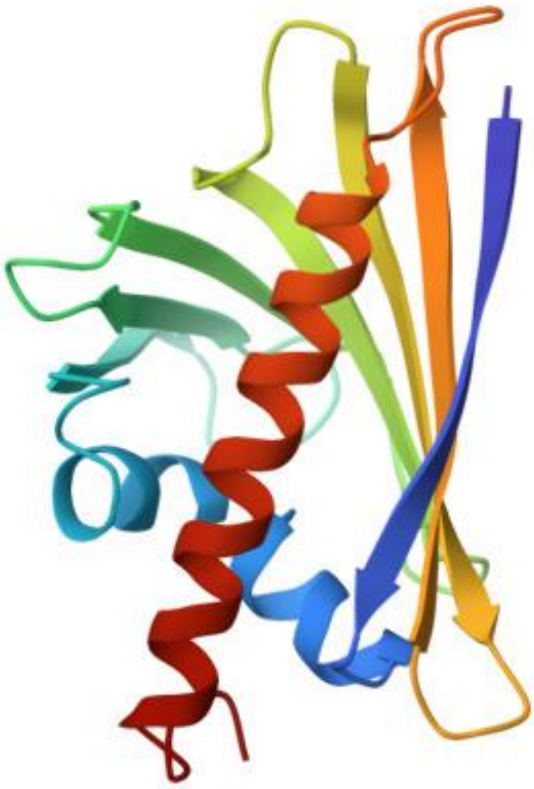
The receptors FcεRI and FcεRII (CD23) as promising target to monitor immunotherapy for birch and soy allergy



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WAGENINGEN
UNIVERSITY & RESEARCH

Rijnstate

The receptors FcεRI and FcεRII (CD23) as promising target to monitor immunotherapy for birch and soy allergy

Setting up a cell free assay to measure the effect of IgG4 antibodies on Bet v 1-IgE complexes binding to CD23 and a study to investigate if the blocking-antibody basophil activation test is a suitable method to measure the effect of allergen-specific immunotherapy on basophil sensitivity and reactivity to Bet v 1

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1027093

MSc Thesis Food Safety

Wageningen University and Research / Rijnstate

Cell Biology and Immunology

March 2024

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Rijnstate

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Abstract

The prevalence of allergic conditions such as birch allergy is increasing worldwide. In addition, most birch allergic patients develop a secondary food allergy for allergic proteins which share homology with the birch allergen Bet v 1. Allergen specific IgE antibodies bind to the receptors FcεRI on mast cells and basophils and FcεRII (CD23) on basophils. IgG4 produced by Allergen-Specific Immunotherapy (AIT) can inhibit IgE binding to CD23. In this study an Enzyme-Linked Immunosorbent Facilitated Antigen Binding (ELIFAB) assay was set up for Bet v 1 to measure the effect of IgG4 antibodies on the binding of allergen-IgE complexes to CD23. When using recombinant IgG4 and IgE monoclonal Antibodies (mAbs) specific for Bet v 1, a significant decrease in complexes binding to CD23 was observed. To my knowledge, this is the first study which found that a mix of three Bet v 1 specific IgG4 mAbs significantly inhibit Bet v 1-IgE complex binding via the low affinity receptor CD23. Furthermore this assay indicates that a mix of IgG4 mAbs is needed to target different epitopes for the inhibition of complexes binding to CD23. Therefore this mix of Bet v 1 specific IgG4 mAbs can be a possible method to treat birch allergy. However before using the ELIFAB in clinical studies, further optimization with sera is needed. Furthermore, IgG4 is able to inhibit IgE-mediated basophil degranulation via FcεRI and the inhibitory receptor FcγRIIb (CD32B). The Indirect Basophil Activation Test (iBAT) and the Blocking Antibody Basophil Activation Test (BA-BAT) were used to investigate the effect of AIT on the basophil sensitivity and reactivity to Bet v 1. The BA-BAT found a significant increase in half maximal effective concentrations (EC50) for patients who received birch immunotherapy. This indicates that the BA-BAT is a promising tool to investigate the effect of AIT for Bet v 1. In addition, Bet v 1 specific IgG and IgE levels can be quantified by an Enzyme-Linked Immunosorbent Assay (ELISA). However, since IgG levels specific for Bet v 1 vary widely in individuals, optimization of the assay is recommended. The results of this study showed that the ELIFAB and BA-BAT which investigate the effect of IgG4 on respectively the receptors CD23 and FcεRI are promising methods for future research to monitor the effect of immunotherapy for birch and soy allergy.

Preface

Most of my university study books which I used for my bachelor and master courses did not stay long in my student room after an exam. However this was not the case for my immunology study book. The immune system is an interesting and complex system of which you never know enough about. Especially the interaction between nutrition and the immune system fascinates me. This was why I started my MSc thesis at the chair group Cell Biology and Immunology about allergies. This thesis project also gave me the possibility to combine research at Wageningen University and Rijnstate hospital, where I also enjoyed my side job since the start of my studies. Therefore this project taught me a lot about the possibilities of multidisciplinary work, which I really liked. In addition, this research showed me many aspects of the fascinating world of human immunology. I will never forget how to create beautiful immune complexes in the lab. I enjoyed working together with a lot of different people in this interesting project. I would like to thank Joost van Neerven, Gosia Teodorowicz and Janneke Ruinemans-Koerts for your enthusiasm and supervision during the past six months, I learned a lot from all of you. I would also like to thank Chantal van Everdingen and Mojtaba Porbahaie for the interesting working discussions. Furthermore I would like to thank the technicians Yvonne Smidt, Monique van Uum, Clemens Elshof and Koen van Riessen for helping me in the lab and Gerben Ferwerda for providing patient samples and giving me inside in allergy treatment in Rijnstate. Finally I would like to thank my lovely housemates Celine, Chiara, Manon, Giselle, Vivian and Meike for their great support and the nice study sessions together.

Abbreviations

AIT	Allergen-Specific Immunotherapy
3D	Three-dimensional
BA-BAT	Blocking Antibody Basophil Activation Test
CH	Constant domain heavy Chain
CL	Constant domain light chain
DC	Dendritic Cells
EBV	Epstein-Barr Virus
EC50	Half maximal effective concentration
EDTA	Ethylene Diamine Tetraacetic Acid
ELIFAB	Enzyme-Linked Immunosorbent Facilitated Antigen Binding
ELISA	Enzyme-Linked Immunosorbent Assay
FAB	Fragment Antigen-Binding
FAE	Fab Arm Exchange
FAP	Facilitated Antigen Presentation
FcγR	Fcy Receptors
iBAT	indirect Basophil Activation Test
IL-13	Interleukin-13
IL-4	Interleukin-4
IL-5	Interleukin-5
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
mAbs	monoclonal Antibodies
MHC	Major Histocompatibility Complex
OAS	Oral Allergy Syndrome
OFC	Oral Food Challenge
OIT	Oral Immunotherapy
PR	Pathogenesis-Related
SAM22	Starvation Associated Message 22
SCIT	Subcutaneous Immunotherapy
SLIT	Sublingual Immunotherapy
T_H2	T Helper 2
TMB	3,3’,5,5’-Tetramethylbenzidine
Tregs	Regulatory T cells
VH	Variable domain heavy chain
VL	Variable domain light chain

1. Introduction

1.1 Allergic reactions

The prevalence of allergic conditions such as asthma, allergic rhinitis, skin allergies is increasing worldwide (Gutowska-Ślesik et al., 2023). Birch pollen is the main type of pollen present in Northern and Central Europe and is a leading cause of allergic rhinitis and perhaps, asthma symptoms (Caillaud et al., 2014; Canova et al., 2013; M. Smith et al., 2014). Due to climate change, the levels of birch pollen and the time of exposure have increased in the last few years (Frei & Gassner, 2008). In addition, many plant food allergens carry homologs of the major birch allergen Bet v 1, therefore birch allergic patients often have IgE against plant derived foods (Wensing et al., 2002).

Between 1997 and 2011, the prevalence of food allergy in children has increased by 50 percent (Ramsey et al., 2019). The largest evidence for the increase in food allergies is found for IgE-mediated food allergies (Tang & Mullins, 2017). The symptoms of food allergies differ in severity and can result in symptoms in almost every organ system (the skin, respiratory tract, gastrointestinal, cardiovascular, and the neurological system (H-K Ho et al., 2014; Sampson et al., 2006). The most severe symptom can cause life threatening reactions such as anaphylactic shock (FDA, 2015).

IgE is produced by B lymphocytes and plasma cells. An increase of IgE levels in bloods indicates the sensitization and predisposition to an allergic reaction. Dendritic Cells (DC) can take up allergens and present it to T cells via Major Histocompatibility Complex (MHC) class II molecules. T cells are then activated which result in proliferation into T Helper 2 (T_H2) cells. T_H2 cells are crucial since they produce the cytokines Interleukin-4 (IL-4), Interleukin-5 (IL-5) and Interleukin-13 (IL-13), which are needed for class switching of the B cell to become IgE producing plasma cells (Curotto de Lafaille et al., 2010; Poulsen & Hummelshoj, 2007). When an IgE-mediated allergic reaction occurs, allergen specific IgE antibodies bind to the IgE-specific receptors FcεRI on mast cells and basophils and CD23 on basophils. Allergen exposure results in cross linking of receptor bound allergen-specific IgE antibodies. This aggregation causes an intracellular signal cascade which will result in the release of histamine and other inflammatory mediators such as cytokines. This will result in an immediate allergic reaction (Li et al., 2022; Saab & Jones, 2022; Wachholz et al., 2005).

1.2 Cross reactivity Gly m 4 – Bet v 1

Cross reactivity occurs when an individual is primarily sensitized and allergic to an allergen but also allergic to another allergen which is structurally similar. In this case, the immune system cannot discriminate between structurally similar allergens, which results in the occurrence of an allergic reaction (Curciarello et al., 2008). Allergens can contain sufficiently similar IgE epitopes and can react with the same IgE molecules. IgE epitopes can be conformational or linear. A linear epitope is composed of continuous epitopes. However, conformational epitopes are composed of discontinuously allocated amino acids, which come closer when the protein is correctly folded. Therefore conformational epitopes are determined by the three-dimensional (3D) structure of the protein (Pomés, 2010; Power et al., 2013; Schein et al., 2010; Varshney et al., 2007). Cross reactivity occurs when their sequence comparisons of allergens reveal a high sequence identity which also indicates that the allergens have a similar 3D fold structure (Negi & Braun, 2017).

One example of cross reactivity is between birch and soy. In 1992, the Bet v 1 homologous protein Gly m 4 from soybean was detected on the messenger RNA level (Crowell et al., 1992). This protein

is formed under stress conditions such as starvation caused by the protein Starvation Associated Message 22 (SAM22). Later research concluded that SAM22 is responsible for the allergic reaction to soybean in birch pollen allergic patients (Kleine-Tebbe et al., 2002). In addition, the structure of Gly m 4 is partly similar to the Pathogenesis-Related (PR) 10 proteins of yellow lupine. The local structure of Gly m 4 that indicates the differences between Gly m 4 and Bet v 1 is also found in the proteins from yellow lupine. Therefore, Gly m 4 can also give insight into the cross reactivity of PR10 proteins (Berkner et al., 2009).

1.3 Birch and soy allergy

Birch pollen can cause allergic rhinitis and asthma. The birch homologous group consists of birch and the other trees of the families *Betulaceae* and *Fagaceae.* 90% of all birch allergic patients response towards the major birch allergen Bet v 1 (Roth-Walter et al., 2014). Bet v 1 is a 17kDA protein and belongs to the group of PR10 proteins (Schenk et al., 2009). Bet v 1 is responsible for cross reactivity to Bet v 1 related PR10 molecules in pollen of early flowering trees and in some plant derived foods (Elisyutina et al., 2019). Approximately 70% of the people with birch allergy develop Oral Allergy Syndrome (OAS), which is defined as a secondary food allergy caused by several vegetables, raw fruits and nuts which include allergic proteins which share 50-80% homology with bet v 1 (Geroldinger-Simic et al., 2011; Laffer et al., 1996; Price et al., 2015).

Soybean is most commonly used as a vegetarian protein source. The consumption of soybean has several nutritional and health benefits. It can for example reduce the risk of breast and prostate cancer and coronary heart disease (Messina, 2016). However, soy can also cause allergic reactions. Currently soy causes 0.2-0.4% of all the allergic reactions (Wiederstein et al., 2023). The risk of a soybean allergy is increasing since the use of soybean has increased largely in food products. There are at least 16 allergic protein fractions which can result in IgE-binding (Wilson et al., 2008). There are two food allergy classes for soy, class 1 and class 2 food allergies. Class 1 causes food allergy via primary sensitization and class 2 causes allergies via cross-reaction via pollen or other leguminous allergens. Gly m 5, Gly m 6, Gly m 7, Gly m Bd 30K and Kunitz trypsin inhibitor belong to the class 1 food allergies while Gly m 3 and Gly m 4 belong to the class 2 allergens (Kattan et al., 2011; Matsuo et al., 2020).

1.4 Low affinity receptor CD23

IgE interaction with allergens results in the release of inflammatory mediators via allergen-mediated cross linking. IgE immune complexes can bind to two receptors, FcεRI, which is a high-affinity receptor and CD23, which binds with a lower affinity (Engeroff & Vogel, 2021). CD23 has been left out in allergy research in the past (Conrad et al., 2007), which is probably because CD23 is involved in different immunological processes (Acharya et al., 2010). CD23 is a member of the calcium-dependent (C-type) lectin family (Engeroff & Vogel, 2021) and CD23 plays an important role in IgE synthesis. However, the exact process causing this effect is still in debate. Down regulation of IgE synthesis can occur in human B cells by direct targeting of CD23 (Payet-Jamroz et al., 2001). This indicates there is a mechanism of positive or negative feedback depending on the concentration of IgE (Fellmann et al., 2015). CD23 cleavage can also cause stimulation of IgE synthesis by working on other cells as soluble CD23. However, the mechanisms for this process are not clear. It is possible that soluble CD23 stimulated IgE synthesis by cross linking membrane IgE and CD21 (McCloskey et al., 2007).

1.5 IgE-Facilitated Antigen Presentation (FAP)

The process of antigen presentation regulated by CD23 can be defined as IgE-Facilitated Antigen Presentation (FAP) (van der Heijden et al., 1993). Primary human B cells cannot directly regulate T cell proliferation, but they can move the IgE immune complexes to human dendritic cells to start T cell proliferation (Engeroff et al., 2018) (figure 1). This T cell proliferation results in the release of proinflammatory T_H2 cytokines already starting at low allergen concentrations. Therefore allergen IgE complexes binding to CD23 can play a role in T cell mediated allergic inflammation in vivo (Van der Heijden et al., 1995). Furthermore, CD23 mediated FAP can also result in IgG responses to unconnected allergens via the mechanism of epitope spreading (Gould & Sutton, 2008). In addition, DC can bind to various unrelated antigens via the Fcγ Receptors (FcγR) which results in antibody response to connected allergens. This mechanism can illustrate the progress of allergen poly sensitivity to different allergens (Engeroff & Vogel, 2021).

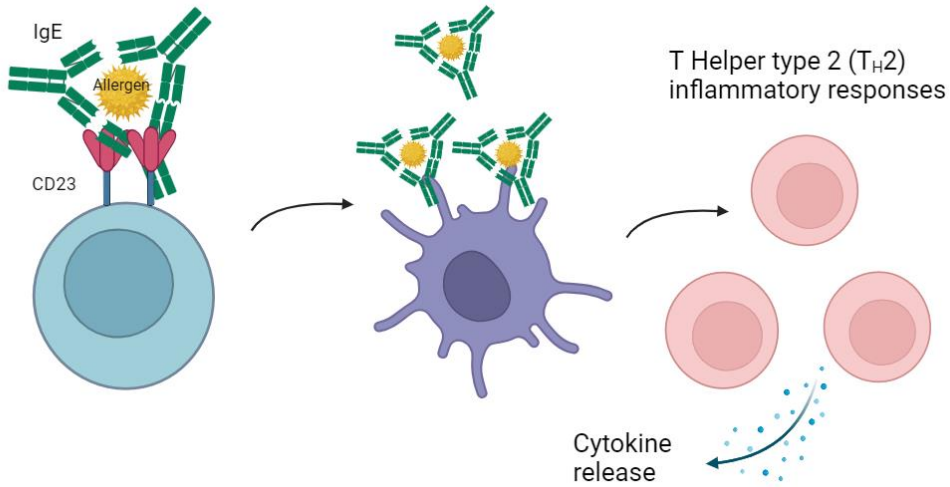

Figure 1. IgE-facilitated Antigen Presentation (FAP). Immune complex binds to CD23 on basophils. B cells move IgE immune complexes to human DC to start T cell proliferation. T cell proliferation results in the release of proinflammatory T_H2 cytokines.

1.6 High affinity receptor FcεRI

FcεRI can be defined as the high affinity IgE receptor and is mostly expressed on mast cells, antigen presenting cells and basophils. IgE can bind to FcεRI which results in the crosslinking of IgE–FcεRI complexes. The formation of these complexes results in the release of mediators such as histamine and leukotrienes in minutes. Furthermore, within hours, also cytokines and chemokines are released which results in prolonged inflammation (Kim et al., 2020) (figure 2). When IgE–FcεRI complexes are formed, signalling cascades are activated which results in the discharge of mediators, which are stored in granules (Nagata & Suzuki, 2022). When an antigen is present, Lyn kinase phosphorylates the Immunoreceptor Tyrosine-Based Activation Motifs (ITAMs) of FcεRIβ and FcεRIγ. This results in a signalling cascade (Sibilano et al., 2014). Furthermore, in the absence of an antigen, IgE can bind to FcεRI and causes phosphorylation of signalling molecules which can also cause mast cells responses in the absence of granulation (Asai et al., 2001).

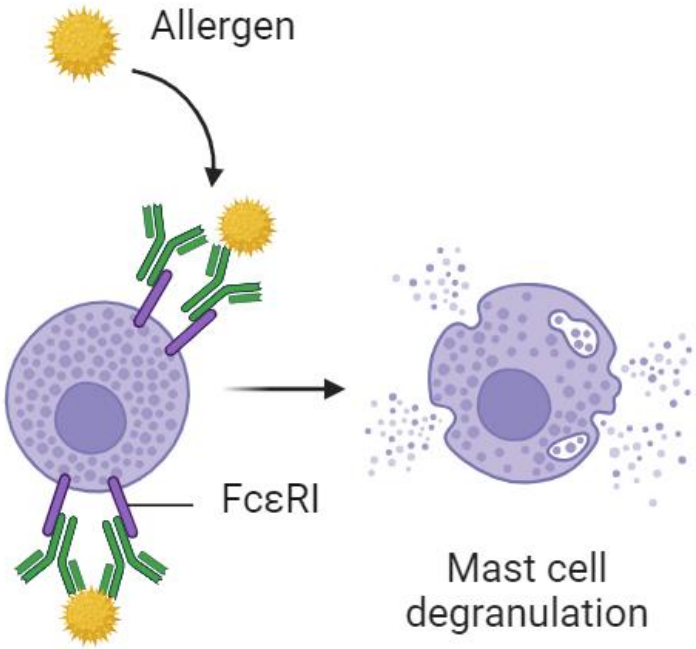

Figure 2. Mast cell degranulation by allergen exposure. IgE can bind to FcεRI which results in the crosslinking of IgE–FcεRI complexes. The formation of these complexes results in the release of mediators such as histamine and leukotrienes in minutes. Furthermore, within hours, also cytokines and chemokines are released witch results in prolonged inflammation

1.7 IgG antibodies

IgG consists out of four different subclasses (IgG1, IgG2, IgG3, IgG4). These subclasses are similar in structure but differ in binding to attachment molecules and receptors, which influences the functionality of the different subclasses. IgG4 has different characteristics which result in efficient inhibition of IgE-Allergen binding (Shamji, Valenta, et al., 2021). IgG4 consists out of approximately 5% of the total IgG in serum, which is the smallest amount of all the IgG subclasses (Qin et al., 2022). In addition, IgG4 levels vary significantly in healthy individuals (Nirula et al., 2011). The structure of IgG consists out of two light chains (25kD) and two heavy chains (-50kd) which are paired together (Angal et al., 1993; Schuurman et al., 2001). The light chain of IgG4 consists out of one constant domain (CL) and one variable domain (VL). However, the heavy chain is composed of three IgG4 specific constant domains (CH, along with CH1, CH3, CH3) and one variable domain (VH). The Fragment Antigen-Binding (FAB) region consists out of the variable domains of the heavy chain and the light chain, which are attached to the CL and CH1 domains. This FAB region is highly specific for one epitope.

