammatory bowel diseases. A special function of the adaptive system is the *Immunological memory*. With the ability to, when once been exposed for an pathogen, set up an even stronger and faster immune response for future, possible responses [12, 13].

### 2.1.1 Innate immune system

The first exposure of a foreign substance occur through penetration of the epithelial layer. The immediate response is directed by phagocytic cells, such as macrophages, neutrophils and dendritic cells, belonging to the innate system. Macrophages and neutrophils engulf pathogens by phagocytosis and eliminate them. If the pathogen express PAMPs, the macrophage gets activated and cytokines, such as IL-1 and TNF, are secreted. IL-1 and TNF will activate the endothelial cells and thus increase vessel permeability nearby blood capillaries.

Furthermore, mast cells will be activated by parts of the complement system, and release histamine. Consequently, histamine will stimulate the endothelial cells further, and permeability will increase, allowing migration of neutrophils into the inflammation focus. The macrophages and dendritic cells, also known as antigen-presenting cells (APCs), take up antigens from the inflammation and express peptides of it on MHC class II molecule. Those cells then express peptides to a local lymph node and the adaptive immune system can be activated. Upon stimulation, dendritic cells

increase their production av MHC class II molecules and thus also the inflammatory response [12, 14].

### 2.1.2 Adaptive immune system

Compared to the innate system, the adaptive system will develop, be more specific and set up an immunological memory when the body exposes for pathogens during the lifetime. The lymphocytes in the adaptive immune system consists of B and T lymphocytes (cells), they are formed from hematopoietic stem cells in the bone marrow. When a peptide from an antigen is expressed on a APC in the lymph node, T cells gets activated which results in different actions depending on subgroup of T cells that develops [12]. Activated $CD8^+$ cytotoxic T cells eliminate infected cells that present viral peptides on MHC class I. $CD4^+$ helper T cells, in other hand, become activated by MHC class II and secrete cytokines as their *Immune effector function*. Another T cell expressing CD4 are the regulatory T cells (belonging to *The immune regulatory*), which in contrast to the conventional $CD4^+$ helper T cells, suppress inflammatory responses instead of activating them. This mechanism is important to prevent development of autoimmunity and allergy [12, 13, 15].

### 2.1.3 B lymphocytes

The main role of the B lymphocytes (cells) is in their formation of plasma cells to produce antibodies against a specific antigen. [12].

#### 2.1.3.1 Maturation of B cells

As shown in figure 2.1, the newly produced immature transitional B cells are released from the bone marrow at which they express CD5 and highly CD24 and CD38. The immature transitional B cells have a functional B cells receptor (BCR), that will be able to recognize its specific antigen. These cells will develop in the spleen to mature naive B cells and consequently lower their CD24 and CD38 expression into $CD24^{lo}CD38^{lo}$ [3, 14]. The naive $CD24^{lo}CD38^{lo}$ B cell circulates in the blood stream until it interacts with its specific antigen in the lymph node. Following activation, with help from the cytokines IL-4 and IL-13 secreted by $CD4^+$ T cells, B cells will differentiate into antibody producing cells, named plasma cells. Memory $CD27^+$ $CD24^{hi}CD38^{lo}$ B cells will also be produced upon activation with the aim to respond fast and effective against antigen in future responses [14, 16, 17].

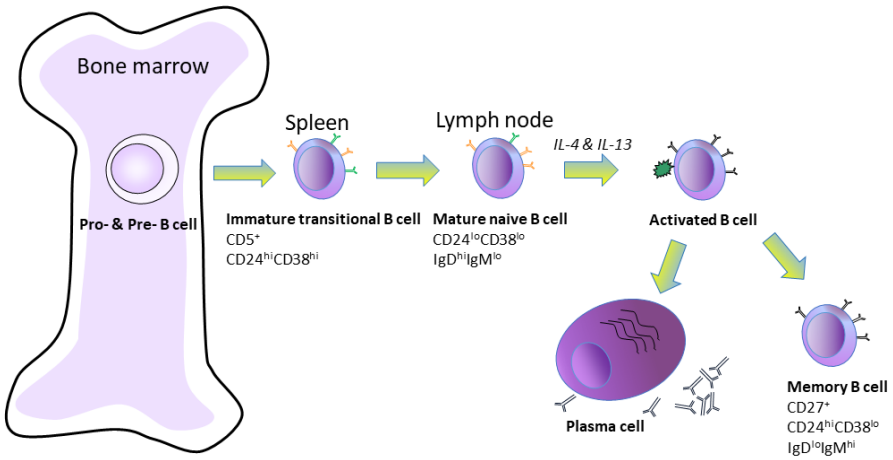


**Figure 2.1:** Pro- and Pre- B cells are developed from hematopoetic stem cells in the bone marrow. As a result, immature transitional B cells are expressing CD5 and highly of CD24 and CD38 when they arrive to the lymph- and blood system. In the spleen, these will develop into mature naive B cells expressing low of CD24 and CD38 and also high of IgD. The mature naive B cell will later on be activated to either a plasma cell secreting antibodies or a memory B cell expressing $CD27^{+}$, $CD24^{hi}CD38^{lo}$ and $IgD^{lo}IgM^{hi}$.

Plasma cells are triggered by the $CD4^{+}$ helper T cells, to produce immunoglobulins (Ig), also known as antibodies. There are different isotypes of immunoglobulins; IgD, IgM, IgG, IgA and IgE, each with an unique function and impact when binding an antigen. As seen in figure 2.1, naive B cells express highly of IgD and memory B cells express highly of IgM, in absence of activation from $CD4^{+}$ T cells. When the B cells are activated, their isotype will class-switch and then express or secrete IgG, IgA or IgE [13]. Immunoglobulins circulate in the bloodstream and the lymphatic system and can bind specific to antigenes. Depending on their isotype they cause different immune resource when binding to their antigen [12, 15]. IgE, for instance, is associated with allergy development and binds to mast cells. When the specific allergen binds to IgE, the mast cell gets activated and histamine is released which trigger an allergic reaction to occur (see figure 2.2) [18].

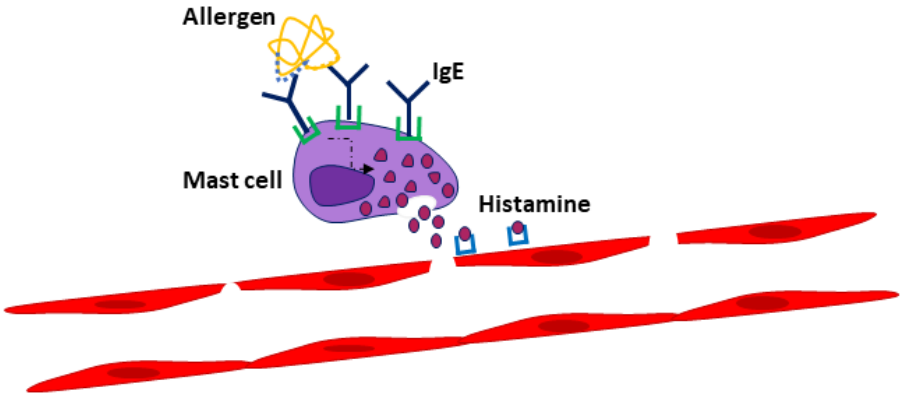


**Figure 2.2:** IgE mediated allergy occurs when the mast cell gets activated and degranulate. To activate the mast cell, allergen binds to IgE attached on cellsurface. Histamine released from the mast cell will affect the endothelial tissue and start the allergic reaction.

## 2.2 Flow cytometry

Flow cytometry is a technique used to measure light scatter, fluorescence, and absorbance from each individual cell. Through this method, it is possible to measure which marker that is expressed by one cell and thus which characteristics it has [20]. To determine cell populations, the biological sample is stained with fluorochromes (fluorescent antibodies) which can be detected at a certain wave length. Each fluorochrome stains for a specific surface marker [21]. If number of cells is of interest, TruCount method can be used. By adding a sample to a TruCount tube which contains fixed and known amount of beads, the cells in the sample can be compared to cell percentage [22]. The sample together with sheath fluid are then inserted to the flow cytometer. The diameter of the tube is decreasing along the path, which results in the cells being forced to line up one by one before entering a laser (see figure 2.3). When measuring absorbance, the excitation light generate side (SSC) and forward scatter (FSC). SSC yield information about the granularity of the cell and FSC about cell size. SSC plotted against FSC gives information of sub populations such as granulocytes and lymphocytes [21].

Fluorescence-activated cell sorting (FACS) is a method followed by flow cytometry, used to separate the cells of interest in the analysed sample. After the laser detection, the specific cells get charged and separates when they exposes to a electrical field [23].

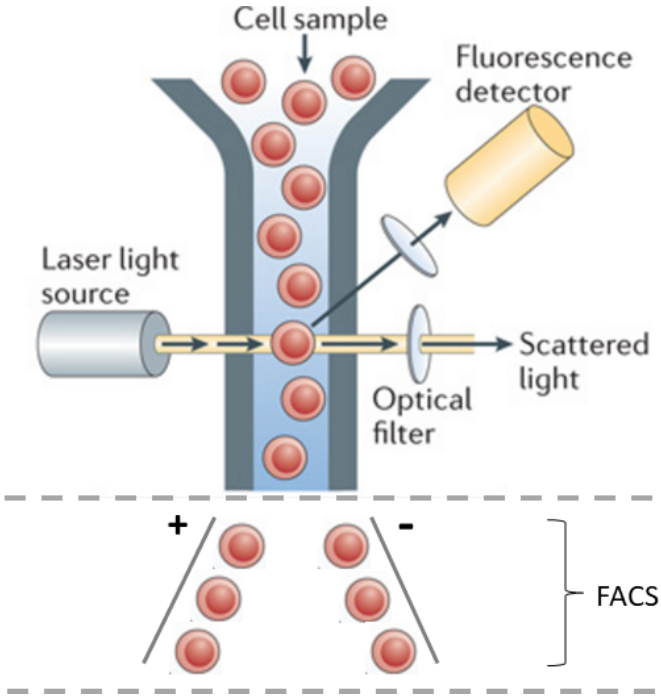


**Figure 2.3:** The sample containing cells marked with different fluorochromes is inserted to the flow cytometer. Each cell is illuminated by a laser before it enters a electric field where it gets charged and separates.

# 3

# Methods

## 3.1 The NICE study

The NICE study consists of 655 families that were recruited during pregnancy. In the cohort, blood samples are collected from infants at birth (cord blood) and 48 hours after delivery and at 4 months of age. In a subcohort to the cohort, consisting of approximately 25 % of the study participants, additional samples are collected at 1 months of age. At 12 months of age, the children visit a doctor to examine allergy development and sensitisation (see figure 3.1). Flow cytometry was performed at samples from children that were included in the subcohort.

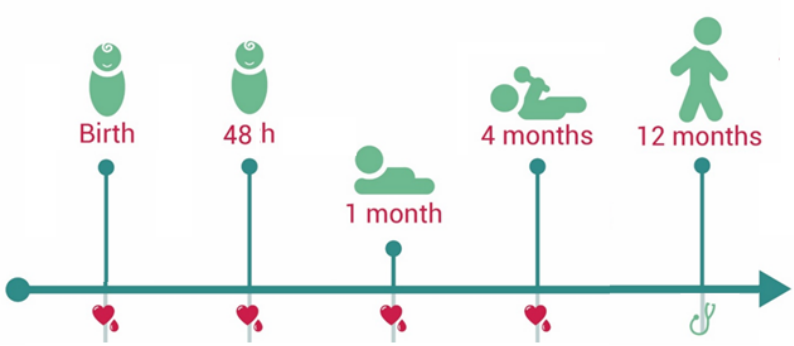


**Figure 3.1:** Blood samples from infants collected at birth, 48h, 1 and 4 months. At 12 months, the children examines regarding allergy development and sensitisation with, for instance, skin prick test.

### 3.1.1 Flow cytometry

To study the B cell phenotype in the blood of newborn children, 50 µl blood were stained with mononuclear antibodies shown in table 3.1 for 20 min in 4 degrees. The red blood cells were lysed with lysis buffer (BD Bioscience, San Diego). Thereafter, the cells were fixated with eBioscience fixation and permeabilisation kit according to the manufactures recommendations. The cells were analysed within 2 days of staining with the flow cytometer BD Accuri C6 (BD Bioscience).

**Table 3.1:** Fluorochromes (fluorescent antibodies) that stains for different surface markers by B cells with aim to detect subpopulations that express that specific marker.

| Monoclonal antibody | Fluorochrome | Supplier |
|---|---|---|
| CD27 | FITC | BD Bioscience |
| CD5 | | BD Bioscience |
| CD20 | PercP | BD Bioscience |
| CD38 | PE | BD Bioscience |
| IgD | | BD Bioscience |
| CD24 | APC | BD Bioscience |
| IgM | | BD Bioscience |

#### 3.1.1.1 Analysis of flow cytometric data

To identify the B cells subpopulations that has been analysed, gates (or encirclements) were set depending on which surface marker the sample is stained for. To identify the lymphocyte population, a gate was set as figure 3.2 shows using forward scatter (FSC) and side scatter (SSC). Next, the B cells were identified as $CD20^+$ (figure 3.3). Thereafter, within the $CD20^+$ B cells, gates for $CD24^{hi}CD38^{lo}$ memory, $CD24^{hi}CD38^{hi}$ transitional and $CD24^{lo}CD38^{lo}$ naive B cells were set (figure 3.4). To identify the population of $CD5^+$ B cell, a control $CD5^+$ B cell group within the lymphocytes was compared to the $CD5^+$ B cells within the $CD20^+$ B cells according to figure 3.5. Identification of $IgD^{hi}IgM^{lo}$, $IgD^{lo}IgM^{hi}$ and class-switched $CD20^+$ B cells were made shown in figure 3.6. The $CD27^+$ memory B cell population was identified by comparing a control of $CD27^+$ cells within the lymphocyte population with the CD27 expression within the B cell population (figure 3.7).

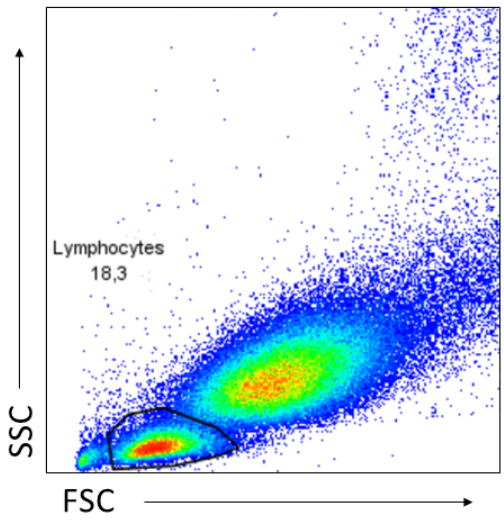


**Figure 3.2:** Lymphocytes were gated using FSC and SSC.

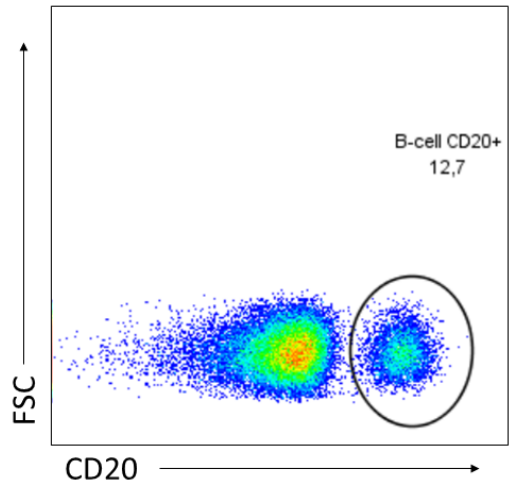


**Figure 3.3:** B cells were identified as expressing $CD20^{+}$, to the far right of the plot. To the left of the dot plot, other lymphocytes can be seen.

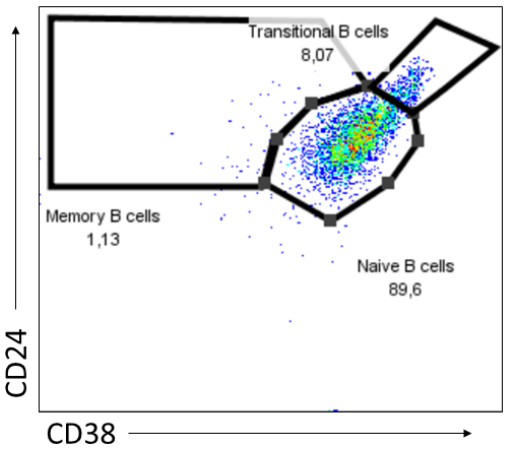


**Figure 3.4:** Within the $CD20^{+}$ B cell population, memory $CD24^{hi}CD38^{lo}$, transitional $CD24^{hi}CD38^{hi}$ and naive $CD24^{lo}CD38^{lo}$ B cells are gated. Low proportion of memory B cells and higher proportion of naive B cells is common among infants.

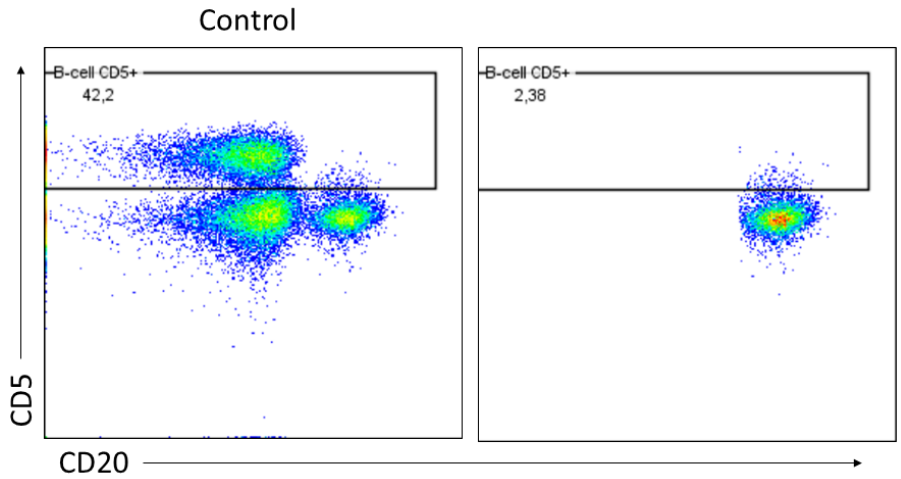


**Figure 3.5:** The gate for $CD5^+$ B cells was determined by comparing the expression of CD5 on the lymphocyte population, in which a clear $CD5^+$ population was observed compared to the $CD20^+$ B cells on the right plot. This gate was then transferred to the B cell, allowing the identification of $CD5^+$ B cell population.