The production of IgG4 has a similar pathway compared to the production of IgE. Also IgG4 is produced when T_H2 cells are activated by a class switch on B cells, which is activated by allergen exposure. The T_H2 cytokines which result in the production of IgE (IL-5, IL-6, IL-9 and IL-13) have a similar outcome on the production of IgG4. It is hypothesized that allergen exposure can result in the switch from IgE to IgG4. In addition, previous research found that especially IL-10 plays an important role in the shift in production from IgE to IgG4 (Qin et al., 2022). IL-10 does not only result in the T cell tolerance but also modifies antibody production, which results in more IgG production compared to IgE production (Nouri-Aria et al., 2004; Rossi et al., 2007). Nevertheless, it is still unclear what is the most important factor for the production of IgE or IgG.

1.8 Allergen specific IgA

Not only IgG results in inhibition of IgE binding to Bet v 1. The same as for IgG4, IgA can compete with IgE for binding to allergens. Sublingual Immunotherapy (SLIT) can result in an increase in allergen specific IgA1,IgA2 and decreases the interaction of IgE with the allergen (Shamji, Larson, et al., 2021). IgA antibodies are not only found in blood but also in local target secretions such as in nasal fluid (Shamji et al., 2019). A study found increased levels of Bet v 1 specific IgA in nasal fluids in children with birch allergy during the pollen season (Gafvelin et al., 2005). In addition, an in vitro study found IL-10 production as a result of cross linking of IgA on monocytes by antigen or anti-IgA. This could be another mechanism of how IgA antibodies cause tolerance by AIT (Pilette et al., 2007).

1.9 Allergen-Specific Immunotherapy (AIT)

At this moment, AIT is the only disease-modifying therapy available (Jutel et al., 2015). This therapy includes exposure with increasing doses of allergens which cause tolerance via Regulatory T cells (Tregs) and the initiation of protective IgG4 antibodies (Boyce et al., 2011; Jutel & Akdis, 2011). There are different administration routes for AIT. Subcutaneous Immunotherapy (SCIT) consists of multiple allergen injections (Ozan Yeğit et al., 2022). In addition, Oral Immunotherapy (OIT) consists of oral allergen exposure and swallowing them immediately. Nevertheless, during SLIT the allergen needs to be kept under the tongue for a specific period of time (Pajno et al., 2018).

In September 2023 a birch immunotherapy study with ITULAZAX® started in Rijnstate hospital in Arnhem. ITULAZAX® is a SLIT which is used for the treatment of AR and/or conjunctivitis caused by pollen from the birch homologous group. This clinical study will investigate whether in vitro biomarkers can predict clinical effects. IgG produced by AIT can inhibit IgE binding to CD23 which results in inhibition of antigen presentation by B-cells (figure 3). Furthermore, studies have found that serum from patients who receive birch pollen immunotherapy inhibits FAP by B cells (Shamji & Francis, 2019; Van Neerven et al., 1999; Van Neerven et al., 2004).

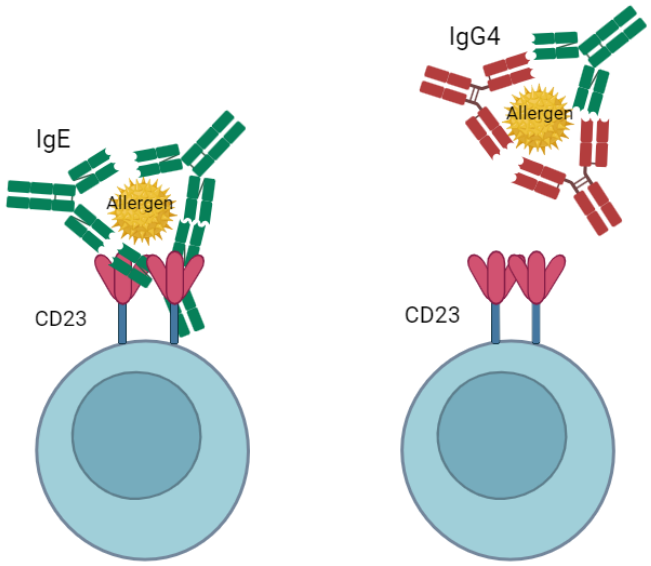

Figure 3. IgE-allergen-complexes are formed on basophils. IgG4 produced by AIT can compete for IgE and therefore inhibit IgE binding by CD23

1.10 Enzyme-Linked Immunosorbent Facilitated Antigen Binding (ELIFAB) assay

IgE receptors, which are present on antigen presenting cells are able to present allergens when specific IgE is present. This will result in T cell activation (Van Der Heijden et al., 1995). Allergen-IgE complexes are formed at mucosal surfaces and therefore a lower concentration of allergen is needed to activate T cell responses (Shamji et al., 2006). The FAB assay is a cell-based assay which measures the inhibition of IgG on allergen-IgE-CD23 complexes (Wachholz et al., 2003). In this assay, the allergen-IgE complexes are incubated with an Epstein-Barr Virus (EBV) transformed cell line. Flow cytometry is used to measure the complexes bound to CD23 on the surface of the cells. This assay requires prolonged culturing and maintenance of viable B cells expressing FcεRII/CD23, which makes the protocol of this assay complex. Therefore, also a cell free Enzyme-Linked Immunosorbent Facilitated Antigen Binding (ELIFAB) assay is developed. The IgE-FAB and the ELIFAB have the same specificity and sensitivity, while the ELIFAB does not require CD23-expressing B cell lines (Shamji et al., 2013). The ELIFAB replaces EBV-transformed B cells with soluble CD23 monomers. The assay makes use of the standard ELISA protocol using a 96 well plate (Shamji et al., 2013). Plates can be read with the use of a standard laboratory microplate absorbance reader; therefore no flow cytometer is needed (Shamji & Francis, 2019).

1.11 Basophil Activation Test

There are different tests for the diagnosis of allergies. The Basophil Activation Test (BAT) is an ex vivo functional assay that is using flow cytometry to quantify basophil degranulation when stimulations with an allergen occurs (Santos et al., 2021). When basophils are exposed to allergens they can degranulate when cross linking occurs of specific IgE on the FceRI receptor. In 1991, the upregulation of the receptor CD63 during basophil activation was discovered (Knol et al., 1991). CD63 is a membrane protein and can be found at the same place as the secretory lysosomal granule that

contains histamine. The movement of CD63 to the cell membrane when degranulation occurs can be measured via flow cytometry (Knol et al., 1991). The BAT defines the amount of stimulated basophils to allergen cross linking IgE and can be used to monitor patients on AIT (Hoffmann et al., 2015). In addition, this assay is becoming more popular since it can also indicate the clinical phenotype of sIgE sensitized patients, which is not possible with only allergen specific IgE levels. In addition, it can replace the Oral Food Challenge (OFC), which is time consuming and patient unfriendly. However, since this is a relatively new diagnostic test, time is needed to set up standardized protocols and clinical validation studies (Santos et al., 2021). However most research on the BAT is performed on peanut, cow's milk and eggs but research on other allergens such as Bet v 1 and Gly m 4 is lacking (Noriega et al., 2021).

1.12 Indirect Basophil Activation Test (iBAT)

Most common the direct BAT is used, which measures the patient's own basophils. Therefore the direct BAT should be performed 24 hours after blood sample collection. Furthermore, in approximately 6-17% of the patients, basophils are not responding to IgE receptor mediated signalling. These patients are called non-responders (Hoffmann et al., 2015). However the Indirect Basophil Activation Test (iBAT) makes use of donor basophils which are sensitized with the patient's IgE. Therefore the timing for performing an iBAT is more flexible and the effect of non-responders is decreased. Nevertheless, the protocol for the iBAT is more time consuming compared to the direct BAT. Furthermore the Blocking Antibody Basophil Activation Test (BA-BAT) can detect the effect of inhibiting factors (such as IgG/IgA) on basophil activation (Ruinemans-Koerts, Uum-Otters, et al., 2022).

In conclusion, IgE cross reactivity can occur between the allergen homologs Bet v 1 and Gly m 4. AIT is a promising therapy which can help allergic patients since it results in the production of protective IgG4 antibodies. However for Bet v 1, the exact role of CD23 and FcεRI on the effect of IgG4 antibodies is not defined yet. Therefore, this study will investigate if IgG4 antibodies produced by AIT inhibit FAP via FcεRI and CD23 for the allergen Bet v 1. Therefore, different experimental studies were performed using the Enzyme-Linked Immunosorbent Assay (ELISA), ELIFAB and iBAT.

2. Research questions and hypotheses

2.1 Hypothesis

The process of antigen presentation regulated by CD23 can be defined as FAP. Immune complexes bind to CD23 which results in the release of proinflammatory T_H2 cytokines. Previous research found that AIT can cause tolerance via Tregs and the initiation of protective IgG4 antibodies. Production of IgG4 initiated by AIT can inhibit IgE binding to CD23 which results in inhibition of FAP by B-cells. Since the ELIFAB is a cell free assay, it could be an accessible method to investigate the formation of Bet v 1-IgE-CD23 complexes and the effect of IgG4 antibodies. Furthermore, IgG4 antibodies can inhibit the process of IgE binding via FcεRI. Since allergy symptoms are a result of the release of histamine and other inflammatory mediators such as cytokines by basophils, the effect of AIT can be monitored by using the BA-BAT.

2.2.1 Research question:

How do IgG4 antibodies stimulated by Allergen-Specific Immunotherapy (AIT) with birch pollen inhibit IgE-facilitated presentation (FAP) via the receptors FcεRI and FcεRII (CD23)?

To answer the research question the following sub-questions were formulated:

2.2.2 Sub questions:

Is it possible to measure the effect of IgG4 antibodies on binding of Bet v 1-IgE complexes to CD23 via the ELIFAB assay?

Can the ELIFAB assay give information about the specific epitope coverage of IgG4 mAbs specific for Bet v 1?

What is a suitable indicator serum for the ELIFAB assay and the BA-BAT?

Can the BA-BAT be used to investigate the effect of AIT on the basophil sensitivity and reactivity to Bet v 1?

Is it possible to quantify Bet v 1-specific IgG4 and IgE antibodies via an ELISA?

3. Material and methods

3.1 Materials

3.1.1 Enzyme-Linked Immunosorbent Facilitated Antigen Binding (ELIFAB) assay

- **Stock samples mAbs IgE, IgG4:** kind gift of Mabylon AG, Schlieren
- **Recombinant Bet v 1** (Inbo, 42290)
- **Recombinant Human CD23/Fc** (R&Dsystems, 123-FE)
- **ELISA 96 wells plate, medium binding**
- **TMB Substrate Solution**
- **ELIFAB washing Buffer:** PBS with 0.05% Tween
- **Blocking buffer:** 1% BSA/PBS solution or Roche Blocking buffer (11112589001)
- **HRP-Conjugated Streptavidin** (ThermoFisher, N100)
- **Biotin Mouse Anti-Human IgE** (BD Pharmingen, 555858) (1:1000)
- **ELISA Stop solution:** 2M H_2SO_4
- **Nunc plates for formation of complexes**

3.1.2 ELISA to measure IgE and IgG specific for Bet v 1

- **Stock samples mAbs IgE, IgG4:** kind gift of Mabylon AG, Schlieren
- **Recombinant Bet v 1** (Inbo, 42290)
- **Goat anti-human IgA-HRP** (Southern Biotech 2050-05) 1:2000
- **Goat anti-human IgG-HRP** (Southern Biotech 2040-05) 1:4000
- **Mouse-anti human IgE-HRP** (southern Biotech 9160-05) 1:2000
- **High binding 96-wells ELISA plates**
- **Nunc plates for making dilutions**
- **Bicarbonate/carbonate coating buffer**
- **ELISA stop solution:** 2M H_2SO_4
- **TMB substrate solution**
- **Wash buffer**: PBS with 0.05% Tween
- **Blocking buffer**: Roche blocking buffer (1111258001)

3.1.3 Indirect Basophile Activation Test (iBAT) Rijnstate

The materials used for the iBAT can be found in appendix section 7.3.

3.2 Methods

3.2.1 Enzyme-Linked Immunosorbent Facilitated Antigen Binding (ELIFAB) assay

3.2.1.1 Coating, washing and blocking

The method of the ELIFAB is visualised in figure 4. First a 96 wells plate was coated with CD23. Afterwards Bet v 1-IgE complexes were added on the plate. IgG4 can compete for binding to CD23. The Bet v 1-IgE complexes binding to CD23 were visualised by biotinylated anti-human IgE and streptavidin-HRP.

For optimization of the ELIFAB assay two coating concentrations of CD23 receptor (2.5 µg/ml and 1 µg/ml) were made in sterile 1 x PBS. An ELISA 96 wells plate medium binding was coated with 50 µl of diluted CD23 and incubated overnight at room temperature. On the next day, the plate was washed twice with 200 µl/well of washing buffer and tap dried. The plate was blocked with 200 µl of blocking buffer (1% BSA/PBS) and incubated at 37°C for 1 hour. During the incubation period with blocking buffer, allergen complexes were prepared with a recombinant Bet v 1 stock sample, three recombinant IgE human-mAbs and three recombinant IgG4 human-mAbs. A mix of the three IgE and IgG4 human-mAbs (ratio 1:1:1) was prepared to allow immune complex formation by different epitopes.

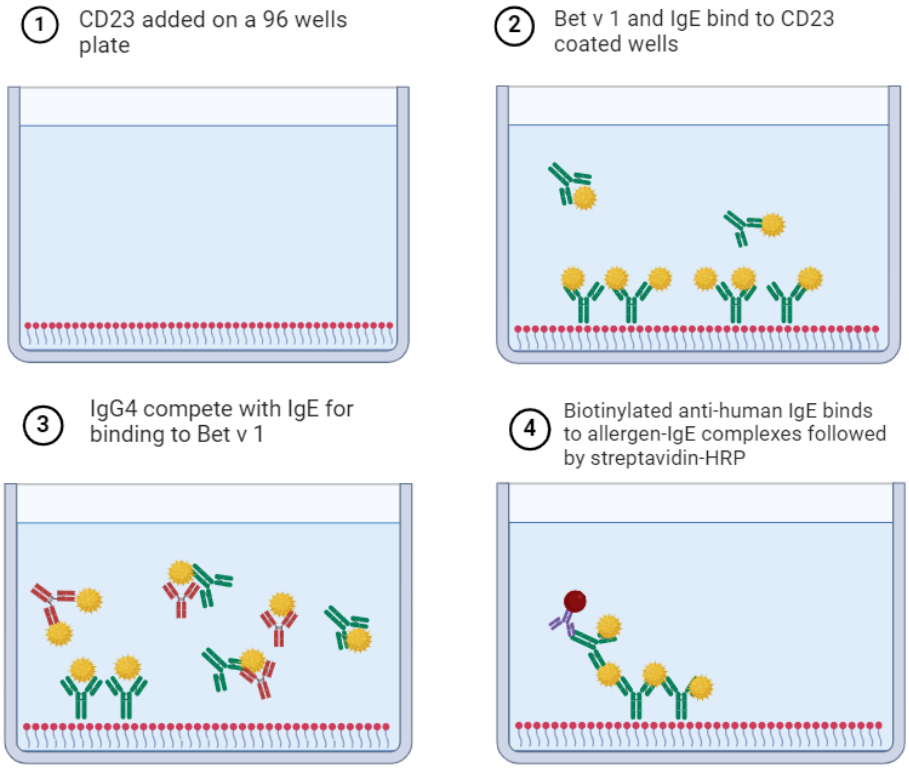

Figure 4. Procedure of the ELIFAB assay. CD23 is coated on a 96 wells plate. Bet v 1-IgE complexes are visualised by biotinylated anti-human IgE and streptavidin-HRP. IgG4 can compete with IgE for binding to Bet v 1 (Shamji & Francis, 2019)

3.2.1.2 Immune complexes Bet v 1– IgE

To investigate the optimal concentration for Bet v 1 and recombinant IgE mAbs, both reagents were diluted in blocking buffer (1% BSA/PBS). Concentrations of Bet v 1 and IgE mAbs were prepared 2x higher than the required final working concentration. To investigate the optimal Bet v 1 concentration for immune complex formation, a serial dilution of recombinant Bet v 1 was prepared (10, 1, 0,1, 0.01, 0.001,0 µg/ml). In addition, IgE samples were diluted to concentrations of 1 and 0.1 µg/ml. Three different recombinant IgE mAbs were diluted in blocking buffer (1% BSA/PBS) and mixed in a ratio 1:1:1. Finally, to form the immune complexes, Bet v 1 samples were mixed with the IgE mAbs in a ratio 1:1. Blocking buffer was used as control when no Bet v 1 was added. The immune complexes were then incubated for 30 min at room temperature.

3.2.1.3 Immune complexes Bet v 1 – IgE – IgG4

To investigate the inhibition of binding Bet v 1-IgE complexes to CD23 by IgG4, three recombinant IgG4 human-mAbs were used. Bet v 1-IgE complexes were formed with an optimal Bet v 1 concentration of 0.01 µg/ml and an IgE concentration of 0.1 µg/ml. Concentrations of Bet v 1 and IgE mAbs were prepared 3x greater than the required final concentration. Three different IgG4 mAbs were mixed in a ratio 1:1:1. IgG4 concentrations of 10 µg/ml, 3.33 µg/ml and 0 µg/ml were prepared in blocking buffer (1% BSA/PBS). To form the complexes, IgE, Bet v 1 and IgG4 were mixed in a ratio 1:1:1. Eventually the complexes were incubated for 30 min at room temperature.

To investigate the effect of different IgG4 epitopes on allergen-complex binding to CD23, a mix of the three different IgG4 mAbs, two different IgG4 mAbs, and the three IgG4mAbs separate were added to the Bet v 1 - IgE immune complexes. For Bet v 1 a concentration of 0.01 µg/ml, for IgE a concentration of 0.1 µg/ml and for IgG4 a concentration of 10 µg/ml was prepared in blocking buffer (1%BSA/PBS). For the control sample only blocking buffer was added instead of IgG4.

3.2.1.4 Making immune complexes from sera

For formation of the Bet v 1-IgE complexes in sera, Bet v 1 was diluted in Roche blocking buffer to a concentration 2x greater than the required final concentration. A serial dilution of recombinant Bet v 1 was prepared (10, 1, 0,1, 0.01, 0.001,0 µg/ml) in Roche blocking buffer. Afterwards, 25 µL of indicator serum was added into the bottom of a separate 96-well round-bottomed plate, followed by 25 µL of Bet v 1 dilution. 25 µL of Roche blocking buffer was used in the negative control wells. The plate was covered and incubated for 30 min at 37 °C.