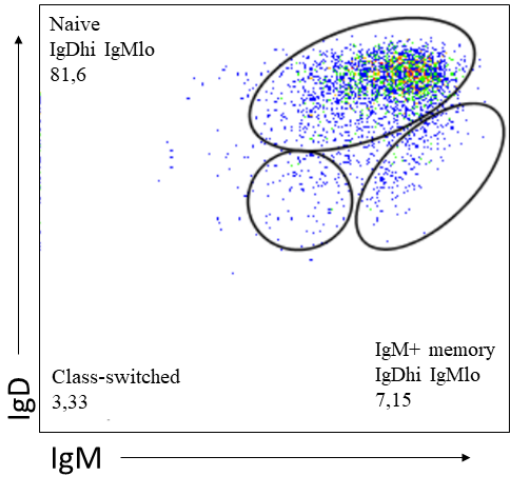


**Figure 3.6:** $IgD^{hi}IgM^{lo}$, $IgD^{lo}IgM^{hi}$ and class-switched $CD20^+$ B cells were gated as shown. $IgD^{lo}IgM^{hi}$ B cells is developed from $IgD^{hi}IgM^{lo}$ B cells and will then be class-switched.

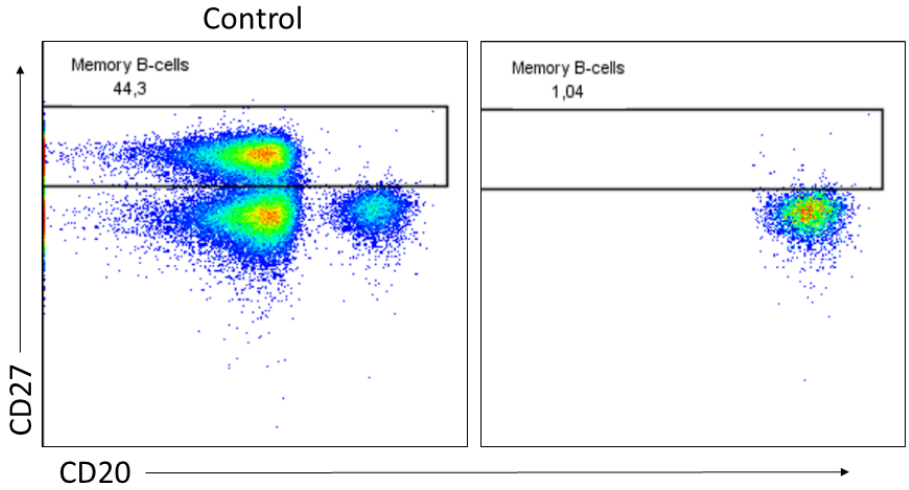


**Figure 3.7:** The gate for $CD27^+$ memory B cells was set by comparing the expression of CD27 on the lymphocyte population, in which a clear $CD27^+$ population was observed. This gate was then transferred to the B cell population, allowing the identification of $CD27^+$ B cell population.

To determine the number of cells/ml blood for each subgroup, analysis of $CD20^+$ B cells with the method TruCount was used. The gate strategy for $CD20^+$ B cells is shown in figure 3.8. The absolute cell number was then calculated with equation 3.1. Analysis on the flow cytometric data from blood samples collected at birth, 48h, 1 and 4 months of age were performed with the use of Flow Jo software version 10.

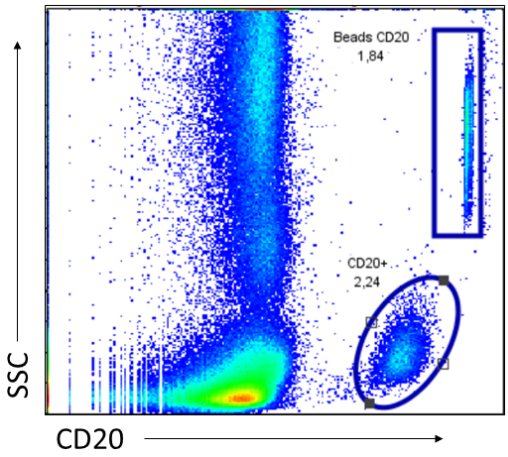


**Figure 3.8:** Gates for beads and $CD20^+$ were set as the figure shows. Amount of beads should be approximately 5000 with aim to receive a reliable amount of cells to analyse.

$$\frac{\text{amount of cells}}{\text{amounts of beads}} * \frac{\text{beads variable}}{\text{blood volume in ml}} \tag{3.1}$$

### 3.1.2 Statistic analysis

To study the relations between the several variables, multivariate factor analysis (SIMCA-P$^+$ software version 16) was applied. This method was used as a first step to observe patterns before further analysis between specific variables were made [13]. Orthogonal projection to latent structures (O-PLS) is a method used to study X-variables relation to a specific Y-variable, with aim to indicate what relationships that would be interesting to study further [24]. In this thesis, the Y-variable consists of the different allergies diagnosed in the NICE study and the flow cytometric data were applied as X-variables. If positive association between Y and X, the bars point at same direction. In contrast, if the bars are pointed at opposite directions, there is a negative association [13].

After O-PLS, further analysis with GraphPad Prism (software version 8) was performed as a complement to the multivariate factor analysis, when analysing specific relationships. Kruskal-Wallis test, followed with Dunn´s multiple comparison test was used to test the relationship between several variables. Univariate analysis was implemented to study the associations between two variables. A correlation coefficient (r) near 1, indicated a high positive correlation. Whereas, r near -1 suggested a inverse relation between the variables. r=0, no correlation between the variables [13]. In this thesis, Spearman´s rank correlation test was used. The significance (p) levels used are shown in table 3.2.

**Table 3.2:** Levels of significance (p) when using Spearman´s rank and Kruskal-Wallis test, followed by Dunn´s multiple comparison test.

| * | ** | *** | **** |
|---|---|---|---|
| $P \leq 0.05$ | $P \leq 0.01$ | $P \leq 0.001$ | $P \leq 0.0001$ |

# 4

# Result & Discussion

## 4.1 Maturation of B cells

First the proportions of naive cells were studied within the B cell population. The majority of B cells in infants, independent of which markers use to detect them, were naive (figure 4.1). However, the percentage of naive B cells detected with the use of IgD and IgM (figure 4.1 a), were higher compared to those detected with CD24 and CD38 (figure 4.1 b). However, the percentage of $IgD^{hi}IgM^{lo}$ increased from birth to 48h of life (p=0.04), and then decreased from 48h to 4 months (p=0.03). The percentage of $CD24^{lo}CD38^{lo}$ B cells stays stable from birth up to 48h, but were then decreased significantly from birth and 48h to 1 and 4 months of life (p<0.0001).

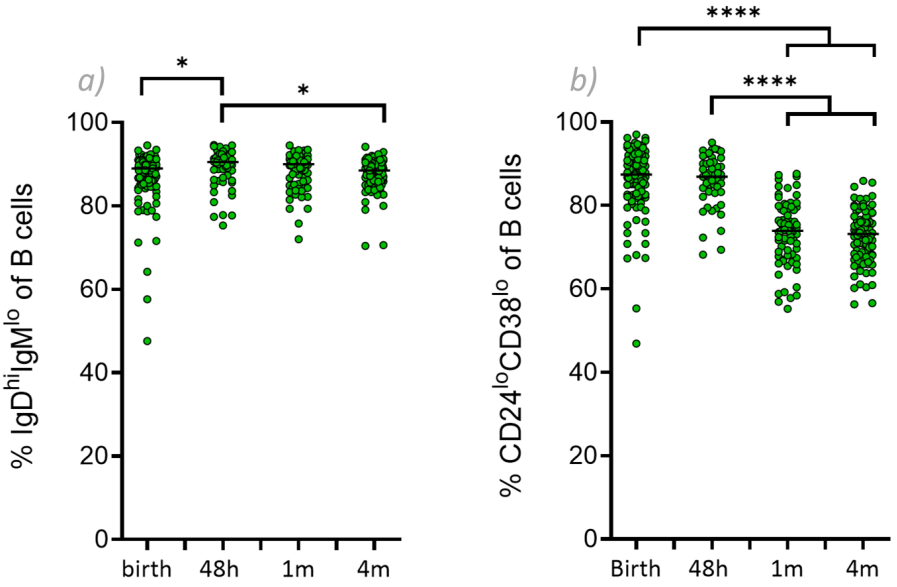


**Figure 4.1:** a) Proportion of $IgD^{hi}IgM^{lo}$ of B cells at birth, 48h, 1 and 4 months. b) Proportion of $CD24^{lo}CD38^{lo}$ B cells at birth, 48h, 1 and 4 months.

Regarding the immature transitional B cells, a quite stable percentage of $CD5^+$ between birth and 48h can be seen in figure 4.2 a. A decrease occurred from 48h to 1 ($p=0.04$) and 4 months ($p<0.0001$) of life. As illustrated in figure 4.2 b, an overall increase of the percentage of $CD24^{hi}CD38^{hi}$ B cells was observed from birth and 48h up to 1 and 4 months ($p<0.0001$) of life.

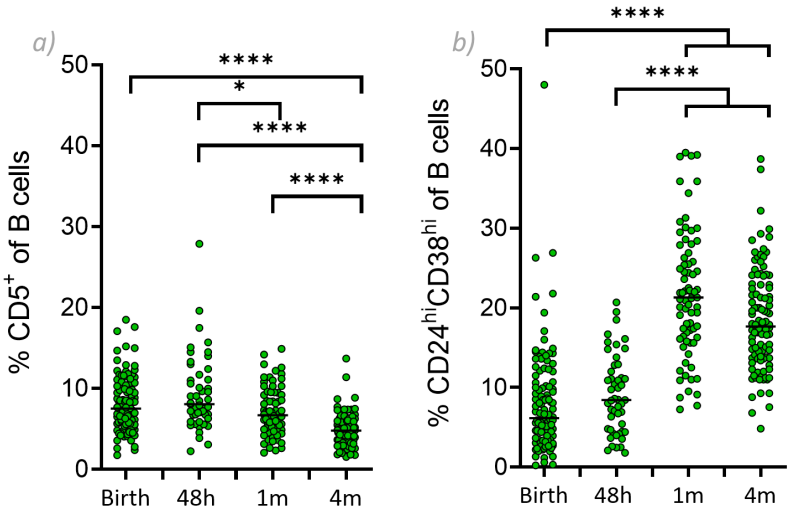


**Figure 4.2:** a) Percentage of $CD5^+$ of B cells at birth, 48h, 1 and 4 months. b) The proportion of $CD24^{hi}CD38^{hi}$ of B cells from birth up to 4 months.

In the farmflora study [17], the authors found an overall higher percentage of $CD5^+$ B cells during the first 3 years of life. Furthermore, the children in the study had their highest percentage of $CD5^+$ B cells at 4 months of age, whereas children in the NICE study seem to peak at birth and the following 48h in life (see figure 4.2 a). The difference in the proportion of $CD5^+$ B cells in the two studies could be due to different fluorochromes used to detect CD5. In the farmflora study APC was used, whereas FITC was used in NICE (see table 3.1). The differences in fluorochromes used, might affect the staining of $CD5^+$ B cells. Either, the FITC fluorochrome is weaker than APC and its possible that low expressing $CD5^+$ B cells are mistaken for negative $CD5^+$ B cells. It might also be that the APC staining is to strong and the low expressing $CD5^+$ B cells in the farmflora study, could in fact be negative for CD5 thus, resulting in higher percentage and number of $CD5^+$ B cells.

The NICE study is the first study to use CD24 and CD38 as surface markers for immature transitional B cells that early in life. In a study by Morbach et al [25], transitional B cells was analysed as $CD24^{hi}CD38^{hi}$ B cells, but at every fifth year from birth up to adult. Thus, it is hard to compare the studies with each other. Therefore, it is difficult to evaluate the increase of percentage of $CD24^{hi}CD38^{hi}$ B cells from birth and 48h up to 1 and 4 months of life.

Figure 4.3 shows the maturation of the percentage of memory B cells from birth up to 4 months when stained for the surface markers IgD and IgM, CD27 as well as CD24 and CD38. First, the $IgD^{lo}IgM^{hi}$ B cells, i.e. the $IgM^+$ memory B cells, were studied (figure 4.3 a). There was an increase of the $IgM^+$ B cells during the first 48h of life ($p<0.0001$). Thereafter, the percentage of $IgM^+$ memory B cells decreased to 3 % at 1 and 4 months of age ($p<0.0001$). These results might reflect an encounter with antigen and consequently activation of B cells into $IgM^+$ memory B cells, already during late pregnancy.

There was an overall increase of $CD27^+$ memory B cells from birth up to 4 months of age ($p=0.006$). However, a decrease was observed between 48h and 1 month ($p=0.03$), that then increased from 1 to 4 months ($p=0.0007$) (figure 4.3 b). Accordingly, the proportion of $CD24^{hi}CD38^{lo}$ B cells increased as well from birth up to 48h ($p=0.0005$), and then continued to increase during the first 4 months ($p<0.0001$) (figure 4.3 c).

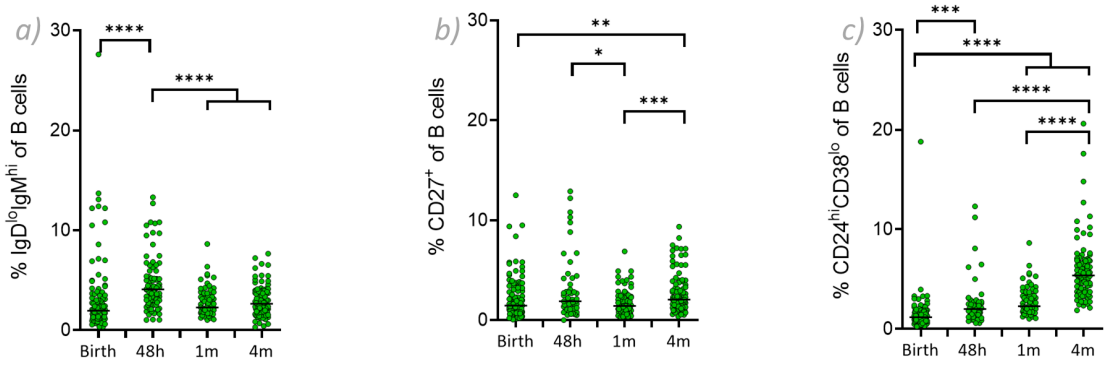


**Figure 4.3:** a) The development of percentage of memory $IgD^{lo}IgM^{hi}$ B cells from birth to 4 months. b) Proportion of $CD27^+$ of B cells from birth up to 4 months. c) Development of percentage of $CD24^{hi}CD38^{lo}$ from birth up to 4 months.

When studying the overall memory B cell population ($IgM^+$ not included), independent of which surface marker were used to identify memory B cells, there was an increase of these B cells population during the first 4 months of life (figure 4.3 b and c). However, in the farmflora study [17], the percentage of $CD27^+$ B cells were quite stable the first 4 months of life. Its possible that this difference is due to $CD27^+$ B cells and not $CD24^{hi}CD38^{lo}$ during early childhood. In the NICE study it is for most the $CD24^{hi}CD38^{lo}$ memory B cells that increase.

An overall increase of the proportion of memory B cells from birth to 4 months of age can be explained by the environmental factors that the child is exposed to, which trigger the immune system to mature. For instance, the bacterial colonisation of the gut occurs during the first year of life [26]. The decrease of the proportion between 48h and 1 month can indicate that other sub populations of B cells are increasing, which is reflected in that we study percentage and not actual number of B cells. Therefore, it would be interesting to analyse how the number of memory B cells are changing from birth up to 4 months.

## 4.2 Correlations between surface markers

In the NICE study different cell surface markers were used to detect same subpopulations of B cells. Although, similar results are theoretically expected independent of the surface markers used. Figures 4.1, 4.2 and 4.3 show that the percentage of naive, transitional and memory B cells differ from each other when studied with different combination of surface markers. This could of course depend on the human factors when analysing the flow cytometric data and evaluating how to set the gates (see figure 3.4 and 3.6). If not done consequently, with proper definitions and controls, the proportion of each subgroup could vary between the different panels. However, a liable reason for the difference of values might in fact be that the surface markers used to identify the subgroups have different functions in the B cells. Thus, their expression might not only be expressed during one phase of the B cells development, but to some extent during another phase of B cell maturation.

Therefore, to compare if the different combination of surface markers used to identify each subgroup could represent the same subgroup, the percentage of naive, transitional and memory B cells identified with different combination of cell surface markers were correlated to each other (figures 4.4, 4.5, 4.6, A.1 and A.2).

As shown in figure 4.4 a, the proportion of $IgD^{hi}IgM^{lo}$ and $CD24^{lo}CD38^{lo}$ correlated highly at birth (r=0.36, p=0.0001). However, the correlation then decreased with age, and did not correlate at all at 4 months of life (figure 4.4 b-d). It is possible that the decrease of correlation with age was depended on that the proportion of $IgD^{hi}IgM^{lo}$ naive B cells also included transitional B cells which also express IgD and IgM [27]. As the proportion of transitional B cells increases with age, the use of IgD and IgM to identify naive B cells might not be optimal.

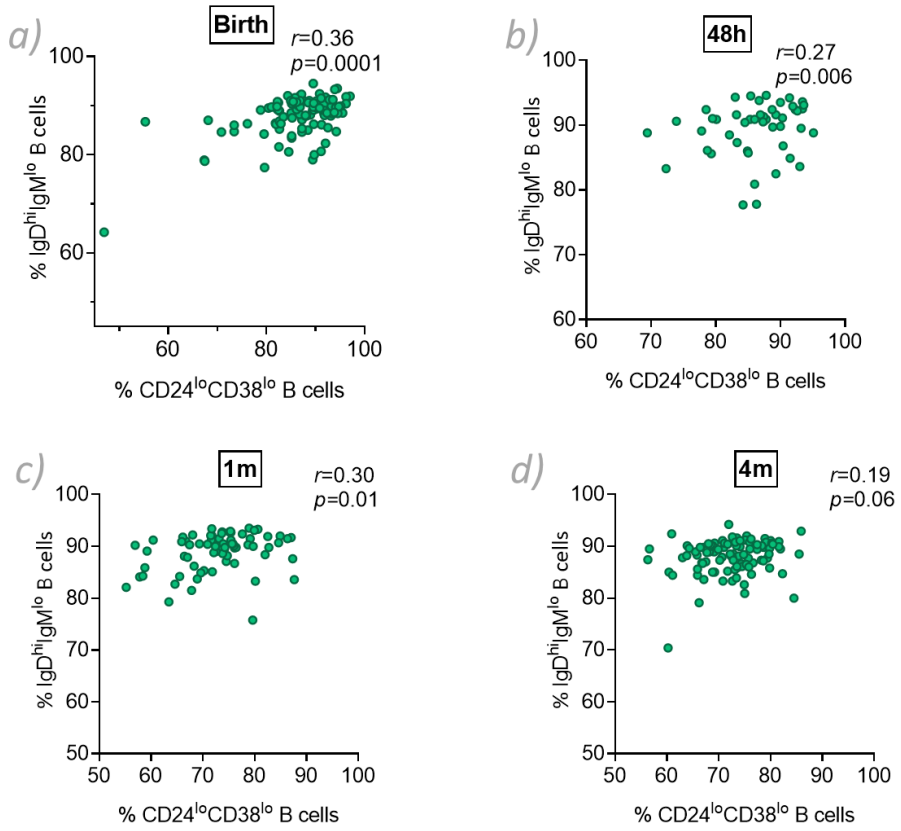


**Figure 4.4:** Correlation between percentage of $IgD^{hi}IgM^{lo}$ of B cells and percentage of $CD24^{lo}CD38^{lo}$ of B cells at birth (a), 48h (b), 1 month (c) and 4 months (d).