3.2.1.5 Transferring immune complexes to the ELISA plate

After formation of the Bet v 1-IgE complexes, the blocked plate was washed three times with washing buffer. Afterwards, 50 µl of the allergen immune complexes were transferred on the 96 wells plate in duplo. As a control, only blocking buffer (1 % BSA/PBS or Roche blocking buffer) was added. The plate was covered with a lit and put on a shaker at 650 rpm for 1 hour at room temperature. After incubation, the plate was washed four times with washing buffer and the biotinylated IgE-specific antibody was diluted 1:1000 in blocking buffer (1% BSA/PBS or Roche blocking buffer). 50 µl of the diluted antibody was added into each well. Later on, the plate was covered and put on the shaker at 650 rpm for 1 hour at room temperature. After incubation, the plate was washed five times with 200 µl washing buffer. Streptavidin peroxidase was diluted in a ratio 1:5000 in blocking buffer (1 % BSA/PBS or Roche blocking buffer). 50 µl of the diluted streptavidin peroxidase was added to the plate and incubated on a shaker at 650 rpm for 30 min at room temperature. Afterwards, the plate was washed six times with washing buffer and 50 µl of

3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to each well. The plate was kept in the dark and the reaction was stopped with 50 µl of 2 N H_2SO_4. Finally, the plate was read on a microplate absorbance reader at 450 nm. The results were expressed as Bet v 1-IgE binding to sCD23 (OD45O).

3.2.2 Sandwich ELISA to measure IgE and IgG specific for Bet v 1

3.2.2.1 Optimization ELISA

The method of the ELISA to measure Bet v 1 specific IgE and IgG is visualised in figure 5. First Bet v 1 was diluted in coating buffer to a concentration of 1 µg/ml. 100 µl of diluted Bet v 1 in coating buffer was added to the high binding 96 well ELISA plate and incubated overnight at 4°C. The next day the plate was washed three times with 200 µl/well washing buffer and tap dried. To block the plate, 250 µl/well Roche blocking buffer was added to each well and the plate was incubated for 1 hour at room temperature. After incubation, the plate was again washed three times with washing buffer. In the meantime, recombinant IgE and IgG4 mAbs were diluted in Roche blocking buffer. Serial dilutions of IgE and IgG4 were made (first experiment: 1, 0.1, 0.01, 0.001 and 0 µg/ml) (second experiment: 0.25, 0.125, 0.0625 and 0 µg/ml) in Roche blocking buffer. After incubation, the plate was again washed three times in washing buffer and the diluted samples were added to the plate (100 µl/well). As a control, a sample with only Roche blocking buffer was added. The plate was then incubated for two hours at room temperature. Then the plate was again washed three times with washing buffer. The antibody goat anti-human IgE (1:2000) and the antibody goat anti-human IgR-grp (1:4000) were diluted in Roche blocking buffer. Hereafter, the plate was again incubated for one hour at room temperature. Finally, the reaction was stopped by adding 100 µl/well stopping buffer (2 N H_2SO_4) to each well. The plate was read at 450 nm on an ELISA plate reader.

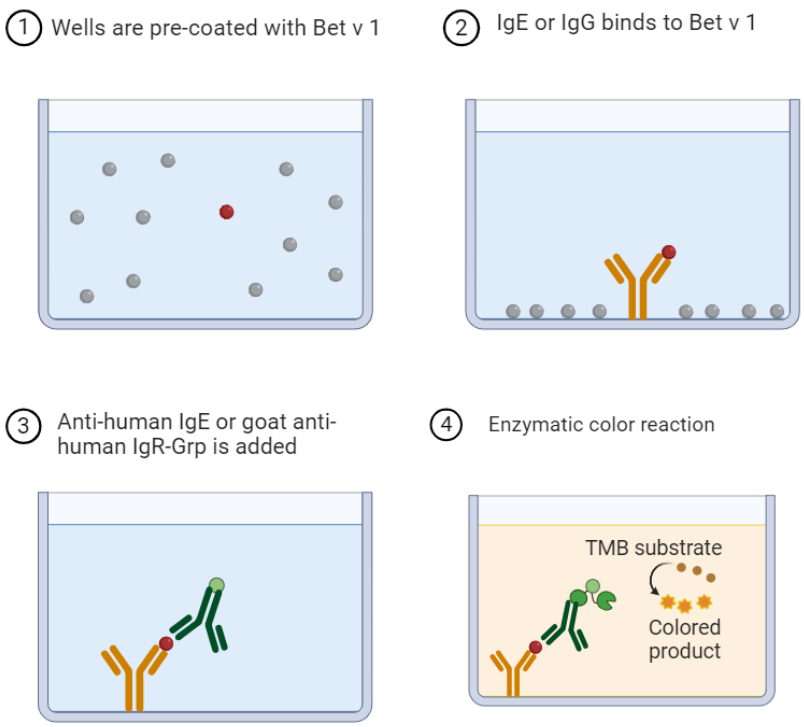

Figure 5. ELISA to measure IgE and IgG specific for Bet v 1. 96 wells plate is coated with Bet v 1. Afterwards, IgE or IgG binds to Bet v 1 and binding is detected via antibody goat anti-human IgE and the antibody goat anti-human IgR-Grp. An enzymatic colour reaction occurs after adding TMB.

3.2.2.2 Measure IgE and IgG specific for Bet v 1 in sera

First Bet v 1 was diluted in coating buffer to concentration of 1 µg/ml. 100 µl of diluted Bet v 1 in coating buffer was added to the a high binding 96 well ELISA plate and incubated overnight at 4°C. The next day the plate was washed three times with 200 µl/well washing buffer and tap dried. To block the plate, 250 µl/well Roche blocking buffer was added to each well and the plate was incubated for one hour at room temperature. After incubation, the plate was again washed three times with washing buffer. In the meantime, a standard curves of IgE mAbs (0.25, 0.125,0.0625,0.3125,0.0145625 µg/ml) and IgG4 mAbs (0.1, 0.01, 0.001, 0 µg/ml) were prepared in Roche blocking buffer. A mix of the three IgE mAbs and the three IgG mAbs (ratio 1:1:1) was used. For measuring IgE, sera was diluted 1:10 and for measuring IgG sera was diluted 1:200. After incubation, the plate was again washed three times in washing buffer and the diluted samples were added to the plate (100 µl/well). As a control, a sample with only Roche blocking buffer was used. The plate was then incubated for two hours at room temperature. Afterwards the plate was again washed three times with washing buffer. The antibody goat anti-human IgE and the antibody goat anti-human IgR-grp were diluted in Roche blocking buffer. For IgE a ratio of 1:2000 was used and for IgG a ratio of 1:4000. Hereafter, the plate was again incubated for one hour at room temperature. Finally, the reaction was stopped by adding 100 µl/well stopping buffer (2N H_2SO_4) to each well. The plate was read at 450 nm on an ELISA plate reader.

3.2.3 Indirect Basophile Activation Test (iBAT) Rijnstate

3.2.3.1 Buffycoat isolation

The method of the iBAT is visualised in figure 6. First buffy coats were isolated. For buffy coat isolation, 8 donor Ethylene Diamine Tetraacetic Acid (EDTA) tubes HbA1c 4 ml (O-positive) were collected. These samples were centrifuged for 10 min at 2200xg. Afterwards, the upper phase (plasma) was removed by a plastic pasteur pipette. 2 ml of saline was added to two falcon tubes. The collected buffy coats were transferred to the falcon tubes and gently mixed by swinging. Afterwards, the tubes were centrifuged again for 10 min at 2200xg. Plasma was removed and two new falcon tubes with 2 ml saline were taken. The buffy coat was transferred to these tubes. Afterwards, the concentration of leukocytes was measured. The buffy coat was then diluted to a final leukocyte count of 15 x 10^9/L. Afterwards, again the concentration of leukocytes was measured. In case the concentration of leukocytes was lower than 15 x 10^9/L, the previous steps were repeated.

3.2.3.2 Stripping of IgE

The isolated buffy coat were first centrifuged at 11°C for 5 minutes at 1000xg. The supernatant was removed and 2 ml of stripping buffer was added. The samples were then gently mixed by swinging. Afterwards, again the tube was centrifuged at 11 °C for 5 min at 1000xg. The supernatant was removed and the pellet was resuspended in 2 ml BSB. Afterwards, again the tube was gently mixed by swinging movements (10-15x) and centrifuged at 11°C for 5 min at 1000xg. The supernatant was removed and the pellet was resuspended with BSB to a final volume of 1500 µl. Finally the tube was again gently swung. Eventually, in a 5 mL falcon tube, the stripped donor cells were mixed with sera from the patient. 130 µl of sera and 500 µl of donor cells were added to the falcon tube. The samples were incubated overnight at 37°C and shaken at 50xg. The next day 770 µl of BSB was added to the sample.

3.2.3.3 Preparation of samples

The next day, dilutions of recombinant Bet v 1 (3, 10, 100, 300, 1000, 3000 and 10000 pg/ml) were prepared in BSB. As a negative control, two tubes with 150 µl BSB were prepared. In all the other tubes 100 µl of BSB was added. For the positive control, two tubes with 50 µl stimulation control (a-FcεR) were prepared. In the other tubes, 50 µl of the diluted Bet v 1 was added. Afterwards, 50 µl of cell suspension was added to all the tubes. Eventually, in each tube, 20 µl of staining reagent was added and the tubes were mixed afterwards. Tubes were then incubated for 15 min in a water bath of 37°C. Lysing buffer was prepared by diluting 5 mL of lysing buffer in 45 mL of demi water and 1.5 mL of lysing buffer was added to the samples. The samples were swinged three times and incubated for 5 minutes at room temperature until a clear suspension was visible. This dilution was centrifuged 5 min at 640 xg. Afterwards, the liquid in the upper part of the tube was removed. Afterwards, 500 µl of washing buffer was added and the tubes were stored in the fridge until measuring. Before measuring in the flow cytometer the samples were mixed for 5 seconds using the vortex. Finally the samples were measured via the FacsCanto II.

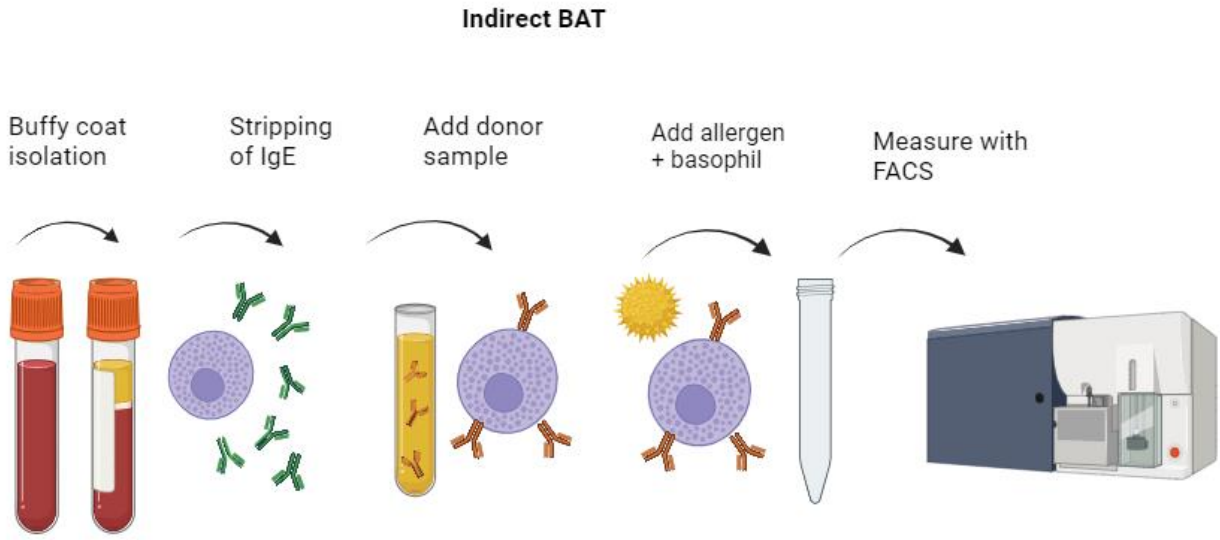

Figure 6. Method of the iBAT. First buffy coats were isolated from 8 donor EDTA tubes. Then the isolated buffy coats are stripped for IgE. The stripped donor cells were mixed with sera from the patient. The next day, dilutions of Bet v 1 were added to the basophils and samples were measured via FACS.

3.2.3.4 Blocking Indirect Basophil Activation Test (BA-BAT) Rijnstate

To investigate the effect of IgG4 on basophil activation, also BA-BATs were performed. For the BA-BAT, the same method as for the iBAT was used. However, instead of adding 100 µl of BSB to all samples, 100 µl BSB% + 10% serum was added (figure 7). This serum was derived from a patient who received immunotherapy for birch. After adding the immunotherapy samples, the samples were incubated 60 minutes in a water bath of 37°C. After incubation the experiment continued the same as for the iBAT described in section 3.3.2. The increase in half maximal effective concentration (EC50) compared to the iBAT was calculated via Nonlin Fit in Graphpad Prism to quantify the inhibition caused by IgG4.

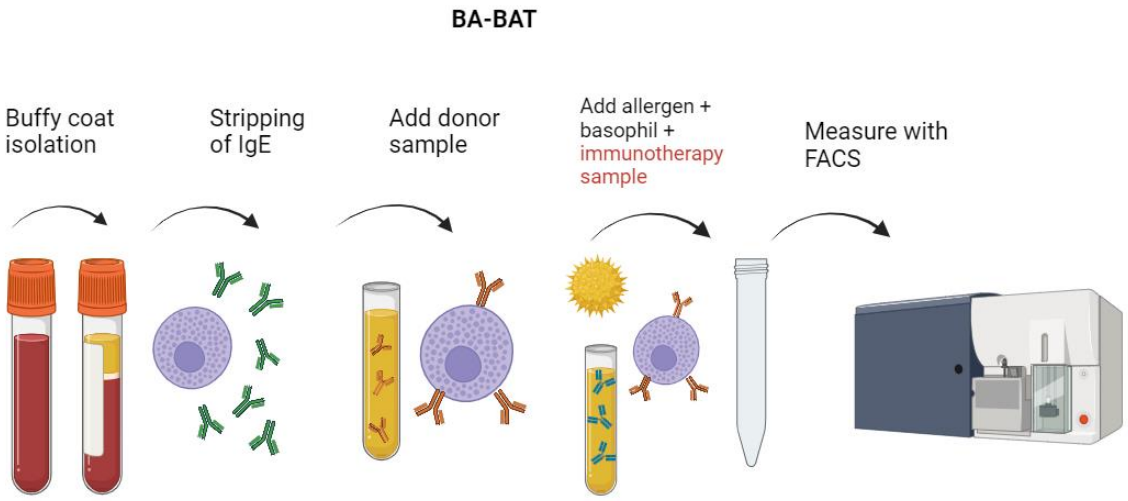

Figure 7. Method of the BA-BAT. First buffy coats were isolated from 8 donor EDTA tubes. Then the isolated buffy coats are stripped for IgE. The stripped donor cells were mixed with sera from the patient. The next day, dilutions of Bet v 1 and a sample from a patient who received immunotherapy were added to the basophils and samples were measured via FACS.

3.2.4 Statistical analysis

Graphpad Prism 10.2.0 was used for statistical analysis and visualisation of the data. First data was checked for normality with a Shiro-Wilk Test. Different statistical tests were performed such as two-way ANOVA, one sample t-test, Wilcoxon test, Paired t test and Pearson correlation to test for significance. Furthermore, the EC50 was calculated by using Non lin Fit.

4 Results

4.1 ELIFAB assay

4.1.1 Binding of Bet v 1 - IgE immune complexes to CD23

Binding of Bet v 1 – IgE immune complexes to CD23 was studied using the ELIFAB assay. However, this assay needed to be optimized first. Figure 8 shows the binding of the immune complex of Bet v 1 and IgE to CD23 expressed as OD values. Two different CD23 concentrations (1 µg/ml and 2.5 µg/ml) were tested for the optimization of the ELISA. In addition, for optimization of the binding of Bet v 1-IgE complexes to CD23 a mix of three IgE mAbs were used in two different concentrations (1 and 0.1 µg/ml). Furthermore, five different rBet v 1 concentrations were investigated (10, 1, 0.1, 0.01 and 0.001 µg/ml). Both IgE concentrations resulted in bell-shaped dose-dependent curves. For the IgE concentration of 0.1 µg/ml a peak in immune complex binding was found around the Bet v 1 concentration of 0.01 µg/ml (graph A and B) while a peak at 0.1 µg/ml was seen for the IgE concentration of 1 µg/ml (graph C and D). The IgE concentration of 1 µg/ml together with a CD23 concentration of 2.5 µg/ml showed higher standard deviations compared to the other concentrations tested (graph D). The higher ODs were observed for the CD23 concentration of 2.5 µg/ml. Based on this outcomes: a CD23 concentration of 2.5 µg/ml, a IgE concentration of 0.1 µg/ml and a Bet v 1 concentration of 0.01 µg/ml were chosen for the formation of Bet v 1-IgE complexes.

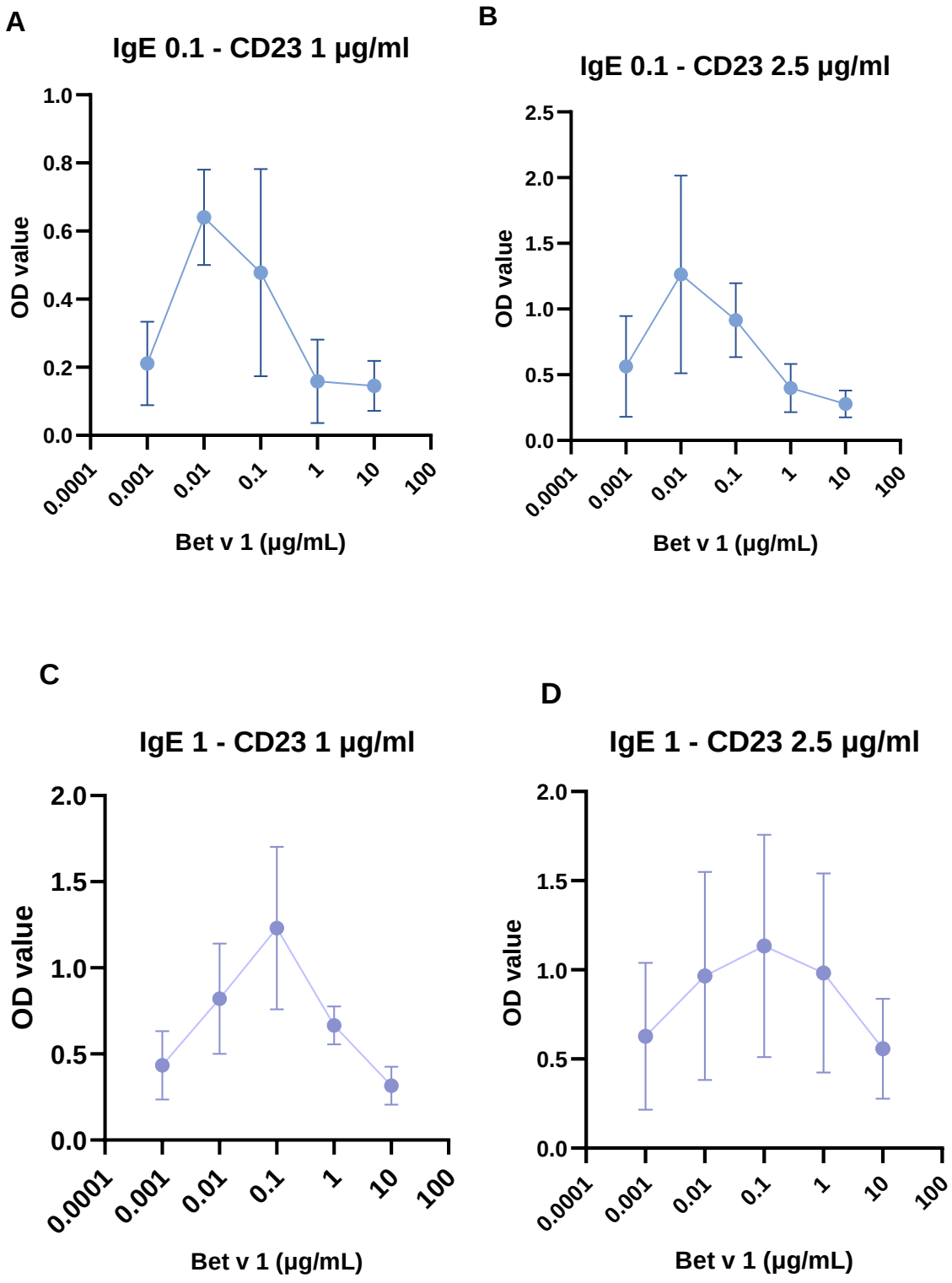

Figure 8. Binding of Bet v 1–IgE complexes to CD23 coated on the ELISA plate expressed as OD measured at 450 nm. Mean and standard deviations are shown for the different Bet v 1 concentrations. Graph A and B show the result of IgE concentration 0.1 µg/ml and a CD23 concentration of respectively 1 µg/ml and 2.5 µg/ml (n=4). Graph C and D show the result of IgE concentration 1 µg/ml and a CD23 concentration of respectively 1 µg/ml and 2.5 µg/ml (n=3)

4.1.2 Inhibition by IgG4 on Bet v 1-IgE complexes binding to CD23

Binding of Bet v 1 – IgE complexes to CD23 was inhibited by IgG4. Figure 9 shows the effect of IgG4 on immune complexes binding to CD23 expressed as percentage. The binding was established as 100% when the immune complex bound to CD23 without the addition of IgG4. Immune complexes were formed based on the results of figure 7. A mix of three different IgG4 mAbs were added to the Bet v 1-IgE complexes. Three different concentrations of the IgG4 mix (1.11, 3.33 and 10 µg/ml) were added. Figure 8 shows an inhibition of immune complexes binding to CD23 for the three different concentrations of IgG4. A higher IgG4 concentration resulted in a lower percentage of immune complexes binding to CD23. An one sample T test was performed to test for significance. A significant difference compared to the control sample (no IgG4) was seen for the IgG4 concentration of 1.11 µg/ml (p=0.0277), 3.33 µg/ml (p=0.0021) and 10 µg/ml (p=0.0027).