As shown in figure 4.5, there was a low correlation between percentage of $CD5^+$ and $CD24^{hi}CD38^{hi}$ at birth, 48h and 4 months of life. Thus, although it has been suggested in a previous study that $CD5^+$ B cells are immature transitional B cells [17], the results in this thesis does not support it. It is possible that the difference in proportion between $CD5^+$ and $CD24^{hi}CD38^{hi}$ that increases with age (see figure 4.2) depends on that the fluorochrome FITC do not stain the weakly expressed CD5 B cells. As discussed in section 4.1, there are also differences in proportion of $CD5^+$ when compared with results from the farmflora study [17].

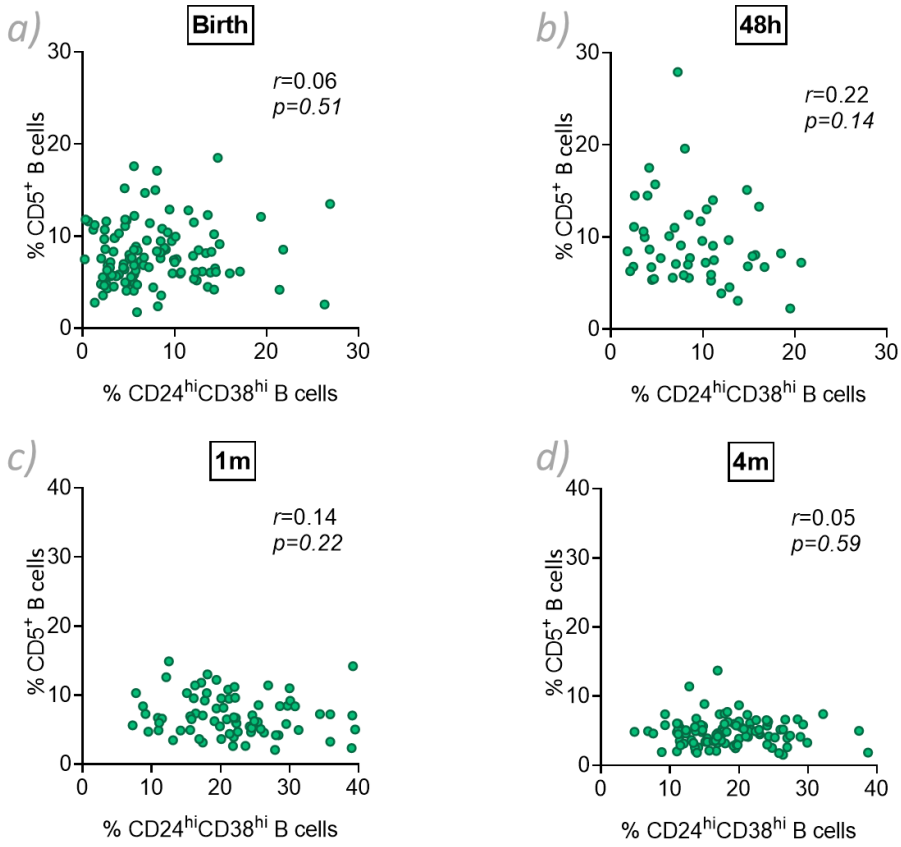


**Figure 4.5:** Percentage of $CD5^+$ of B cells correlated with percentage of $CD24^{hi}CD38^{hi}$ of B cells at birth (a), 48h (b), 1 month (c) and 4 months (d).

At birth, the percentage of $CD27^+$ and the percentage of $CD24^{hi}CD38^{lo}$ are correlated with r=0.31 and p=0.001 (figure 4.6). The correlation is even higher at 48h with r=0.46 and p=0.0009. At 1 and 4 months the correlation is lower with r=0.27 and p=0.03, respectively, and r=0.24 and p=0.01, respectively.

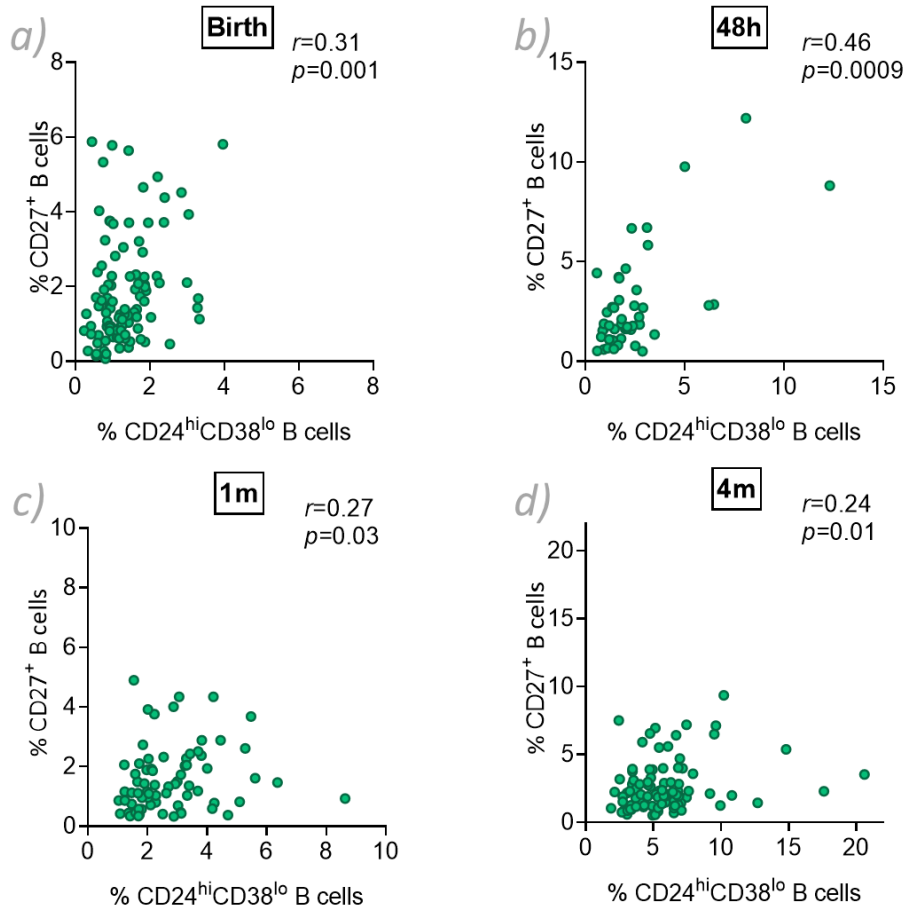


**Figure 4.6:** Correlation between percentage of $CD27^+$ of B cells and percentage of $CD24^{hi}CD38^{lo}$ of B cells at birth (a), 48h (b), 1 month (c) and 4 months (d).

When comparing the relation between the proportion of memory B cells identified with CD27 against CD24 and CD38, there was a significant correlation between the populations (figure 4.6). However, there was no correlation when studying the surface markers $CD24^{hi}CD38^{lo}$ against $IgD^{lo}IgM^{hi}$ or $CD27^+$ against $IgD^{lo}IgM^{hi}$ (appendix figures A.1 and A.2). This most probably is due to that $IgD^{lo}IgM^{hi}$ B cells are $IgM^+$ memory B cells, i.e. B cells that have been activated in absence of $CD4^+$ T cells and thus not gone through class-switch recombination. CD27 is expressed by class-switched cells and thus, the two cell populations should not correlate although they both are memory B cells.

To conclude the correlation tests between percentage of different surface markers for subpopulations of B cells, the correlation that showed significance from birth up to 4 months was the percentage of $CD27^+$ correlated with the percentage of $CD24^{hi}CD38^{lo}$ (figure 4.6). These two surface markers could thus be used together to detect memory B cells. The percentage of $IgD^{hi}IgM^{lo}$ and $CD24^{lo}CD38^{lo}$ did show significance up to 1 month, and p=0.06 at 4 months (figure A.1). If further analysis can be done on data from when the children are older, maybe $IgD^{hi}IgM^{lo}$ and $CD24^{lo}CD38^{lo}$ can be used together to detect naive B cells.

From the TruCount analysis of samples at birth, correlation test between the number of subpopulations could also be performed. Number of naive B cells identified as $IgD^{hi}IgM^{lo}$ was correlated with number of $CD24^{lo}CD38^{lo}$ (figure 4.7 a). The same was done with the number of transitional B cells between the surface markers $CD5^{+}$ and $CD24^{hi}CD38^{hi}$ (figure 4.7 b), and for the number of memory B cells that identified as $CD27^{+}$, $CD24^{hi}CD38^{lo}$ and $IgD^{lo}IgM^{hi}$ (figure 4.8).

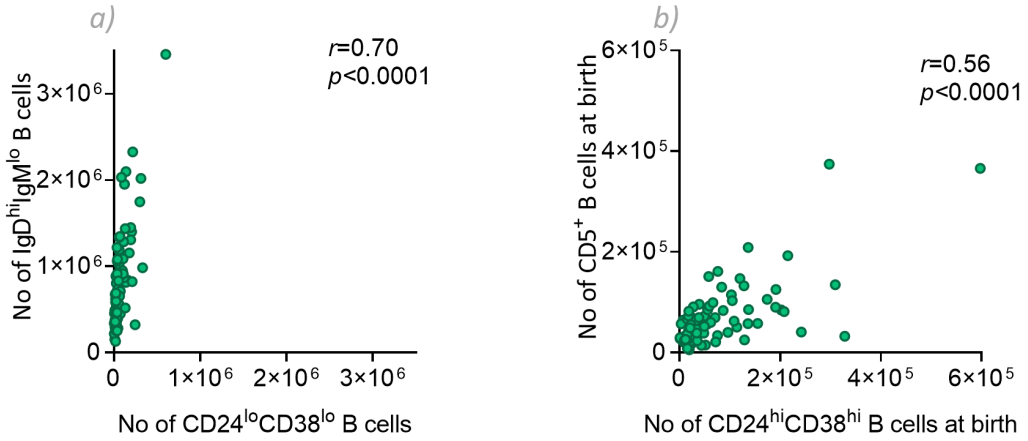


**Figure 4.7:** a) Correlation between number of $IgD^{hi}IgM^{lo}$ and number of $CD24^{lo}CD38^{lo}$ at birth. b) Correlation between number of $CD5^{+}$ and number of $CD24^{hi}CD38^{hi}$ at birth.

Interestingly, there was a high correlation between the number of naive B cells stained for $IgD^{hi}IgM^{lo}$ and $CD24^{lo}CD38^{lo}$ as seen in figure 4.7 b ($r=0.70$, $p<0.0001$). The same was observed regarding $CD5^{+}$ B cells compared to $CD24^{hi}CD38^{hi}$ B cells ($r=0.56$, $p<0.0001$).

As shown in figure 4.8, there was high correlations between the three different surface markers for number of memory B cells, i.e. $IgD^{lo}IgM^{hi}$, $CD27^{+}$ and $CD24^{hi}CD38^{lo}$ B cells.

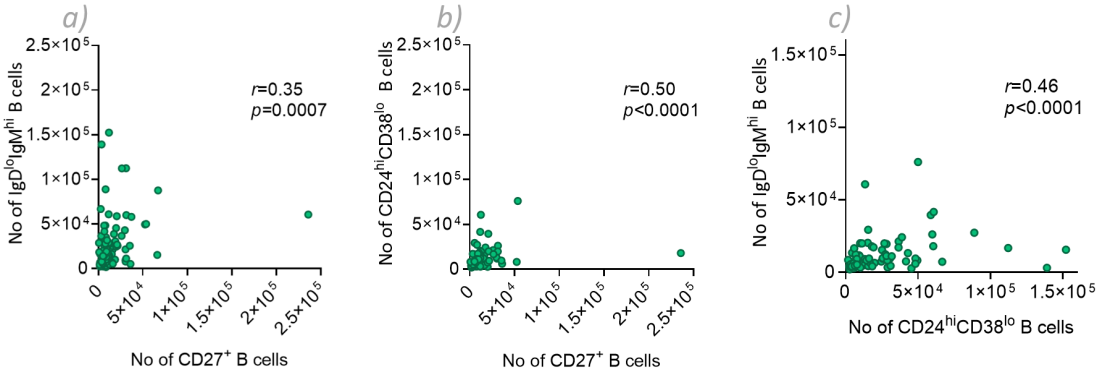


**Figure 4.8:** a) Correlation between number of $IgD^{lo}IgM^{hi}$ and number of $CD27^{+}$ at birth. b) Correlation between number of $CD24^{hi}CD38^{lo}$ and $CD27^{+}$ at birth. c) Correlation between number of $IgD^{lo}IgM^{hi}$ and $CD24^{hi}CD38^{lo}$ at birth.

Thus, although the different surface marker combinations used to identify naive, transitional or memory B cells did not show the same maturation pattern or correlate well when studied in percentage during the first 4 months of life (figures 4.1, 4.2, 4.3), they did correlate strongly when studying the number of cells. To better understand the differences found regarding the correlations when studying the percentage of B cell population in comparison to the number of B cell population at birth, it is important to study the number of different B cells populations and the different staining combinations at the later time points as well.

## 4.3 Allergy presence at 12 month

As of today, 452 of the 655 children included in the NICE study have been examined regarding allergy development and sensitisation. Of these children, 6.2 % were found to have eczema, 2.4 % were diagnosed with food allergy and 6.2 % were found to have asthma. 14.6 % have one or several allergies (see table 4.1).

**Table 4.1:** The table shows amount of children at 12 months of age that got examined by a doctor regarding allergy development and sensitisation. Also how many (amount and percentage) that were found to have any allergy.

| **Allergy diagnosis at 12 months** | **No of Children n=452** | **%** |
|---|---|---|
| Any allergy | 66 | 14.6 |
| Eczema | 29 | 6.2 |
| Food allergy | 11 | 2.4 |
| Asthma | 29 | 6.2 |

According to the atopic march, the proportion of children with eczema is used to be high at a age of 1-1.5 year, and will then decrease with age. The proportion of children with food allergy is also expected to be at its highest at 1.5 years of age, but not as high as the proportion of eczema [5]. That could explain the higher percentage of eczema compared to food allergy in the NICE study (table 4.1). The prevalence of asthma is usually most common when the children reached the age 10-15 years [5]. The high percentage of asthma in NICE is interesting as it reaches the prevalence of eczema in the children.

In the NICE study, 195 of the children are included in the subcohort in which there are results regarding B cell maturation. Thus, not all of the children diagnosed with allergy, will be included in the subcohort.

To analyse if B cells maturation from birth up to 4 months have any impact on allergy development at 12 months of age, O-PLS analysis was made to see any relations between the different allergies (table 4.1) and flow cytometric data at birth, 48h, 1 and 4 months. The figures A.3, A.4 and A.5 in Appendix A.1 shows that there is no clear relationships between the variables. Consequently, seen from the

O-PLS results, no significance will probably be seen when comparing different allergies with flow cytometric data from birth up to 4 months.

Figure 4.9 shows the number of children with and without eczema at 12 months of age and their percentage of transitional $CD24^{lo}CD38^{lo}$ B cells during their first 4 months of life. As predicted from the O-PLS analysis, no significance can be seen between children diagnosed with eczema and children with no allergy development at 12 months. Additionally, as the figure shows, there is a low number of children diagnosed with eczema in the subcohort study. Thus, the results in this thesis cannot conclude if there indeed is a difference of levels of transitional B cells during the first 4 months of life in children that develop eczema at 12 months of age.

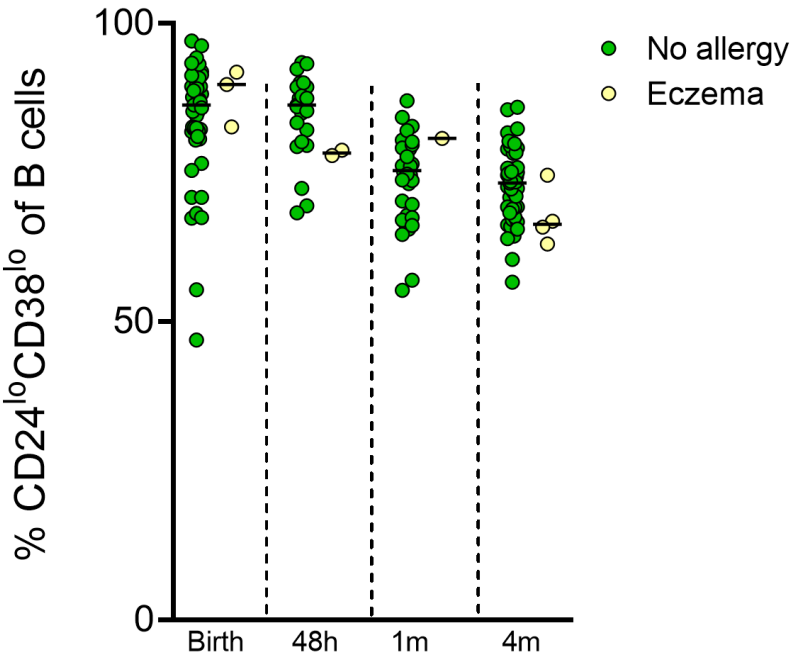


**Figure 4.9:** Percentage of $CD24^{lo}CD38^{lo}$ of B cells at birth, 48h, 1 and 4 months among children with eczema compared with children with no allergies at 12 months.

To conclude, a complement of the amount of immunological data by the subcohort needs to be done to get more data to compare with and thus, a more reliable result. It also would be interesting to do the analysis with the number of subpopulations and also, with data up to 12 months of age, when the children is examined for allergy development.

# 5
# Conclusion

In this thesis, I found that the percentage of different subpopulations of B cells from birth up to 4 months of life, could differ depending on what surface staining combination was used.