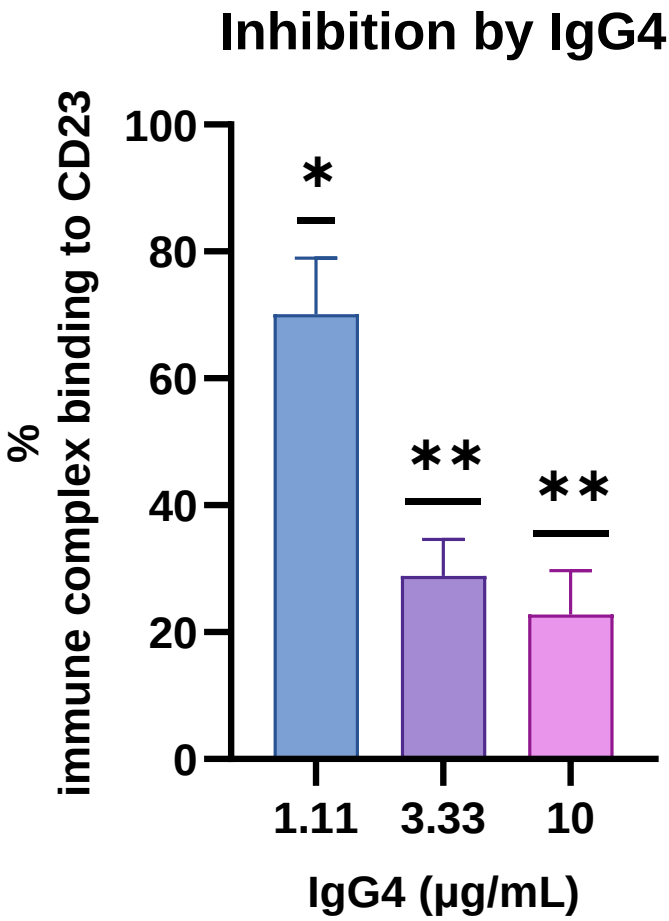

Figure 9. Effect of IgG4 on the immune complex binding to CD23 expressed as percentage. The binding was established as 100% when the immune complex bound to CD23 without the addition of IgG4. The mean and standard deviations from three separate experiments are shown (n=3). Three different IgG4 concentrations (1.11, 3.33 and 10 µg/ml) were investigated.

Furthermore, Figure 10 shows the effect of the three different IgG4 mAbs to investigate if different IgG4 epitopes are needed for the inhibition of allergen-IgE complexes binding to CD23. Immune complexes were formed based on the results of figure 1. A CD23 concentration of 2.5 µg/ml, an IgE concentration of 0.1 µg/ml and a Bet v 1 concentration of 0.01 µg/ml was used. The highest inhibition is seen when a mix of three IgG4 antibodies is used. Lower inhibition is seen when two and respectively one antibody is used. Significance differences were found when adding IgG4 antibodies compared to adding no IgG4. An one sample T test was performed which resulted in a significance difference for the mix of IgG4 (p=0.004), IgG 1+2 (p=0.0011), IgG 2+3 (p=0.0216) and IgG 1+3 (p=0.0200). However, no significance difference was seen for the IgG4 antibodies alone (IgG1; p=0.1376, IgG2; p=0.0960, IgG3; p= 0.1643). This indicates that a mix of IgG4 mAbs is needed to target different epitopes for the inhibition of allergen-IgE complexes binding to CD23.

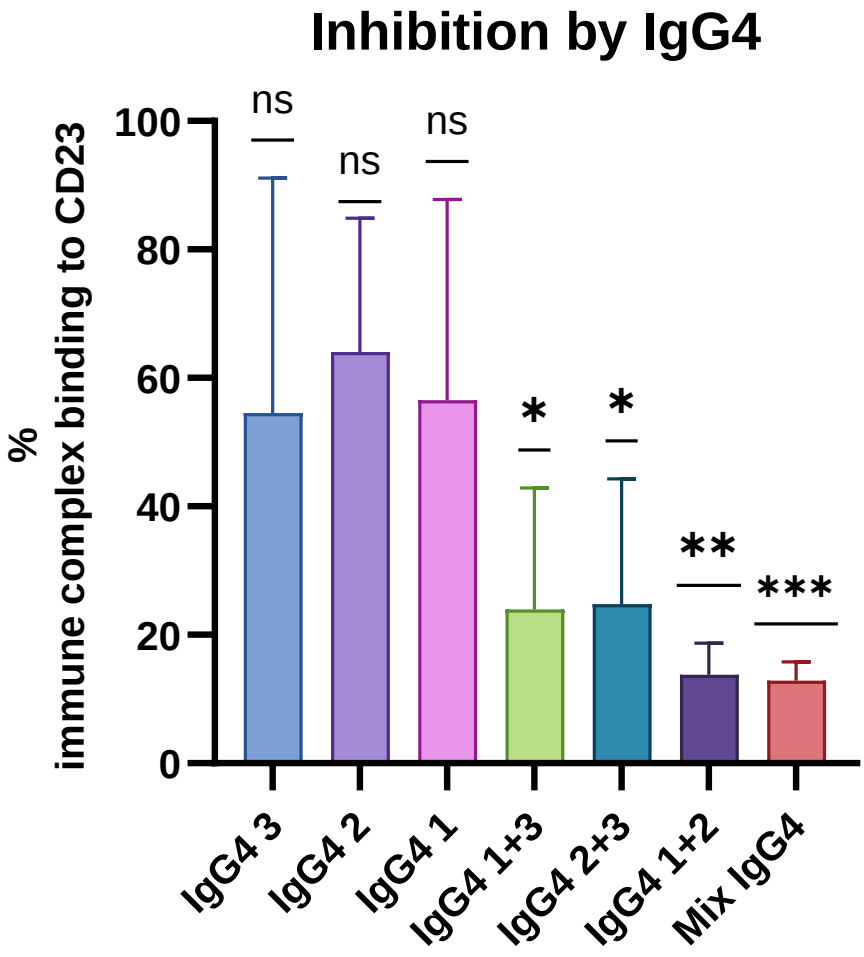

Figure 10. Effect of IgG4 on the %immune complex binding to CD23. IgG4 1,2 and 3 indicate the separate IgG4 mAbs. The mean and standard deviations from three separate experiments are shown (n=3). The effect of a mix of three IgG4 mAbs, two IgG4 mAbs and all the mAb separately are shown. 100% of binding was set on immune complex binding to CD23 without adding IgG4.

4.1.3 Test indicator sera for ELIFAB assay

After optimization of the ELIFAB assay with recombinant mAbs, the ELIFAB assay was optimized for different indicator sera. This sera contained high Bet v 1 specific IgE levels and could be used as a reference point for the decrease of complexes binding to CD23. Before using the ELIFAB assay with patient sera, a suitable indicator serum should be identified. This indicator serum should contain high allergen-specific IgE levels. Furthermore, it should be checked if the serum has binding with different concentrations of the allergen. It is optimal to reach a binding between 70-80%. Figure 11 shows binding of Bet v 1 -IgE immune complexes to CD23 for four different sera derived from birch allergic patients. The birch specific IgE concentrations of the sera TP51,TP14, TP32 and TP47 were respectively 5.22, 22.2, 40.1 and 85.5 kU/L. A slight increase in binding was seen at a Bet v 1 concentration of 0.01 µg/ml. However, this effect was small since already high binding was observed at a Bet v 1 concentration of 0 µg/ml. Therefore another experiment was performed were the sera was diluted 2x and another blocking buffer (Roche blocking buffer) was used (figure 12). Diluting the sera 2x times lowered the background and gave a clearer immune complex binding compared to the non diluted sample. However, still a high background level was observed. Therefore, another experiment was performed were the sera was diluted either 2x or 5x times and half of the samples was centrifuged (15000g for 20 min) (figure 13). Centrifugation showed a slightly higher increase compared to the non-centrifuged sample. The lowest background value was seen for the 5x diluted non-centrifuged sample. However, a lower increase in immune complex binding was seen for this sample. The highest increase in allergen immune complex binding to CD23 was seen for 2x diluted and centrifuged samples.

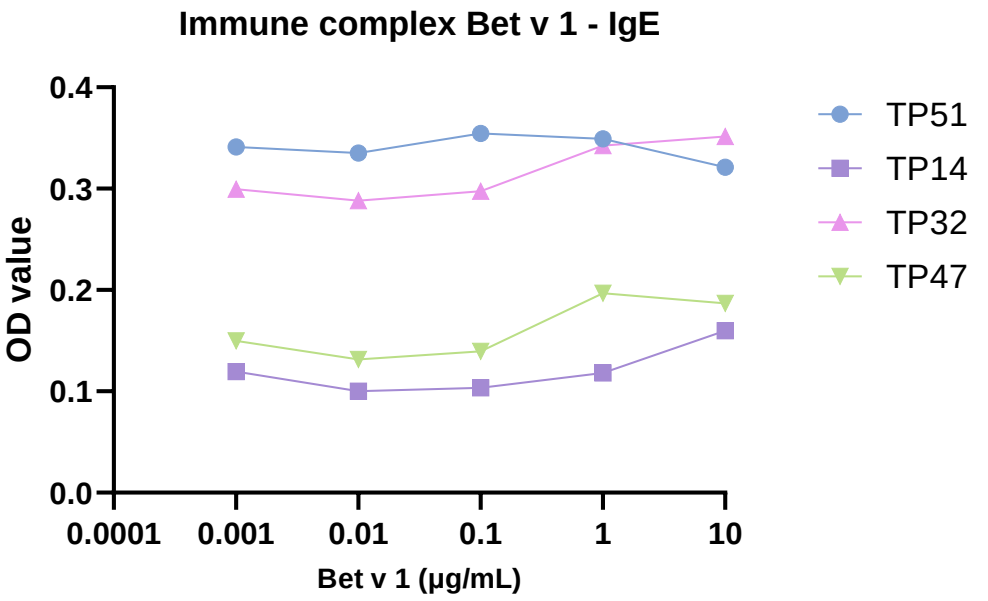

Figure 11. Immune complex Bet v 1-IgE binding to CD23 for different Bet v 1 concentrations (µg/ml). Four different samples from birch allergic patients were tested (n=4).

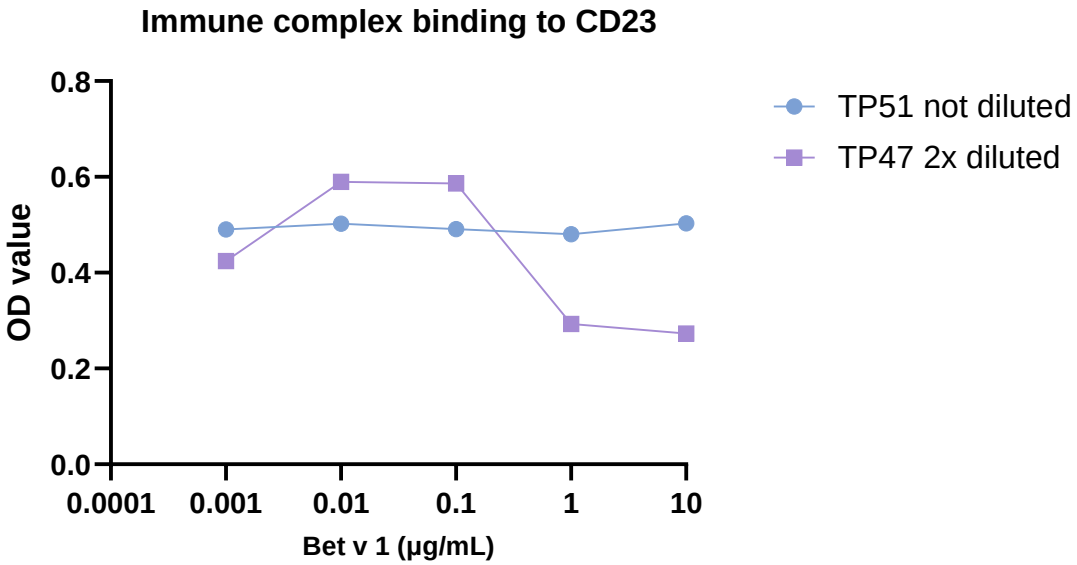

Figure 12. Immune complex Bet v 1 -IgE binding to CD23 for different Bet v 1 concentrations (µg/ml). Two different samples from birch allergic patients were tested (n=2).

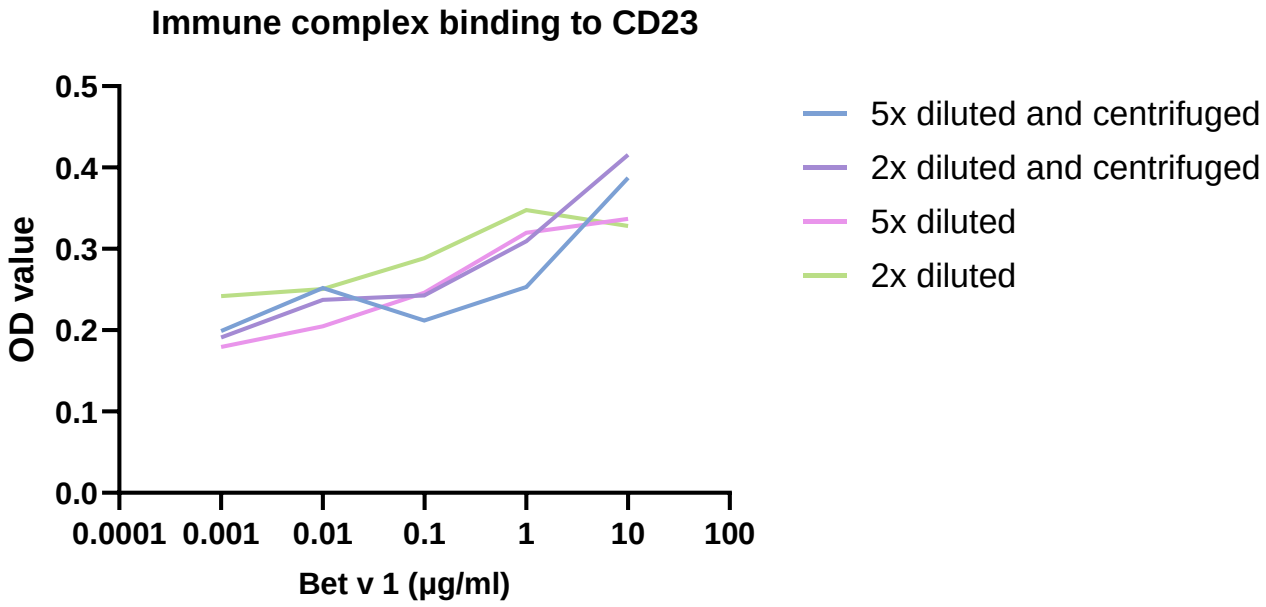

Figure 13. Immune complex Bet v 1-IgE binding to CD23 for different Bet v 1 concentrations (µg/ml). A sample from a birch allergic patient was tested for different dilutions (2x and 5x). Both samples were tested with and without centrifugation. Sera TP32 was used for all the different conditions (n=4).

4.2 Basophil Activation Test

4.2.1 Indirect Basophil Activation Test (iBAT)

First, for optimization of the iBAT, basophils were incubated with different concentrations of Bet v 1 (3, 10 100, 300, 1000, 3000, 10000 pg/mL) and a serum from a birch allergic patient (TP15). The birch specific IgE concentrations for the TP sera were measured in Rijnstate. The TP15 sample has an birch specific IgE concentration of 22.5 kU/L. The iBAT for TP15 was repeated three times with a different set of basophils. Figure 14 shows the mean and standard deviations of the % activated basophils (%CD63) for different Bet v 1 concentrations (3, 10 100, 300, 1000, 3000, 10000 pg/mL). The maximum basophil reactivity reaches approximately 80%. The iBAT showed a dose response curve for the different Bet v 1 concentrations. An increase in activated basophils (%CD63) was seen for higher Bet v 1 concentrations.

In addition, figure 15 shows the % activated basophils (%CD63) for different Bet v 1 concentrations (3, 10 100, 300, 1000, 3000, 10000 pg/mL) for two different sera from patients who received birch immunotherapy (BP002 + BP003). The experiment for sera BP003 was performed twice but the experiment for sera BP002 was performed once. Experiments were performed with different sets of basophils. The maximum basophil reactivity reaches approximately 70%. The iBAT showed a dose response curve for the different Bet v 1 concentrations. An increase in activated basophils (%CD63) is seen for higher Bet v 1 concentrations.