Thus, correlation tests between surface markers stains for the same subpopulation was performed to examine if the different surface markers could represent the same subpopulation of B cells. I found that the cell surface marker CD27 was comparable to the combination of CD24 and CD38, and thus both could be used equally to study the percentage of memory B cells. However, the different surface marker combinations used to detect naive and transitional B cells, did not correlate well and suggests that they are not equal when to study these two populations.

Eczema and asthma were the diagnosed allergies with highest prevalence at 12 months. According to the atopic march, the presence of eczema should be greatest. The diagnosis of asthma could therefore be questioned.

O-PLS showed low relationships between the different allergies and immunological data. Consequently, no significance could be seen when comparing $CD24^{lo}CD38^{lo}$ between children with eczema and no allergy.

Further analysis with number of cells, complementary of data and at data when the children is older should be done to see if the development of B cells subpopulations have any impact on allergy development.

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# A

# Appendix

## A.1 Correlation results

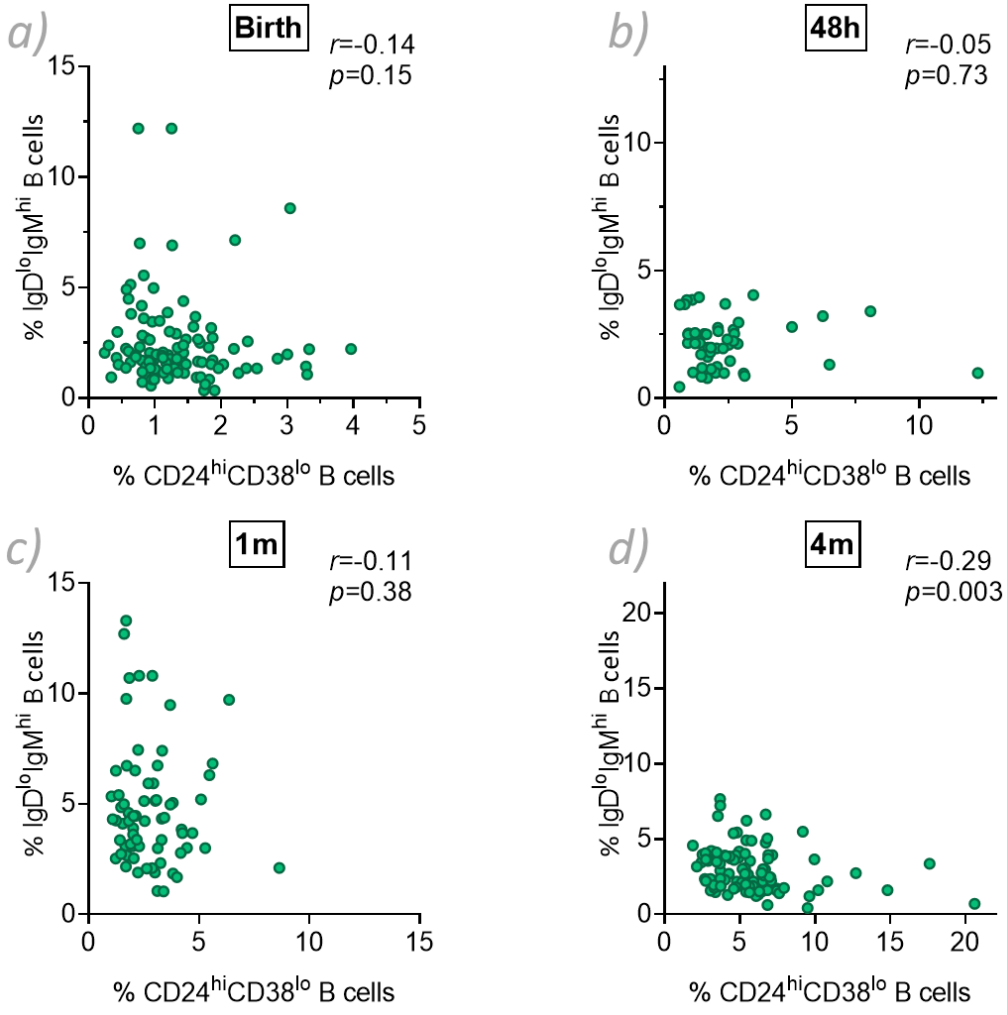


**Figure A.1:** Percentage of $IgD^{lo}IgM^{hi}$ of B cells correlated with percentage of $CD24^{hi}CD38^{lo}$ of B cells at birth (a), 48h (b), 1 month (c) and 4 months (d).

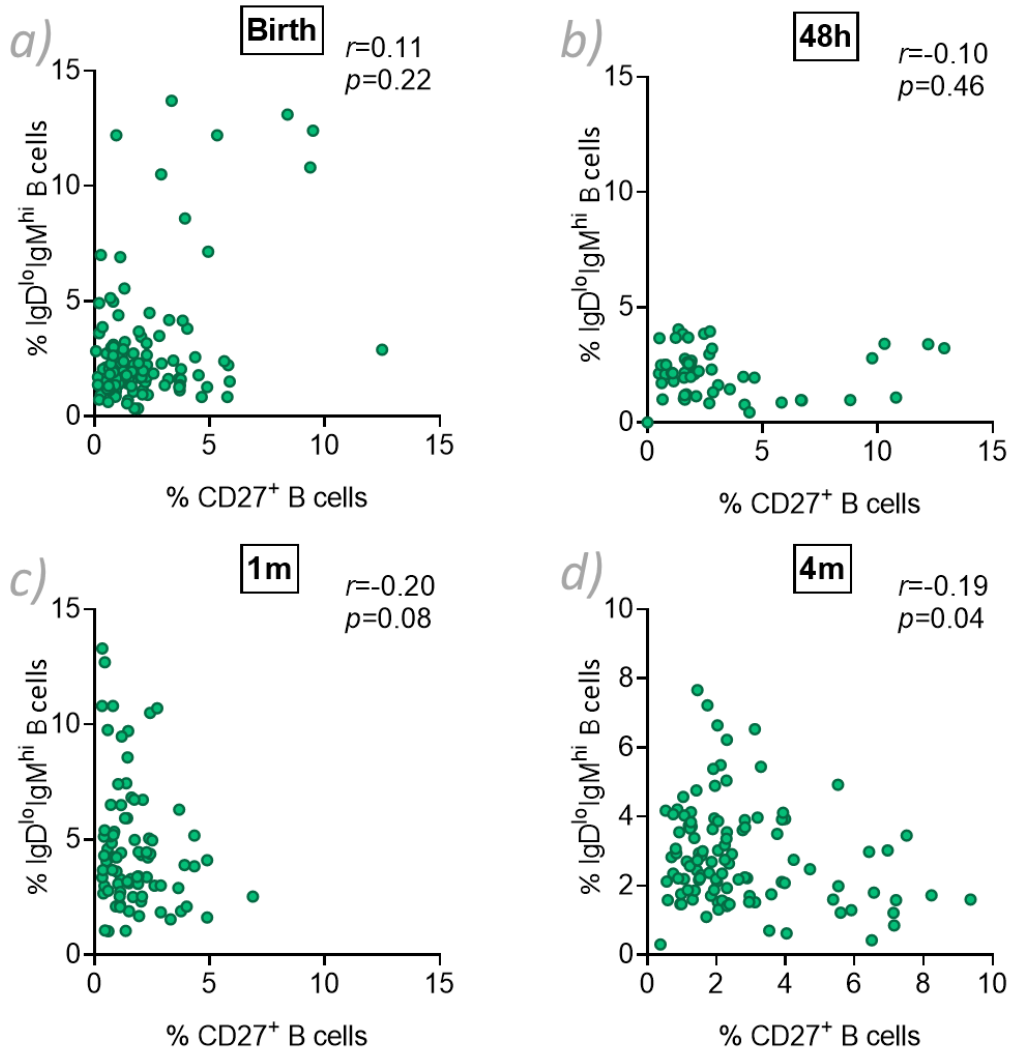


**Figure A.2:** Correlation between percentage of $IgD^{lo}IgM^{hi}$ of B cells and percentage of $CD27^{+}$ of B cells at birth (a), 48h (b), 1 month (c) and 4 months (d).