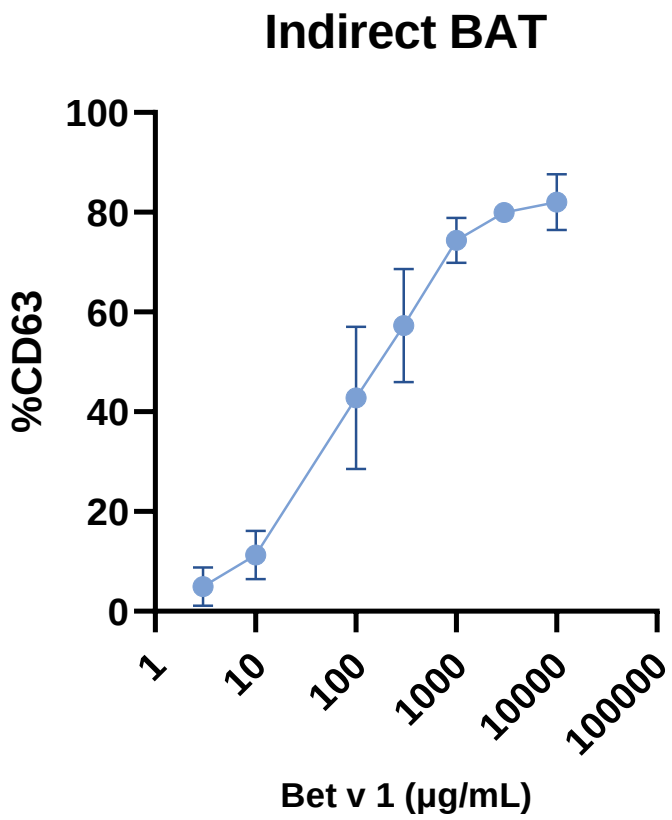

Figure 14. Results of the indirect BAT**.** The % activated basophils (%CD63) for different bet v 1 concentrations (3, 10 100, 300, 1000, 3000, 10000 pg/mL) are shown. Mean and standard deviations of the three separate experiments are indicated (n=3). Three different sera from birch allergic patients were used (TP15).

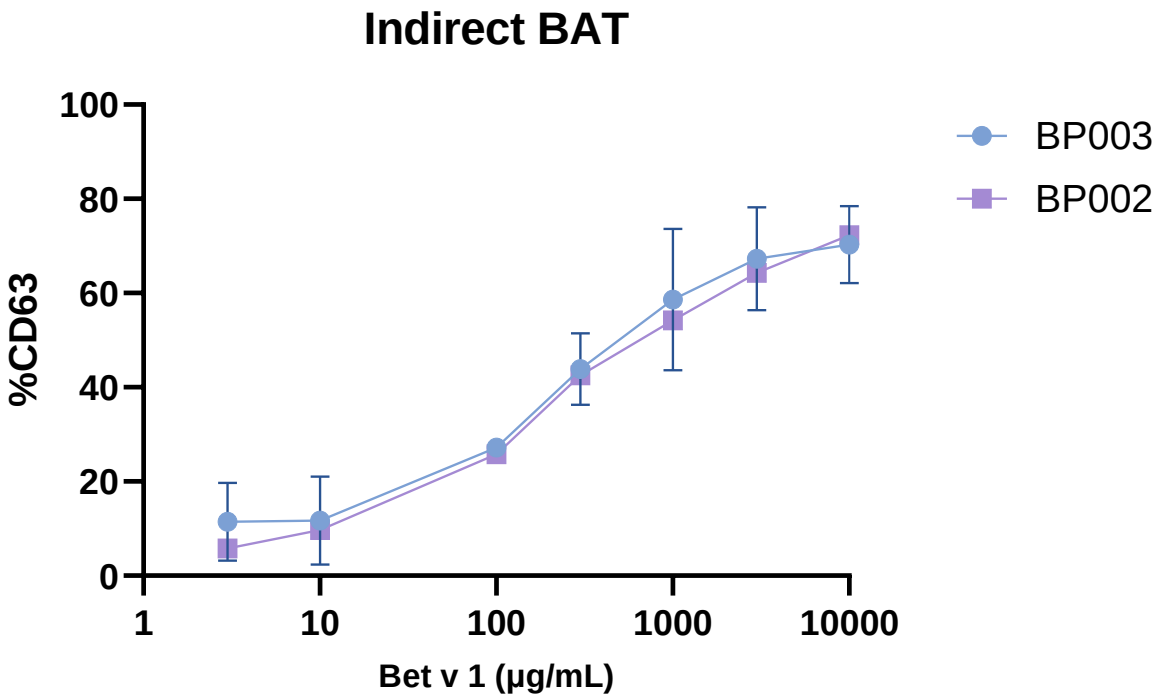

Figure 15. Results of the iBAT**.** The % activated basophils (%CD63) for different Bet v 1 concentrations (3, 10 100, 300, 1000, 3000, 10000 pg/mL) are shown. Mean and standard deviations of the three separate experiments are indicated (n=3). Two different sera from a patients who received immunotherapy were used (BP002 + BP003). BP003 was used two times (n=2) and BP002 ones (n=1).

4.2.2 Blocking Antibody Basophil Activation test (BA-BAT)

Since the iBAT showed a clear dose response curve for Bet v 1, the next step was to investigate the effect of AIT on basophil activation. For the BA-BAT, the sample TP15 was used as indicator sera. The immunotherapy samples BP003 and BP002 were used for inhibition. In addition also the samples BP003 and BP002 were used as indicator sera since no t=0 samples (before immunotherapy) from these patients were available. Therefore it was possible to investigate the effect of using a standard indicator sera versus an indicator sera derived from the same patient. Figure 16 shows the results of the BA-BAT compared to the iBAT. For the iBAT, basophils were incubated with either TP15 (graph A or B) or an immunotherapy sample (graph C and D) and different concentrations of Bet v 1 (3, 10 100, 300, 1000, 3000, 10000 pg/mL). Inhibition was performed with immunotherapy sera BP002 or BP003. All BATs showed a dose response curve for the different Bet v 1 concentrations. However, a shift to the right is seen for the BA-BAT compared to the iBAT for the different Bet v 1 concentrations. The maximal basophil activation is a measure for the basophil reactivity. A decrease in maximal basophil activation was seen for the inhibition by BP002 but not for the inhibition by sera BP003. All experiments were performed once expect for experiment D, which was performed twice. The same mix of basophils was used for either experiment A, B,C or D. However, different basophils

were used for the duplicate of experiment D. The observed shift to the right for the BA-BAT indicates the inhibition of basophil activation (%CD63) as a result of the birch immunotherapy.

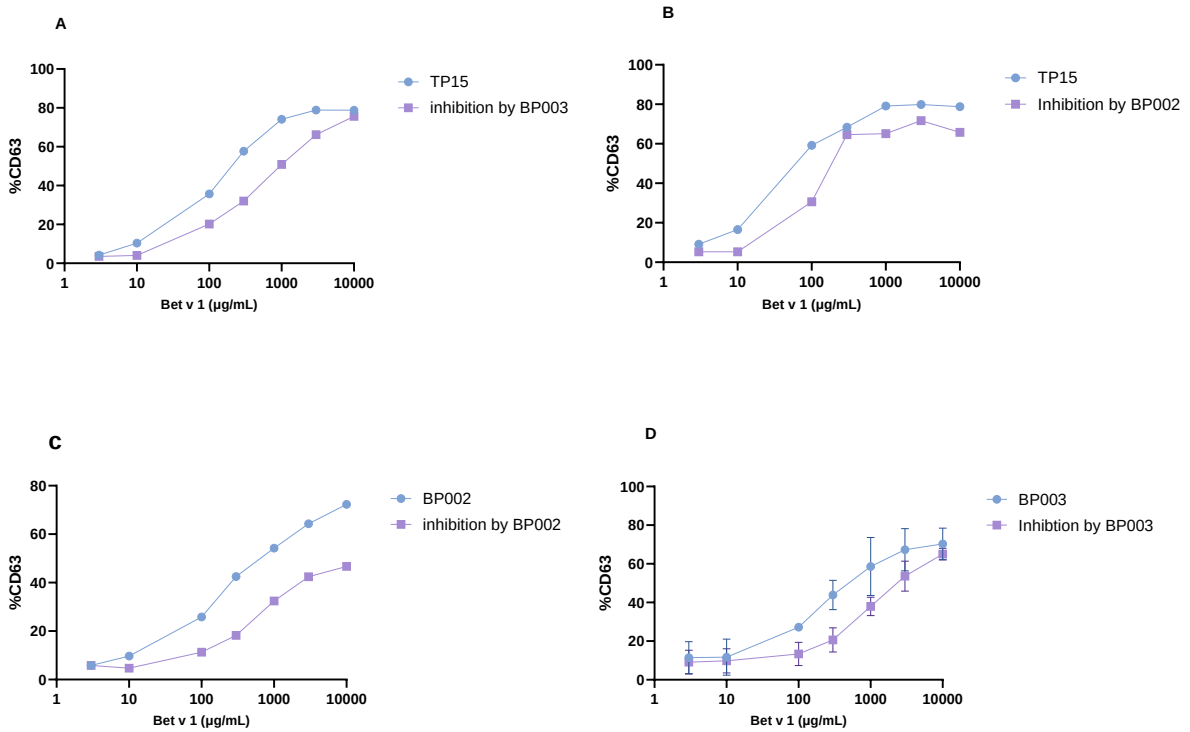

Figure 16. Results of the iBAT (blue line) and the BA-BAT (purple line)**.** The % activated basophils (%CD63) for different Bet v 1 concentrations (3, 10 100, 300, 1000, 3000, 10000 pg/mL) are shown. For the iBAT, basophils were incubated with either TP15 (graph A or B) or an immunotherapy sample (graph C and D) and inhibition was performed with immunotherapy sera BP002 or BP003.

In addition, the EC50 was calculated for each sample based on the dose response curves of both BATs (figure 17). The EC50 is the concentration inducing the half-maximal basophil activation and is an indicator for the basophil sensitivity. The EC50 was calculated to investigate the effect of the BA-BAT on the EC50 for Bet v 1 (figure 16). All BA-BATs resulted in an increase in EC50 for Bet v 1. The highest increase was found when for both the iBAT and the BA-BAT the BP003 sample was used. Using the TP15 sample for the iBAT resulted in a lower increase. This effect was also seen for the BP002 sample. A paired T test was performed for the different experiments. For TP14+BP002 a Wilcoxon test was performed since the data was not normally distributed. Significant differences were found for BP003+BP003 (p=0.021), TP15+BP003 (p=0.0142), BP002+BP002 (p=0.0054) and TP14+BP002 (p=0.0156).

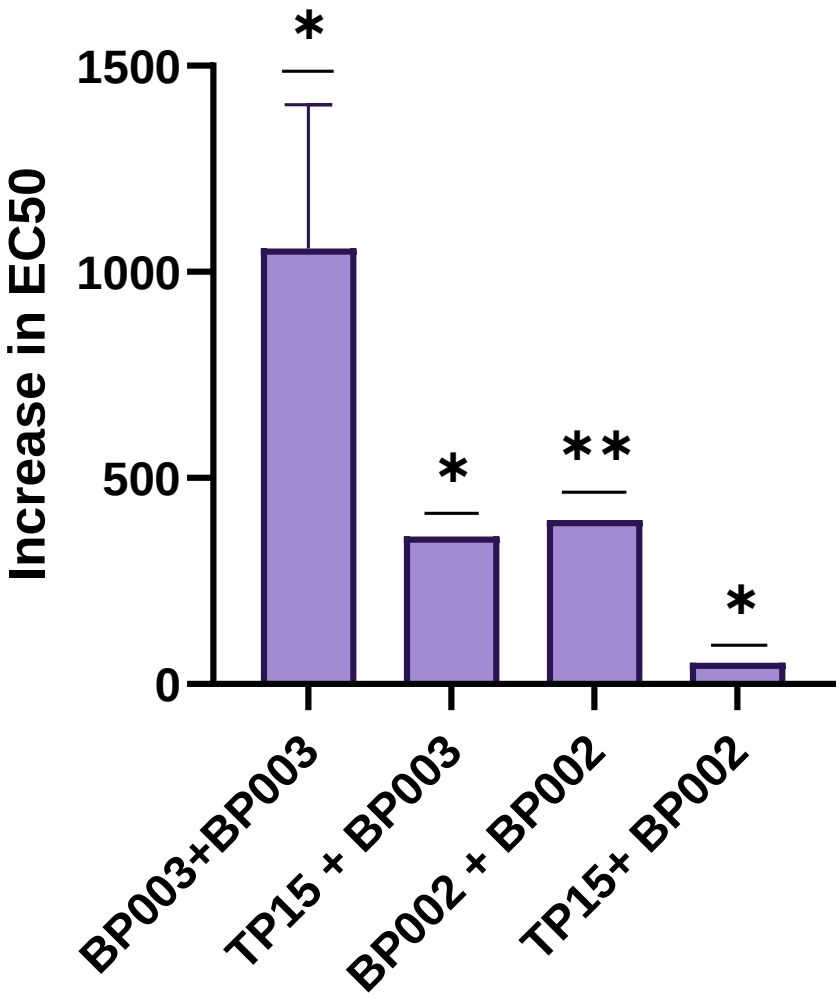

Figure 17. Increase in EC50 (for Bet v 1) for the BA-BAT compared to the iBAT. For the iBAT basophils were incubated with either TP15 or an immunotherapy sample (BP002 + BP003) and inhibition was performed with immunotherapy sera BP002 or BP003. TP15 is a sample from a birch allergic patient and BP002 and BP003 are samples from patients who received birch immunotherapy. All experiments were performed ones (n=1). Except for BP003+BP003 which was performed twice (n=2).

4.3 ELISA to measure Bet v 1 specific IgE and IgG

To test if ELISA is a suitable method to measure IgE and IgG specific for Bet v 1, first the ELISA was tested with mAbs IgG4 and IgE antibodies (figure 18). A straight line was observed until around 0.2. Higher concentrations resulted in a plateau. In addition, figure 19 shows the results from a similar experiment. However, lower concentrations for IgE and IgG were tested, which resulted in a straighter curve. To measure IgG and IgE in sera, standard curves were made with mAbs IgE and IgG4 antibodies based on the results of figure 18 and 19. Figures 20 and 21 show the results of IgE and IgG measurement in sera. Five samples from patients who received immunotherapy (BP001-BP005) and

five samples from a birch allergic patient (TP14,TP15,TP32,TP47,TP51) were tested. Birch specific IgE levels for the sera TP14,TP15,TP32,TP47,TP51 were also measured in Rijnstate. The concentrations measured were respectively 22.2, 22.5, 40.1, 85.5 and 5.22 kU/L. For the IgE measurement using ELISA only two TP sera were tested since a limited amount of sera was available. No significant Pearson correlation was found for the Bet v 1 specific IgG and IgE values (p=0.5422). Furthermore the ratio IgG/IgE was calculated and is shown in figure 22. For Bet v 1 specific IgG a wide spread of values was found, which was mostly the result of one outlier (TP32). Higher concentrations for IgG were found compared to IgE.

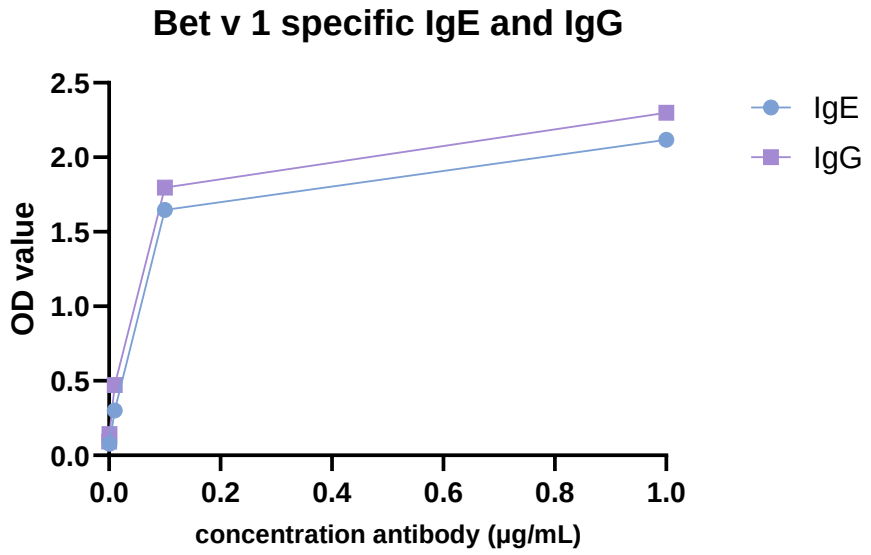

Figure 18. Measurement of IgE and IgG binding to Bet v 1 by ELISA. Dilutions of IgE and IgG4 mAbs IgE and IgG4 were used for measurements (1, 0.1, 00.1, 0.001, 0 µg/ml)

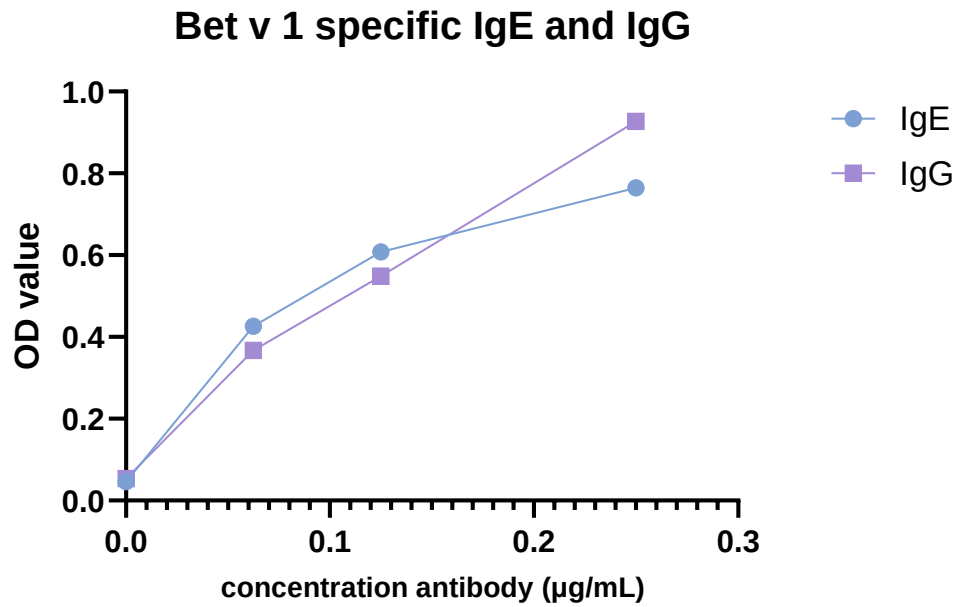

Figure 19. Measurement of IgE and IgG binding to Bet v 1 by ELISA. Dilutions of recombinant antibodies IgE and IgG4 were used for measurements (0.25, 0.125, 0.0625, 0 µg/ml)

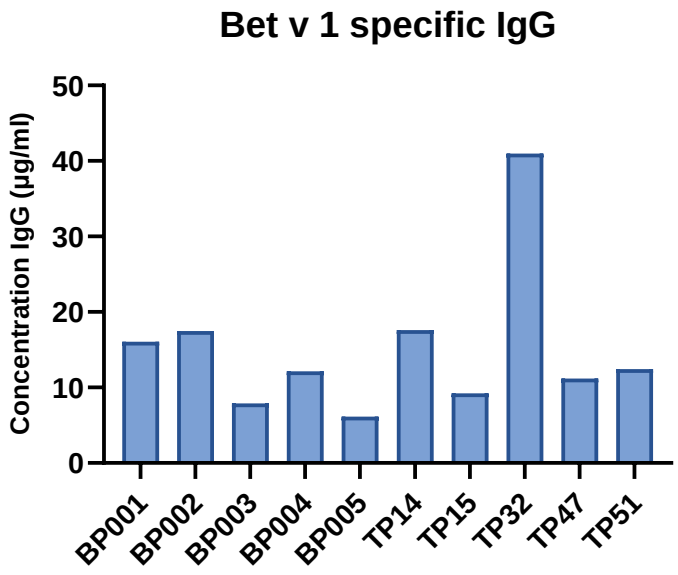

Figure 20. IgG measurement in sera (n=10) in patients who received birch immunotherapy (n=5) and in sera from birch allergic patient without immunotherapy (n=5).

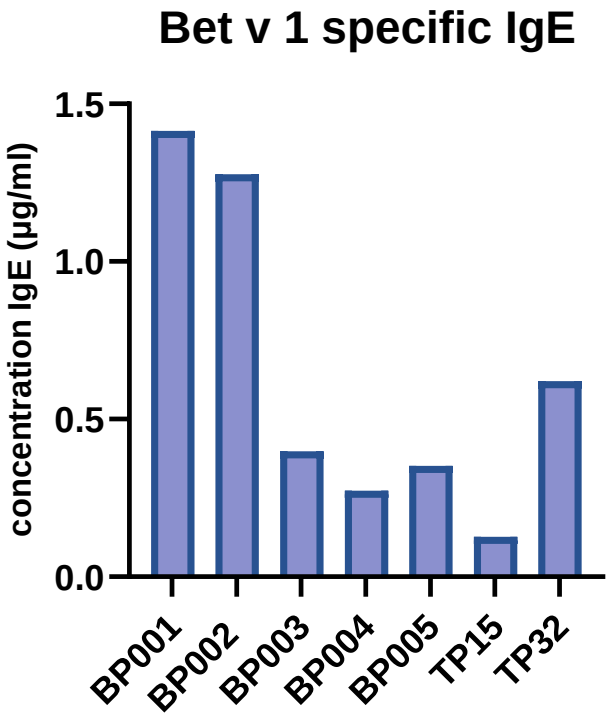

Figure 21. IgE measurement in sera (n=7) in patients who received birch immunotherapy (n=5) and in sera from birch allergic patient without immunotherapy (n=2).

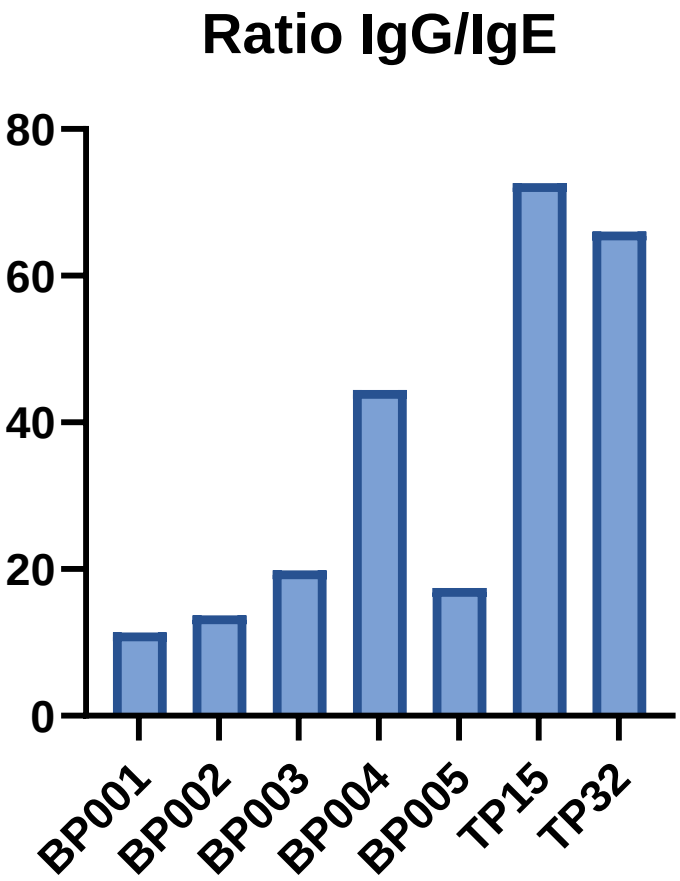

Figure 22. Ratio IgG/IgE for the five immunotherapy samples (n=5) and for the birch allergic patients (n=2).

5 Discussion

In this study the ELIFAB assay was set up for the allergen Bet v 1. When using recombinant IgG4 and IgE mAbs specific for Bet v 1, a significant decrease in allergen-IgE complexes binding to CD23 was observed. To my knowledge, this is the first study which found that a mix of three Bet v 1 specific IgG4 mAbs significantly inhibit allergen-IgE complex binding via the low affinity receptor CD23. Furthermore, it was found that a mix of IgG4 mAbs is needed to target different epitopes for the inhibition of allergen-IgE complexes binding to CD23. Therefore this mix of Bet v 1 specific IgG4 mAbs can be a possible method to treat birch allergy. However, before using the ELIFAB in clinical studies, further optimization with sera is needed. Moreover, IgG4 is able to inhibit IgE-mediated basophil degranulation via FcεRI and via the inhibitory receptor CD32B. The iBAT and the BA-BAT were used to investigate the effect of AIT on the basophil sensitivity and reactivity to Bet v 1. The BA-BAT found a significant increase in EC50 values for patients who received birch immunotherapy. This indicates that the BA-BAT is a promising tool to investigate the effect of AIT for Bet v 1. In addition, Bet v 1 specific IgG and IgE levels can be quantified by an ELISA. However, since IgG levels specific for Bet v 1 vary widely in individuals, optimization of the assay is recommended.

For optimization of the ELIFAB, the assay was first tested with recombinant Bet v 1 and recombinant IgE and IgG mAbs. Bell shaped curves were seen for all different IgE and CD23 concentrations investigated. Previous research confirmed that immune complexes are formed when an optimal antigen: antibody ratio is present (Pierson et al., 2007; Taborda et al., 2003). When there is lack or overabundance of antibodies, this can destroy the optimal structure of immune complexes to require the stimulation of effector actions (L. L. Lu et al., 2017). It has been speculated that for the formation of immune complexes optimal antibody-FcR crosslinking is needed. Poor antibody-FcR crosslinking can be a result of low antibody levels or when antibody levels are too high. Too high antibody levels can result in the formation of small immune complexes which are not able to bind to the receptor (Taborda et al., 2003). Therefore the binding of immune complexes to receptors shows a typical bell shaped curve (L. L. Lu et al., 2017). The results of this study found a higher optimal binding of immune complexes to CD23 at a higher Bet v 1 concentration (0.1 µg/ml instead of 0.01 µg/ml) when a higher IgE concentration (1 µg/ml instead of 0.1 µg/ml) was used. This is probably because an IgE concentration of 1 µg/ml results in the formation of smaller immune complexes. Therefore optimal concentrations for the formation of immune complexes was set at a Bet v 1 concentration of 0.01 µg/ml and a IgE concentration of 0.1 µg/ml.

Similar experiments which used flow cytometry instead of ELISA found similar bell shaped curves when forming immune complexes with Bet v 1 and IgE (Meulenbroek, 2013; Pree et al., 2010; Shamji et al., 2006). In addition, the MSc thesis project of Alex Overman found peaks at similar Bet v 1 concentrations when analysing Bet v 1-IgE complexes with the use of flow cytometry. However, most studies explained this bell shaped response for binding of allergen-IgE complexes to the FcεRI receptor (Fehrenbach et al., 2009; Gimborn et al., 2005; Huber, 2013). However, a few studies found this typical bell shaped curve for the receptor CD23. The study of Pree et al. (2010) found a similar bell shaped curve for CD23 for the allergen Phl p when using flow cytometry. Furthermore, the study of Shamji et al. (2006) also found a bell shaped curve for Bet v 1-IgE complexes binding to CD23. However, these results were obtained using the cell based FAB assay and not yet confirmed using the ELIFAB assay. However, the ELIFAB assay was also tested for different allergens such as the Japanese cedar pollen allergen, Ves v 5, Bet v 1, peanut extract and cat allergen (Fukano et al., 2019; Möbs et al., 2015; Shamji et al., 2013). In these studies also similar bell shaped curves were found.

This indicates that also for the receptor CD23 an optimal antigen: antibody ratio is essential for the formation of allergen-immune complexes.

As a second step of the project the binding of Bet v 1–IgE complexes to CD23 was blocked by recombinant IgG4 mAbs. The blocking experiments showed that an increased dose of IgG4 causes a decrease in Bet v 1–IgE complex binding to CD23. IgG4 has several specifications which can cause this effect. First of all, IgG4 are dynamic molecules (Kolfschoten et al., 2007; Labrijn et al., 2011). IgG4 consists out of two different isomers which differ in their disulphide bonds of hinge cysteines. Studies found that IgG molecules can switch randomly with other IgG4 molecules which cause formation of monovalent-bispecific antibodies, this process is called Fab Arm Exchange (FAE) (Aalberse & Schuurman, 2002; Van Der Velden et al., 2001). Because of FAE, IgG4 is unable to adequately cross link the allergen and form allergen-immune complexes. Furthermore, IgG4 is not able to attach to an antigen with both fab arms, which causes a lower affinity. IgG4 has also a lower affinity to activate the FcγR. However, IgG4 has a high affinity for the CD32B receptor (Shamji, Valenta, et al., 2021). This is the result of a number of amino acid substitutions in the hinge/CH2 regions of IgG4 (Y. Lu et al., 2007; R. I. F. Smith et al., 1995).

Furthermore, IgG4 can also inhibit IgE-mediated basophil degranulation (Lambin et al., 1993). This effect is caused by two mechanisms. IgG4 can compete with IgE and can bind at the same time with the inhibitory receptor CD32B. However, the mechanism of how the interaction with CD32B results in the inhibition via FcεRI is unclear (James & Till, 2016). It is found that aggregated IgG4 results in the binding of IgG4 F(ab')2 complexes to CD32B on cell surfaces (Bruhns et al., 2009). However, it should be taken into account that IgG4 forms small complexes in contrast to IgG1 (van der Zee et al., 1986), which is probably caused by the process of FAE (Kolfschoten et al., 2007). The interaction with CD32B can hinder downstream signalling through FcεRI. This results in prevention of the release of histamine and other mediators (Bruhns et al., 2005; Daeron et al., 1995). Furthermore, it is found that IgG4 produced by AIT can inhibit IgE-FAP by B cells in vitro (James et al., 2011; Nouri-Aria et al., 2004; van Neerven et al., 1999). It seems that the functional inhibitory effect of IgG4 causes the success of AIT and not the absolute concentrations in serum since often the levels of IgG4 do not correlate with clinical responses (James & Till, 2016). This is in line with my results. The sample with the higher Bet v 1 specific IgG level (BP002) did not result in a higher increase in EC50 for the BA-BAT.

Treatment with allergen IgG mAbs can result in the reduction of allergic symptoms (Pauli et al., 2008) and therefore Bet v 1 IgG mAbs can be a possible treatment option for allergies. However, previous studies found that individual clones of IgG mAbs unsuccessfully block binding of IgE to Bet v 1. Therefore, a mix of IgG clones is needed which target different epitopes to prevent IgE-mediated basophil degranulation (Gadermaier et al., 2018; Osman Mirza et al., 2000; Spangfort et al., 2003). For example, a study found that individual mAbs did not often result in blocking of Bet v 1 binding to IgE. However, a mix of IgG mAbs resulted in >80% blocking in 9 out of 10 cases (Zhang et al., 2018). However, although four mAbs were able to bind at the same time to Bet v 1, a follow up study demonstrated that three mAbs were adequate to inhibit allergic cell activation. This demonstrates that specific epitope coverage is already enough to inhibit an allergic response (Atanasio et al., 2022). However, these studies were performed targeting the high affinity receptor FcεRI. Only one study which used Fel d 1-neutralizing IgG antibodies found inhibition of allergen-IgE complex via CD23 (Shamji, Singh, et al., 2021). My results demonstrate the same effect for Bet v 1. A mix of three mAbs resulted in >80% blocking while this was not the case for the mix of two mAbs or the mAbs separately. To my knowledge, this is the first study which found that Bet v 1–specific IgG4 mAbs inhibit allergen-IgE complex binding via the low affinity receptor CD23.

After successful optimisation of the ELIFAB assay with recombinant IgE and IgG mAbs, the second step was to introduce, optimize and test an indicator sera. The ELIFAB assay makes use of an indicator serum which should contain high allergen specific IgE levels. This indicator sera can be used as a reference point for the decrease of complexes binding to CD23. Therefore the serum of time point zero (before start of AIT) could be used but also a pooled serum instead of a serum from a single patient (Shamij, 2019). Furthermore, this same indicator sera should also be used for performing the BA-BAT. Previous research which investigated the effect of birch AIT with the use of the BA-BAT used a time point zero serum as indicator sera (Würtzen et al., 2021). In this study, it was investigated which indicator sera is most suitable to monitor the effect of birch AIT.

For the ELIFAB assay, four different sera with different birch specific IgE levels were tested for formation of immune complexes. For all four different sera a slight increase in immune complex binding to CD23 was observed for Bet v 1 concentrations starting at 0.01 µg/ml. However, a high background value (OD around 0.3) was observed even in controls where no Bet v 1 was included. Therefore, different dilutions of sera were tested. However, this did not result in a clear decrease in background value. This high background value could be explained by aggregated IgE which can also bound to CD23 (Engeroff et al., 2020). Therefore one sample was centrifuged to remove possible IgE aggregates. Nevertheless this did also not reduce the background signal. In addition another possible method would be to purify IgE from sera (Badloe et al., 2022). Additionally it is recommended to test more indicator sera (from another batch) for the formation of immune complexes. Furthermore, higher CD23 concentrations could be tested for optimal binding of immune complexes to CD23. The protocol of Shamji et al. (2019) recommends testing a range of CD23 concentrations until 30 µg/ml. Moreover, the study of Fukano et al. (2019) used an optimal concentration of 20 µg/ml for the ELIFAB assay while in this study a maximal concentration of 2.5 µg/ml was used. On top of that, another study tested different CD23 concentrations for the allergen Phl p and an increase in allergen-immune binding to CD23 started from 3.2 µg/ml (Shamji et al., 2013).

Besides the ELIFAB assay, also the iBAT was examined in this study. For the iBAT, the results of this study found a dose response curve for Bet v 1. This indicates that an increase in activated basophils (%CD63) is seen for higher Bet v 1 concentrations. Therefore the iBAT can be a possible method for the diagnosis of birch allergy. There are two ways to express basophil activity, basophil reactivity and basophil sensitivity. Basophil reactivity can be defined as the maximum of basophils (%CD63) that respond to a stimulus and basophil sensitivity can be defined as the allergen concentration when half of the reactive basophils give a response. In this study, basophil sensitivity was calculated by establishing the concentration of Bet v 1 at which 50% of basophils respond (EC50) (Hoffmann et al., 2015b; Santos et al., 2021). Often a bell shaped curve is seen which reaches a plateau at high concentrations. This is the result of IgE-FcεRI complexes which causes receptor aggregation. This aggregation depends on the reactiveness of the allergen and on the affinity of IgE for the allergen. There are several factors that have an impact on the shape of the dose response curve. For example the affinity of the allergen to IgE, epitope diversity of IgE, density of epitope specific IgE and intrinsic properties of the basophils (Chirumbolo et al., 2008). Furthermore several factors can influence the outcome of the BAT such as patient medication, material for the BAT, antibodies for staining and the analysis via flow cytometry (Santos et al., 2021). The direct BAT needs to make use of fresh basophils which makes this test less flexible. Furthermore this also causes a higher risk of nonresponding basophils. Since only frozen samples were available, the indirect BAT was used in this study. Nevertheless, research indicates that the iBAT shows a similar reliability compared to the direct BAT for the diagnostics of peanut allergy (Ruinemans-Koerts, Brouwer, et al., 2022). This indicates that this method could also be suitable for the diagnosis of birch allergy.

In this study an increase in EC50 for Bet v 1 was found for the BA-BAT compared to the iBAT. For the BA-BAT, Bet v 1 was pre-incubated with patient serum before adding to the basophils. This was done to detect the effect of inhibiting factors (such as IgG/IgA) on basophil activation. The increase in EC50 indicated that IgG antibodies produced after birch AIT result in a deceased binding of IgE to FcεRI and that the BA-BAT can be a suitable tool to investigate the effect of AIT on the basophil sensitivity to Bet v 1. However no clear effect on basophil reactivity was found. The increase in EC50 found for the BA-BAT can be explained by IgG4. IgG4 is able to inhibit IgE signalling via FcεR1. This effect is caused by structural features of IgG4 causing low affinity to FcγR and high affinity to CD32B as explained in detail in the paragraphs above. The study of Würtzen et al. (2021) used a similar method. In this study, also the iBAT and BA-BAT were used to investigate the effect of birch AIT on basophil activation. Blood samples from 12 patients who received birch AIT and 7 placebo patients were analysed for basophil activation. Allergens were incubated with either pre or post-IT serum and the EC50 was calculated. Significance increases in EC50 were found after AIT treatment (Würtzen et al., 2021).

Moreover, this method was also used for the allergens Ara h2 and Ara h6. In this study a decrease in basophil sensitivity (higher EC50) was found. Furthermore, in this study also heated serum was preincubated with Ara h2/h6. The dose response curve of this BAT was similar to the normal BAT. This confirms that the inhibition by serum is not a result of non-specific factors but caused by immunoglobulins which lose their antigen-binding when exposed to high temperatures. Therefore the BA-BAT is a useful method to determine the effect of allergen immunotherapy-induced IgG/IgA on IgE-mediated basophil and T-cell activation (Ruinemans-Koerts, Uum-Otters, et al., 2022). Since my results for Bet v 1 found increased levels of EC50 for the BA-BAT compared to the iBAT, it could be established that the BA-BAT is a possible tool to investigate the effect of birch immunotherapy on basophil sensitivity.

For the BA-BAT the sample TP15 was used as indicator sera. The immunotherapy samples BP003 and BP002 were used for inhibition. In addition also the samples BP003 and BP002 were used as indicators since no t=0 samples from these patients were available. Therefore it was possible to investigate the effect of using a standard indicator sera versus an indicator sera derived from the same patient. A higher increase in EC50 was found when taking BP002 or BP003 as indicator sera. A lower increase in EC50 was found when incubating the basophils with the standard indicator serum (TP15). For a future clinical study, it is therefore recommended to incubate the basophils with its patients own sample (t=0) instead of an standard indicator sera since this study showed this can clearly influence the results.

To investigate the effect of birch immunotherapy it is of interest to quantify the levels of Bet v-1 specific IgG. In this study, ELISA was used to quantify IgG Bet v 1 antibodies produced after AIT. However, most studies are using OD values when measuring Bet v 1 specific IgG (Akinfenwa et al., 2021; Brazhnikov et al., 2023; Gepp et al., 2016). In this study, a standard curve from recombinant IgG4 was made to quantify IgG4 levels. However, it should be taken in account that the standard curve was made to detect IgG4 while total IgG was measured in the sera. Furthermore, the standard curve already reached a plateau after a concentration of 0.2 µg/ml. Therefore the measured IgG concentrations are probably not that precise. Furthermore, no reference sample without IgG was measured. Only the blocking buffer served as a control. In addition, the standard curve was made in blocking buffer and therefore it does not completely reflect IgG levels in sera. Literature suggests that Bet v 1 specific IgG levels vary widely. Studies found Bet v 1 specific IgG levels are ranging from approximately 0 to 25 µg/ml (Strobl et al., 2023; Subbarayal et al., 2013). In both studies, high standard deviations were observed, especially for IgG4. This is comparable with the outlier which

was found for the TP32 sera. However in another other study lower Bet v 1 IgG4 levels were observed until around 2 µg/ml (Guhsl et al., 2015).

Also IgE levels specific for Bet v 1 were measured in immunotherapy samples using ELISA. No significant correlation was found for Bet v 1 specific IgE and IgG values. Other studies also did not find a significant correlation between Bet v 1 specific IgE and IgG values (using OD values). A low correlation was found between Bet v 1 specific IgE and IgG4 but not between IgE and IgG1 (Brazhnikov et al., 2023). This could be explained by the fact that the class switch from IgE to IgG4 results from the same pathway (Eriksen et al., 2023). Therefore, the IgG:IgE ratio could be a more essential parameter to measure the effect of immunotherapy. It established that an increased IgG/IgE ratio can be the result of administration of recombinant allergen (Fujita et al., 2012). Therefore in this study, also the ratio IgE/IgG was calculated.