## A.2 O-PLS results

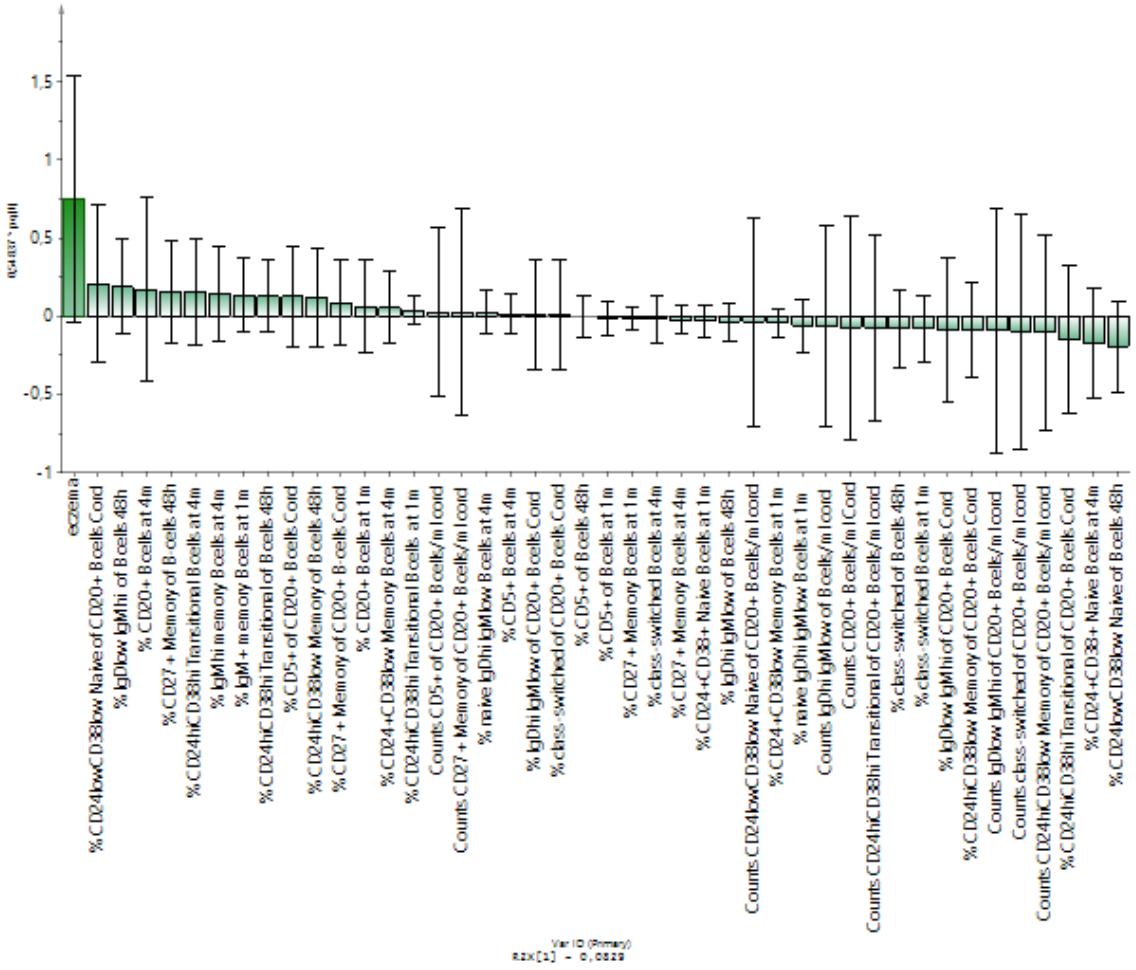


**Figure A.3:** Orthogonal projection to latent structures (O-PLS) analysis using eczema as Y-variabel and several immunological data on the X-variables.

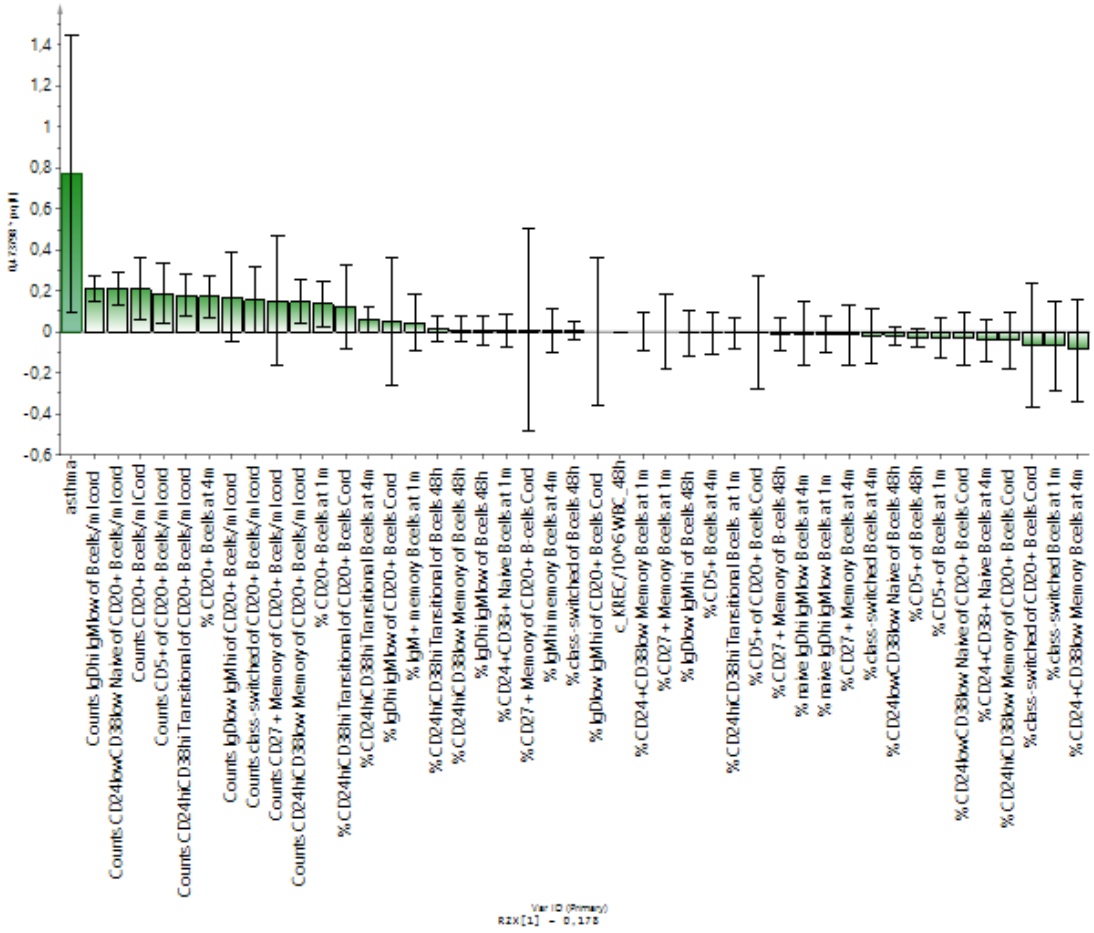


**Figure A.4:** Orthogonal projection to latent structures (O-PLS) analysis using asthma as Y-variabel and several immunological data on the X-variables.

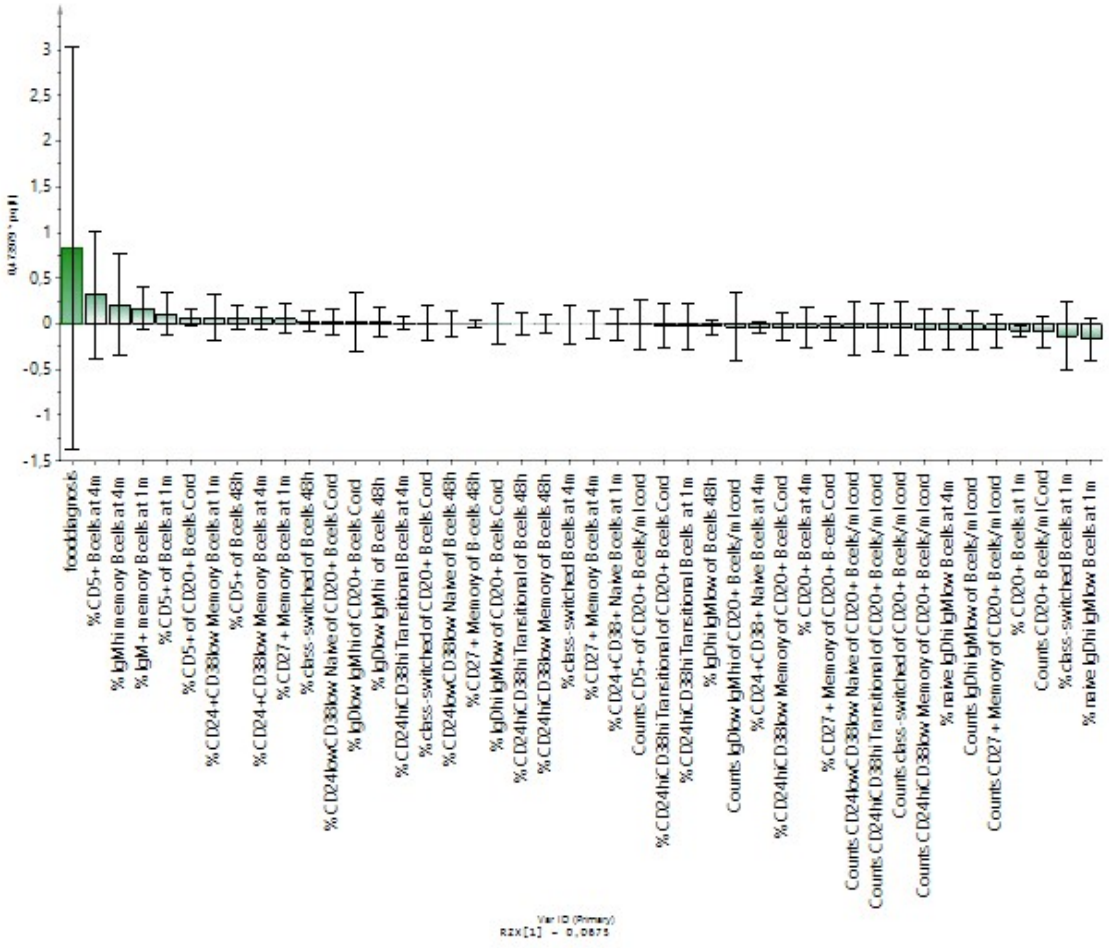


**Figure A.5:** Orthogonal projection to latent structures (O-PLS) analysis using food allergy as Y-variabel and several immunological data on the X-variables.