However the question how IgE and IgG can recognise different epitopes on Bet v 1 remains unexplained. At this moment, little is known about the epitope diversity of immunoglobulin subclasses produced during AIT (Gepp et al., 2016). Limited information about the specificity of IgE and IgG for Bet v 1 epitopes is available. For Bet v 1, IgE antibodies almost only recognize the folded Bet v 1 allergen but do not react to the unfolded recombinant Bet v 1 fragments or to Bet v 1 fragments which lack the secondary structure (Vrtala et al., 1997). However, this difference in binding is less investigated for the IgG antibody. For other allergens, it was found that Der p 1 (house dust mite) specific IgE and IgG antibodies can possibly bind the same recombinant allergen fragments (Chen et al., 2008). Nevertheless, another study found that IgE mainly binds to the conformational epitopes on house dust mite allergens (Huang et al., 2019). A recent study found that IgG, IgG1 and also IgG4 antibodies mostly bind to unfolded Bet v 1 fragments and peptides whereas IgE binds to conformational epitopes (Brazhnikov et al., 2023).

This study has several strengths and limitations. First of all, multiple repeats of the ELIFAB experiments were performed which shows high reproducibility of the results. In addition, different assays were tested to show the effect of IgG antibodies on immune complex binding to CD23 and FcεRI. Furthermore, since different IgG4 mAbs were available, the effect of different epitopes could be investigated. However, a limited number of sera was used in this study and no t=0 samples were available for the immunotherapy sera. Furthermore, only the allergen Bet v 1 was included and not the allergen Gly m 4. Therefore cross reactivity was not investigated in detail. Furthermore, Bet v 1 specific IgA measurements were not included since very high background values were found. In addition, only three different sera were tested for the iBAT since the iBAT is a very time consuming method. This should be taken into account when performing a future study with a high number of participants. Moreover, the ELIFAB was mostly tested with recombinant mAbs and not with human sera. When testing the ELIFAB with human sera, high background values were found. First this high background should be lowered to show clear binding of immune complexes to CD23.

The results of this study could be used for the birch immunotherapy study with ITULAZAX® in Rijnstate hospital. Based on the results of this study, there are several recommendations for this trial. First of all, it is recommended to test more indicator sera for the ELIFAB for the formation of immune complexes binding to CD23. This indicator sera could be used as reference before adding an immunotherapy sample. Before using the ELIFAB assay in the trial, immunotherapy samples should be tested instead of using recombinant IgG4 antibodies. Furthermore, better validation of the iBAT with different donors is recommended. The EC50 before and after AIT could be established to show the effect of birch immunotherapy. It is recommended to use a t=0 sample as reference and not the same standard sample for all the patients. Furthermore, for the BA-BAT also the area under the

curve can be calculated to investigate the basophil reactivity (Hoffmann et al., 2015). Also the ratio IgE/IgG could be determined before and after birch immunotherapy treatment. To validate the IgG and IgE measurements, values could also be measured with the use of ImmunoCAP. Furthermore Bet v 1 specific IgA antibodies could be measured since IgA can also compete with Bet v 1 specific IgE. Finally, both the ELIFAB, the ELISA and the BA-BAT should be tested for the allergen Gly m 4.

To conclude, this study found that the ELIFAB assay is a promising method to investigate the effect of IgG4 antibodies on allergen-IgE complexes binding to CD23. To my knowledge, this is the first study which found that Bet v 1–specific IgG4 mAbs significantly inhibit allergen-IgE complex binding via the low affinity receptor CD23. Furthermore this assay clearly shows the need of different IgG4 epitopes for inhibition of immune complex binding to CD23. Therefore a mix of the three Bet v 1 specific IgG4 antibodies can be a possible treatment option for birch allergy. This inhibition by IgG4 can be explained by the mechanism of FAE. Furthermore, IgG4 is not able to attach to an antigen with both fab arms and has a low affinity to activate the FcγR. Moreover, IgG4 can inhibit IgE-mediated basophil degranulation since IgG4 can compete with IgE and can bind at the same time with the inhibitory receptor CD32B. However, more validation is needed to show the effect of an indicator or immunotherapy sera in the ELIFAB assay instead of using recombinant IgG4 and IgE mAbs. These validations are needed before using the ELIFAB assay in future clinical studies. Moreover, ELISA can be used to quantify IgG4 antibodies in sera. However, since IgG4 levels specific for Bet v 1 vary widely in individuals, optimization of the assay is recommended. In addition, the Bet v 1 IgG:IgE ratio could be a more essential parameter to measure the effect of AIT since often the levels of IgG4 do not correlate with clinical responses. On top of that, the BA-BAT can be used to investigate the effect of AIT on basophil sensitivity and reactivity. It is therefore recommended to use a t=0 sample (before start of AIT) as indicator serum. In conclusion, the results of this study show that the ELIFAB and BA-BAT which investigate the effect of IgG4 on respectively the receptors CD23 and FcεRI are promising methods for future research to monitor the effect of immunotherapy for birch and soy allergy.

6 References

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7. Appendix

7.1. ELIFAB protocol

Materials

- **Stock samples mAbs IgE, IgG4:** kind gift of Mabylon AG, Schlieren
- **Recombinant Bet v 1** (Inbo, 42290)
- **Recombinant Human CD23/Fc** (R&Dsystems, 123-FE)
- **ELISA 96 wells plate, medium binding**
- **TMB Substrate Solution**
- **ELIFAB washing buffer:** PBS with 0.05% Tween
- **Blocking buffer:** 1% BSA/PBS solution or Roche Blocking buffer (11112589001)
- **HRP-Conjugated Streptavidin** (ThermoFisher, N100)
- **Biotin Mouse Anti-Human IgE** (BD Pharmingen, 555858) (1:1000)
- **ELISA Stop solution:** 2M H_2SO_4
- **Nunc plates for formation of complexes**

Coat Plate with sCD23

1. A stock solution of sCD23 is made by reconstituting 50 µg of lyophilized sCD23 protein (R&D) in 100 µL sterile PBS
2. Make CD23 aliquots. Add 10 µL in each Eppendorf tube
3. Prepare dilutions of stock sCD23 in PBS. First add buffer (PBS) then the stock.
4. Pipette 50 µL of diluted sCD23 to each well of a 96-well microtiter plate
5. Cover plate and leave overnight at room temperature

Block Plate with 1% BSA

1. Wash the plate prepared in Subheading “Coat Plate with sCD23” twice with 200 µL/well of ELIFAB buffer
2. Add 200 µL of blocking buffer to each well. Cover plate and incubate for 1 h at 37 °C. Be sure that the time lag between the BSA blocking and allergen complex incubations is minimal

Prepare allergen complexes

1. See separate protocol (section 7.1.2)

Adding Allergen Complex into sCD23-Coated Plate

1. Wash the BSA-blocked plate three times with 200 µL ELIFAB washing buffer
2. Transfer all preincubated allergen complex onto the sCD23- coated plates. Add 50 µL to each well. Cover the plate and leave on shaker at 650 rpm for 1 h at room temperature.

Conjugate (Antibody Detection)

1. Wash the plate four times with 200 µL ELIFAB washing buffer
2. Dilute the biotinylated IgE-specific detection antibody 1:1000 (BD bioscience) in blocking buffer (1% BSA/PBS and pipette). Add 50 µL into each well.
3. Incubate covered plate on shaker at 650 rpm for 1 hour at room temperature.

Streptavidin Peroxidase

1. Wash the plate five times with 200 µL ELIFAB washing buffer
2. Dilute streptavidin peroxidase 1:5000 in blocking buffer (1% BSA/PBS)
3. Pipette 50 µL into each well and incubate on a shaker at 650 rpm for 30 min at room temperature

Chromogenic Substrate (TMB)

1. Wash the plate six times with ELIFAB washing buffer
2. Add 50 µL of TMB substrate solution into each well.
3. Leave to incubate for up to 10 min in the dark, monitor the process. Stop reaction when colouring goes to fast.
4. Stop reaction with 50 µL per well of 2M H_2SO_4.
5. Read plate on a microplate absorbance reader at 450 nm.
6. Results are expressed as allergen-IgE binding to sCD23 (OD450).

7.1.2 Formation of immune complexes

Materials

- **Stock samples mAbs IgE, IgG4:** kind gift of Mabylon AG, Schlieren
- **Recombinant Bet v 1** (Inbo, 42290)
- **Eppendorf Tubes**
- **Blocking buffer:** 1% BSA/PBS solution

Prepare allergen complexes Bet v 1- IgE

1. To test the optimal Rec Bet v 1 concentration, make a serial dilution of stock Rec Bet v 1 concentrations (eg. 10, 1, 0,1, 0.01, 0.001,0 µg/ml) in blocking buffer (1% BSA/PBS). Dilute to a working dilution 2x greater than the final concentration
2. Dilute mAbs IgE samples in blocking buffer to a working dilution 2x greater than the final concentration
3. After making the dilutions, mix three different human-mAbs IgE samples in the same ratio in Eppendorf tubes (ratio 1:1:1).
4. Make complexes by mixing the Bet v 1 dilutions with the IgE in a ratio 1:1 in Eppendorf tubes
5. Incubate complexes for 30 min at room temperature

Prepare complexes Bet v 1-IgE-IgG4

1. Based on the protocol to make allergen complexes with IgE, select the optimal Bet v 1 concentration for making the allergen complexes. Dilute Rec Bet v 1 in blocking buffer ((1% BSA/PBS). Dilute to working dilution 3x greater than the final concentration.
2. Dilute IgE and IgG4 samples in blocking buffer (1% BSA/PBS) to working dilution 3x greater than the final concentration.
3. Mix three different human IgG4 samples in the same ratio in Eppendorf tubes (1:1:1). Do the same for the IgE samples.
4. Make complexes by mixing the Bet v 1 samples, the IgE and the IgG4 samples in a ratio 1:1:1 in Eppendorf tubes.
5. Incubate complexes for 30 min at room temperature

7.1.3 Prepare allergen complex with indicator serum

1. To remove aggregates indicator serum can be centrifugated at 15000g for 20 min
2. Add 25 µL of indicator serum into the bottom of a separate 96-well round-bottomed plate (Nunc), followed by 25 µL of Bet v 1 dilution. 25 µL of blocking buffer(Roche) can be used in negative control wells.
3. Prepare allergen of interest (standard curve) in blocking buffer to a concentration 2x greater than the required final concentration.
4. Cover plate and incubate allergen/serum for 1 h at 37 °C

7.2 ELISA protocol: Measure IgA, IgE and IgG in sera

Materials:

- **Stock samples mAbs IgE, IgG4:** kind gift of Mabylon AG, Schlieren
- **Recombinant Bet v 1** (Inbo, 42290)
- **Goat anti-human IgA-HRP** (Southern Biotech 2050-05) 1:2000
- **Goat anti-human IgG-HRP** (Southern Biotech 2040-05) 1:4000
- **Mouse-anti human IgE-HRP** (southern Biotech 9160-05) 1:2000
- **High binding 96-wells ELISA plates**
- **Nunc plates for making dilutions**
- **Bicarbonate/carbonate coating buffer**
- **ELISA stop solution:** 2M H_2SO_4
- **TMB substrate solution**
- **Wash buffer**: PBS with 0.05% Tween
- **Blocking buffer**: Roche blocking buffer (1111258001)

Methods:

- Coat plate with Bet v 1 in coating buffer (100µl/well)
- Cover plate with lid and incubate overnight in the fridge (4℃)
- Next day: wash plates 3x with ±200 µl wash buffer and tap dry (same for every washing step)
- Add 250 ul/well Roche blocking buffer and incubate for 1h at room temperature (RT)
- After 1 h of incubation with blocking buffer the plates can be washed 3x with wash buffer
- Prepare in the meantime samples by diluting sera in blocking buffer
- Diluted samples 100 µl /well can be added to the plate (include blanco's with buffer only)
- Incubated for 2h at room temperature
- Plates are washed 3x with wash buffer
- Detection antibody Goat anti-human IgA-HRP is diluted 1:2000; Goat anti-human IgG-hrp is diluted 1:4000 and goat anti-human IgE 1:2000 in Roche blocking buffer. Add 100 µl/well
- Plates are washed 3x with wash buffer
- 100 µl/well TMB is added per well and developed for ±10'
- 100 µl/well 2M H_2SO_4 is added per well to stop the reaction
- Read stopped plate at 450 nm with 620 nm as reference on ELISA plate reader

Bijlage
Werkwijze + Registratie Indirecte BAT (aftekenlijst)

Behorend bij document(en): Analysevoorschrift Indirecte BAT (documentnummer)

Datum:…..&…..-…..-20…..

Monsternr: Patiëntnaam: geboortedatum:

Opgeslagen in Flow:

Buffycoat isolatie opgewerkt door:

Verzamel 6 EDTA buizen, waarvan alle aangevraagde bepaling gedaan zijn, met bloedgroep O positief (dubbele controle op bloedgroep en volledig afgewerkte aanvraag)
Anonimiseer de buizen door het monsteretiket te verwijderen.
Plak dit monsteretiket op een vel papier en bewaar dit twee dagen in het mapje monsters gebruikt voor BAT (PP202 tafel HbA1c)

Centrifugeer de donor-bloedbuizen 10 minuten bij 2200g (programma 1).

Vul twee 5 ml falcon tubes met 2 ml fysiologisch zout en label deze buizen als "FZ" (NaCl 0,9% max 1 week houdbaar).

Pipetteer het supernatant (plasma) met een plastic pasteurspipet af.(tot plm. 3 mm boven het celniveau) in een 50 ml afvalbuisje.

Pipetteer daarna de buffycoats over in de twee "FZ" falcon buisjes. Het is niet erg als er wat erytrocyten of plasma bij zit.

Dop de buisjes af en meng zorgvuldig (kantelen).

Centrifugeer de twee falcon buisjes (10 min bij 2200xg - programma 1).

Zet de centrifuge (leeg) weer aan op programma 6 om hem af te koelen tot 11 ºC voor het strippen.

Vul een nieuw 5 ml falcon tube met 2 ml fysiologisch zout en label deze buis als WBC1.

Pipetteer het supernatant opnieuw af (tot 3 mm boven het celniveau) in een afvalbuisje.

Pipetteer beide buffycoats over naar buisje "WBC 1".

Dop de buis af en meng zorgvuldig (kantelen).

Meet de leuko concentratie en noteer dit bij "WBC 1" (open cap, aspiration sensor off).

Bereken, m.b.v. het Excel programma de verdunning die nodig is om op een WBC concentratie tussen 12,5 en **15,0 x 109/l** te komen.
Noteer deze volumina onderaan deze lijst (. . . . . µl buffycoat + ... µl FZ)

Maak deze verdunning in een 2 ml falcon buisje en label deze buisje "WBC2".

Dop de buis af en meng zorgvuldig (zwenken)

Meet de suspensie opnieuw om de concentratie te controleren en noteer dit bij "WBC 2"

Herhaal zo nodig stap 16 t/m 19. (dit wordt dan "WBC 2b")

Strippen van IgE

Voor start: ontdooi 5 ml BSB. (voor stap 19 t/m 34) noteer datum van maken: d.d. . . . . serum patiënt.
Zet de 37°C hybridisatie stoof/shaker aan.

Centrifugeer [WBC 2] bij 11°C voor 5 minuten bij 1000xg (progr. 6 strippen iBAT).

Pipetteer het supernatant af (tot 5 mm boven het celniveau).

Voeg 2 ml koude stripping buffer toe, meng zorgvuldig (10 à 15 keer)
Noteer datum van maken onderaan deze lijst.

Centrifugeer dit bij 11°C voor 5 minuten bij 1000xg (progr. 6 strippen iBAT).

Pipetteer het supernatant af (tot 5 mm boven het celniveau).

Resuspendeer het pellet in 2 ml BSB.

Dop de buis af en meng zorgvuldig (10 à 15 keer).

Centrifugeer dit bij 11°C voor 5 minuten bij 1000xg (progr. 6 strippen iBAT).

Schakel de centrifuge uit of over op ander, niet koelend, programma.

Pipetteer het supernatant af (tot 5 mm boven het celniveau).

Resuspendeer het pellet tot een totaalvolume van ± 1500 µl met BSB. Doe dit door het volume te vergelijken met een buisje waar exact 1500 µl in zit.

Dop de buis af en meng zorgvuldig (10 á 15 keer kantelen).

Basofielsensibilisatie

Meng de gestripte donorcellen met serum van de patiënt in een Falcon tube van 5 ml volgens onderstaand schema. Geef Falcon tube de naam "[BAT registratie nr] + WBC3"

	Overnacht
Serum/plasma	130 µL
donorcellen	500 µL

Incubeer overnacht in de hybridisatie stoof/shaker bij 37 °C en zwenk op stand 50.
(De plastic houder moet vastzitten met een plakbandje zodat deze niet verschuift).

Volgende dag

Meet de leukocyten om de concentratie te controleren.

Voeg, bij een leucoconcentratie van 10-15 x10^9/l, 770 ml BSB toe aan de buis.

Indien afwijkende leucoconcentratie en meer dan 22 buizen bereken dan m.b.v. het Excel bestand en de leucocyten conc. de toe te voegen hoeveelheid BSB. Overleg met senior

Noteer de hoeveelheid toegevoegde BSB onderaan deze lijst.

BSB	770 µL

Activatie en meting

Voor start: ontdooi 2,5 ml BSB
allergeen (van elk 2 vials; zie werkschema)
FcεRI en fMLP
Zet het waterbad bij de morfologie aan.
Haal lysingbuffer, indien nog restant vorige keer aanwezig, uit koelkast om op kamertemperatuur te komen of maak nieuw voor punt 43.

Oplossen allergenen

Nummer de allergeen vials volgens het werkschema.

Centrifugeer de allergenen kort (3 sec) in de galaxy minstar centrifuge.

Los de allergenen op volgens de in het werkschema aangegeven hoeveelheid BSB. Neem steeds nieuwe pipettip.

Laat de vials 10 minuten staan.

Zet ondertussen de Falconbuizen klaar voor stap 41 en 42

VORTEX en centrifugeer de ALLERGENEN EN controles

Vortex de opgeloste allergeen vials en de *FcεRI en fMLP* controles (5 sec)

Centrifugeer kort (3 sec) in de galaxy minstar centrifuge.

Maken verdunningsreeks allergenen

Maak de verdunningsreeksen van de overige allergeen conc. volgens het werkschema. Etiketteer hiervoor Falconbuizen.

Voeg eerst in alle buisjes de BSB toe en daarna het allergeen.

Gebruik telkens nieuwe tips.

Vortex de oplossing voor iedere doorverdunning.

Analyse

Nummer Falconbuizen met stift. Pipetteer onderin de buizen volgens het werkschema:

- **BSB** in alle buisjes
  - **150 µl in de blanco's** (sluit de blanco buisjes af met een dopje)
  - **100 µl in de overige buisjes**.
- Voeg aan de positieve controles **50 µl stimulation control toe (FcεRI en fMLP*)***
- Voeg aan de overige buizen **50 µl allergeen** van de juiste conc. toe.
- Voeg aan alle buizen **50 µl celsuspensie** toe. Zwenk de buis met donorbloed tussendoor.
- Pipetteer in elke buis (in de vloeistof) **20 µl staining reagens**. Neem steeds een nieuwe pipetpunt.
- Meng de buizen vanuit de losse pols en voorzie ze van een dopje.

Incubeer 15 minuten in een waterbad van 37° **AANZETTEN FLOWCYTOMETER** (zie ook Bedieningsvoorschrift FACSCanto II (037348))

- Zet de flowcytometer aan (hfdst 7.1.1 opstartprocedure)
- Fluidics startup aanzetten op flowcytometer (hfdst 7.1.1 opstartprocedure)
- Maak buisjes voor spoelen klaar. (FACSClean™, FACSRinse™ en aqua dest)
- Spoelprocedure flowcytometer (hfdst 7.4.4 spoelprocedure)
- Indien nog niet uitgevoerd de dagelijkse CS&T check uitvoeren (hfdst 7.1.6 dagelijkse performance check met CS&T beads)

Bereiden lysing buffer

- Bereid, indien geen restant van vorige keer aanwezig, lysing buffer door 5 ml 10 x lysing geconcentreerde buffer uit de Bühlmann kit te verdunnen tot 50 ml met steriel aqua dest.

Lyseren en wassen

Voeg 1,5 ml lysing buffer, van kamertemperatuur, toe. (25 ml dispenseer pipet)
Zwenk dit minimaal 5x.
Incubeer 5 minuten bij kamertemperatuur totdat de oplossing helder is geworden.
Centrifugeer 5 minuten bij 640xg, geremd (programma 5 BAT). Controleer!
Zet de buisjes voorzichtig terug in het rekje.
Giet de bovenstaande vloeistof in één vloeiende beweging zo veel mogelijk af.
Dep de buizen af op een absorberend doekje.
Spoel de wasbak schoon.
Voeg 500 µL wasbuffer toe en plaats de dopjes op de buisjes.
Vortex de buizen 5 seconden.
Plaats, indien niet direct gemeten kan worden, de buizen in de koelkast tot de meting.

Meten op flowcytometer

Meet alle buizen op de FACSCanto flowcytometer.
(zie ook Bedieningsvoorschrift FACSCanto II (037348) hfdst 7.3 analyse)

Meng alle buizen kort op de vortex voordat ze in de carrousel geplaatst worden.

Sla alle meetresultaten op (hfdst 7.4.2 opslaan van meetresultaten)
naamformat: panel jjjjmmdd bijvoorbeeld:
BAT koemelk 2021-11-17 21046577003 of iBAT koemelk 2021-11-17 C28

Buffycoat isolatie uitgevoerd door:	

Leucocyten concentratie WBC 1	$x10^9/l$

Verdunning WBC 1	µl WBC1	µl Fys. zout

Leucocyten concentratie WBC 2	$x10^9/l$

	Gemaakt d.d.
BSB	Dag 1 isolatie: Dag 2 BAT
Stripping buffer	
Lysing buffer	

	Datum/tijd in	Datum/tijd uit
Incubatie in stoof		

Leucocyten concentratie WBC 3	$x10^9/l$

Toegevoegde µl BSB	µl

Bedieningsvoorschrift
FacsCanto II (Research)

DOEL en TOEPASSINGSGEBIED

FacsCanto II is een flowcytometer. Door middel van de (indirecte) Basofiele Activatie Test (BAT) wordt de activatie van basofielen gemeten na stimulatie met allergenen.

ALGEMENE GEGEVENS VAN HET APPARAAT

Zie Bedieningsvoorschrift FacsCanto II 2 Algemene gegevens van het apparaat.

DEFINITIES en termen

Zie Bedieningsvoorschrift FacsCanto II 3 Definities en termen.

Specificaties

Zie Bedieningsvoorschrift FacsCanto II 4.1 Meetprincipe; Zie Bedieningsvoorschrift FacsCanto II 4.2 Achtergrond; Zie Bedieningsvoorschrift FacsCanto II 4.3 Gebruikersinstellingen.

VEILIGHEID en milieuaspecten

Zie Bedieningsvoorschrift FacsCanto II 5.1 Bescherming apparatuur en omgeving; Zie Bedieningsvoorschrift FacsCanto II 5.2 Bescherming van medewerkers; Zie Bedieningsvoorschrift FacsCanto II 5.3 Afvalverwerking.

HULPSTOFFEN en hulpmiddelen

Zie Bedieningsvoorschrift FacsCanto II 6.1 Hulpstoffen; Zie Bedieningsvoorschrift FacsCanto II 6.2 Bereiding hulpstoffen; Zie Bedieningsvoorschrift FacsCanto II 6.3 Hulpmiddelen.

WERKWIJZE

Apparatuur

Opstartprocedure

- Zet de flowcytometer aan met behulp van de groene knop aan de linkerkant van het apparaat, de opstart duurt 9 minuten.
- Zet de PC aan.
- User id is Administrator, password is BDIS#1 (CST operator, welcome#1).
- Kies voor het **<FACSdiva>** programma (voor zelf te definiëren experimenten).
- Kies **<Administrator>** een paswoord is niet nodig.
- Er wordt nu een verbinding gemaakt tussen de flowcytometer en de computer, zie rechtsonder.
- Het bolletje verandert van geel naar groen. Linksonder wordt aangegeven hoelang het opwarmen van de lasers nog duurt. Tijdens het opwarmen van de lasers kan alvast opgestart worden.
- Indien pop-up kies **<use CST settings>**.
- Ga in de menubalk naar **<Cytometer>**.
- Klik op **<Fluidics Start Up>** en **<OK>**.
- Na ongeveer 5 minuten is de flowcytometer klaar met de spoelprocedure.
- Als ook het bolletje rechtsonder groen is, is de flowcytometer klaar voor gebruik.
- Klik eventueel boven in de taakbalk op **<View>** om de volgende tabbladen te openen: **<Browser>, <cytometer>, <inspector>, <worksheet>** en **<aquisition dashbord>.**

Vervangen van reagentia

Het niveau van de diverse reagentia wordt op de flowcytometer in het midden van het scherm weergegeven door middel van groene indicatie niveaus.
De flowcytometer geeft een alarm bij een tekort van een van de volgende reagentia; BD Sheath Fluid, FACSclean, FACSshutdown.
- Als de FACS bezig is met een meting, druk op **<Stop Acquiring>**.
- Verwijder de snelkoppeling door op de metalen tab te drukken.
- Trek de sensor uit het vat.
- Verwijder de dop van het reagensvat en plaats deze op het nieuw aan te sluiten vat.
- Sluit de snelkoppeling weer aan
- Verwijder de luchtbellen, kies **<Cytometer>**, **<Cleaning Modes>**, **<Prime After Tank Refil>**.
- Na voltooien van het primen klik op **<Ok>**.
- Vervolg de meting.

Legen van het afval vat

Wanneer het afvalvat vol is geeft de flowcytometer een alarm, handel als volgt:
- Als de FACS bezig is met een meting, druk op **<Stop Acquiring>**.
- Verwijder de snelkoppeling door op de metalen tab te drukken.
- Trek de sensor er recht uit. (Wanneer de cytometer aan het meten is staat het afvalvat onder druk. Verbreek altijd de verbinding met de fluidics cart (trek grijze kabeltje uit de aansluiting aan de fluidics

cart) voordat het vat wordt geleegd. Wacht in ieder geval 30 seconden voor het verwijderen van de dop van het vat (je ziet de vloeistof stoppen met stromen door het doorzichtige slangetje).
- Verwijder de dop van het afvalvat.
- Leeg het vat, de inhoud mag geloosd worden op het riool.
- Sluit de dop en sluit de snelkoppeling weer aan.
- Hervat de meting.

Kalibratie

Zie voor kalibratie 9.1 Maken van een baseline en meten van compensaties
Kalibratie frequentie: Het maken van een baseline gebeurt bij een nieuw lotnummer CS&T IVD beads of na technisch onderhoud waarbij filters, PMT's zijn veranderd.

Inklaren nieuwe lotnummers monoclonale antistoffen

Zie Bedieningsvoorschrift FacsCanto II 7.1.5 Inklaren nieuwe lotnummers monoclonale antistoffen.

Spoelprocedure flowcEL (FACS Clean, aQUA DEST, FACS RINSE, AQUA DEST)

Nadat de Flowcytometer is ingeschakeld en de fluidics start-up is gedaan en voordat de Flowcytometer wordt uitgeschakeld dient deze te worden gespoeld.

- Vul 3 Falcon buisjes met resp. 1 ml FACSClean, aqua dest, Detergent Solution.

- Ga naar **<Cytometer>**, **<Cleaning Modes>**, **<Clean Flow Cell>**.
- Installeer een buisje met FACSClean™ onder de SIT (Sample injection tube). De zwarte houder om naald dient weggeklapt te worden.
- Klik op **<OK>**.
- Klik op **<OK>** na de melding Cleaning is complete.
- Herhaal deze spoelprocedure met aqua dest, Detergent Solution™ en nogmaals met aqua dest.

- Voer een bubble filter purge en een degas flow cell uit, ga naar **<Cytometer>**, **<Cleaning Modes>**, **<Bubble Filter Purge and Degas Flow Cell>**.

REAGENTIA EN HULPSTOFFEN

Reagentia

Artikel	**Flow2Cast kit**
Omschrijving	Flow2Cast kit voor BAT
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	FK-CCR
Verpakking	100 testen/kit
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	2-8°C.
Voorraad	Arnhem PP-202-KK1 (voorraad in koelcel)
Bereiding	Zie 7.4.

Deze kit bevat

Artikel	**Stimulation buffer (BSB)**
Omschrijving	Basofiele stimulatie buffer
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	B-CCR-STB
Verpakking	Flesje (lyophilized)
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	Ongeopend 2-8°C.
Voorraad	Arnhem PP-202-KK1
Bereiding	Zie 7.4.

Artikel	**Stimulation control anti-FcεRI mAb (B-CCR-STCON)**
Omschrijving	Stimulatie controle anti-FcεRI mAb
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	B-CCR-STCON
Verpakking	1 lyophilized vial.
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	Ongeopend 2-8°C.
Voorraad	Arnhem PP-202-KK1
Bereiding	Zie 7.4.

Artikel	**Stimulation control fMLP (B_CCR_FMLP)**
Omschrijving	Stimulatie controle fMLP
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	B-CCR-FMLP
Verpakking	1 lyophilized vial
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	Ongeopend 2-8°C.
Voorraad	Arnhem PP-202-KK1
Bereiding	Zie 7.4.

Artikel	**Staining reagent**
Omschrijving	Kleur reagens (anti-CD63-FITC en anti-CCR3-PE mAb)
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	B-CCR-SR
Verpakking	Flesje à 2,2 ml
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	2-8°C.
Voorraad	Arnhem PP-202-KK1.
Bereiding	Klaar voor gebruik

Artikel	**Lysing reagent 10 x concentrated**
Omschrijving	Lyses reagents 10 x geconc.
Firma	BÜHLMANN Laboratories AG
Artikelnummer	B-CCR-LYR
Verpakking	Fles à 25 ml.
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	2-8°C.
Voorraad	Arnhem PP-202-KK1.
Bereiding	Zie 7.4.

Irriterend

Artikel	**Wash buffer**

Omschrijving	Wasbuffer
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	B-CCR-WB
Verpakking	Fles à 100ml.
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	2-8°C.
Voorraad	Arnhem PP-202 KK1.
Bereiding	Klaar voor gebruik

Overige reagentia (niet aanwezig in de kit)

Artikel	**Stripping buffer**
Omschrijving	Stripping buffer
Firma	Zelf gemaakt (Reagentia van Merck)
Artikelnummer	(Art. 1.06346.0500, Art. 1.04936.0500)
Verpakking	50mL Greiner tube
Houdbaarheid	1 jaar (om besmetting te voorkomen, gebruik 1 maand)
Bewaarcondities	2-8°C.
Voorraad	Arnhem PP-202 KK1.
Bereiding	Zie 7.4.

Artikel	**Gedestileerd water**
Omschrijving	Gedestileerd water
Firma	Braun, Germany (via apotheek)
Artikelnummer	N.v.t.
Verpakking	Fles à 100ml.
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	18-28°C
Voorraad	Arnhem, PP-202
Bereiding	Klaar voor gebruik.

Artikel	**Natriumchloride 0.9%**
Omschrijving	Natriumchloride 0.9%
Firma	Braun, Germany (via apotheek)
Artikelnummer	N.v.t.
Verpakking	Fles à 100ml.
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	18-28°C
Voorraad	Arnhem, PP-202
Bereiding	Klaar voor gebruik.

Controles

Artikel	**Stimulation control anti-FcεRI mAb (B-CCR-STCON)**
Omschrijving	Stimulatie controle anti-FcεRI mAb
Firma	BÜHLMANN Laboratories AG, Switzerland

Artikelnummer	B-CCR-STCON
Verpakking	1 lyophilized vial.
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	Ongeopend 2-8°C.
Voorraad	Arnhem PP-202-KK1
Bereiding	Zie 7.4.

Artikel **Stimulation control fMLP (B_CCR_FMLP)**

Omschrijving	Stimulatie controle fMLP
Firma	BÜHLMANN Laboratories AG, Switzerland
Artikelnummer	B-CCR-FMLP
Verpakking	1 lyophilized vial
Houdbaarheid	Tot vervaldatum.
Bewaarcondities	Ongeopend 2-8°C.
Voorraad	Arnhem PP-202-KK-1
Bereiding	Zie 7.4.

Bereiding reagentia – controles – standaarden

Artikel **Stripping buffer**

Bereiding	Weeg 20.0 g Sodium dihydrogen phosphate monohydrate (NaH2PO4•H2O, 137.99 g/mol, Merck Art. 1.06346.0500) en 0.37 g potassium chloride (KCl, 74.55 g/mol, Merck Art. 1.04936.0500) af op aparte vloeipapiertjes en voeg dit toe aan een erlenmeyer met ongeveer 700mL aquadest en mix. Corrigeer de pH naar 3.55 met sodium hydroxide (NaOH, 5M, apotheek) or hydrogen chloride (HCl, 5M, apotheek). Vul dan de erlenmeyer met aquadest aan tot 1 liter. Verdeel de stripping buffer over 12 50 mL Greiner buizen. Etiketteer de buizen voor iedere maand één.
Houdbaarheid	1 jaar
Bewaarcondities	2-8°C
Bewaarplaats	Arnhem PP202-KK1

Artikel **Stimulation buffer (BSB)**

Bereiding	Los de inhoud van het flesje op in 50 ml steriel water (water afmeten met 50 ml steriele Greiner buis) Uitvullen in 10 steriele greiner bio-one buizen (5x5ml, 4x6mL voor pinda; 4x5mL, 4x6.5mL voor hazelnoot)), flesje met restant invriezen tot kit op is. (gebruik steriele filtertips van 1000 ul of gieten)
Houdbaarheid	6 maanden.
Bewaarcondities	-20°C
Bewaarplaats	Arnhem PP202-DV2

Artikel **Stimulation control anti-FcεRI mAb (B-CCR-STCON)**

Bereiding	Los de inhoud van de vial op in 1.5 ml stimulation buffer (BSB)

Uitvullen in 11 steriele epjes à 120 ul, flesje met restant invriezen tot de kit op is.

- verbruik is 2x50 ul per run, circa 10 runs/kit
- vermijd extra invries/ontdooi stappen
(gebruik steriele filtertips van resp. 1000 en 200 ul)

Houdbaarheid	6 maanden
Bewaarcondities	-20°C
Bewaarplaats	Arnhem PP202-DV2

Artikel **Stimulation control fMLP (B-CCR-FMLP)**

Bereiding

Los de inhoud van de vial op in 1.5 ml stimulation buffer (BSB)
Uitvullen in 10 steriele epjes à 120 ul, flesje met restant invriezen tot de kit op is.

- verbruik is 1x50 ul per run, circa 10 runs/kit
- vermijd extra invries/ontdooi stappen
(gebruik steriele filtertips van resp. 1000 en 200 ul)

Houdbaarheid	6 maanden
Bewaarcondities	-20°C
Bewaarplaats	Arnhem PP202-DV2

Artikel **Lysing reagent 10 x concentrated**

Bereiding

Verdun 5 ml lysing reagent 10 x concentrated tot 45 ml met gesteriliseerd water in een 50 ml steriele Greiner buis.

Houdbaarheid	tot vervaldatum.
Bewaarcondities	2-8°C.
Bewaarplaats	Arnhem PP202-KK1

Werkschema Indirect BAT Boompollen

Bloedafname **Datum:** **Etiket bloedafname:**

Tijd:

Analyse BAT **Datum:**

Buis nr	Buis code	Allergeen/controle	Verdunning	BSB (ul)	Allergeen/ controle (ul)	EDTA Bloed (ul)	Staining reagent (ul)
1	PBa	Neg Contr. 1		150		50	20
2	PBb	Neg Contr. 2		150		50	20
3	PC1a	a-FcεRI 1a		100	50	50	20
4	PC1b	a-FcεRI 1b		100	50	50	20
5	A-3	n Bet v1 (3 pg/ml)	60 ul 10 + 140 ul BSB	100	50	50	50
6	A-10	n Bet v1 (10 pg/ml)	20 ul 100 + 180 BSB	100	50	50	20
7	A-100	n Bet v1 (100 pg/ml)	20 ul 1.000 + 180 ul BSB	100	50	50	20
8	A-300	n Bet v1 (300 pg/ml)	20 ul 3.000 + 180 ul BSB	100	50	50	20
9	A-1.000	n Bet v1 (1.000 pg/ml)	20 ul 10.000 + 180 ul BSB	100	50	50	20
10	A-3.000	n Bet v1 (3.000 pg/ml)	60 ul 10.000 + 140 ul BSB	100	50	50	20
11	A-10.000	n Bet v1 (10.000 pg/ml)	Werkoplossing 10.000	100	50	50	20

Werkoplossing: 10,000 pg/ml **zie lotnr. registratie**
Epjes met minimaal **130 ul** (=> 150) vullen!)

Remarks:

- Final concentration = start concentration x 0.227 bijv. 100 ng/ml x (50/220) = 22.7 ng/ml

Preparation times:

- Allergens:
- Add blood:

Add to each tube:

- 100 µl BSB (150 µl BSB in neg. contr.)
- 50 µl of the different controls/allergens (see table above)
- 50 µl blood
- 20 µl staining reagent
- Total = 220 ul

Werkschema BAT Boompollen

Bloedafname **Datum:** **Etiket bloedafname:**

Tijd:

Analyse BAT **Datum**

Run naam:

Buis nr	Buis code	Allergeen/controle	Flowrate H=High	Events	Measure time	Q2 (% stimulated basophils)
1	PBa	Neg Contr. 1	H	550		
2	PBb	Neg Contr. 2	H	550		
3	PC1a	a-FcεRI 1a	H	550		
4	PC1b	a-FcεRI 1b	H	550		
5	A-3	n Bet v1 (3 pg/ml)	H	550		
6	A-10	n Bet v1 (10 pg/ml)	H	550		
7	A-100	n Bet v1 (100 pg/ml)	H	550		
8	A-300	n Bet v1 (300 pg/ml)	H	550		
9	A-1,000	n Bet v1 (1,000 pg/ml)	H	550		
10	A-3,000	n Bet v1 (3,000 pg/ml)	H	550		
11	A-10,000	n Bet v1 (10,000 pg/ml)	H	550		

Werkschema Inhibition BAT Boompollen

Bloedafname **Datum:** **Etiket bloedafname:**

Tijd:

Analyse BAT **Datum:**

Buis nr	Buis code	Allergeen/controle	Verdunning	BSB (ul) + 10% serum (ul)	Allergeen /controle (ul)		EDTA Bloed (ul)	Staining reagent (ul)
1	PBa	Neg Contr. 1		150		Blocking gedurende 60 minuten bij 37 °C	50	20
2	PBb	Neg Contr. 2		150			50	20
3	PC1a	a-FcεRI 1a		100	50		50	20
4	PC1b	a-FcεRI 1b		100	50		50	20
5	A-3	n Bet v1 (3 pg/ml)	60 ul 10 + 140 ul BSB	100	50		50	50
6	A-10	n Bet v1 (10 pg/ml)	20 ul 100 + 180 BSB	100	50		50	20
7	A-100	n Bet v1 (100 pg/ml)	20 ul 1.000 + 180 ul BSB	100	50		50	20
8	A-300	n Bet v1 (300 pg/ml)	20 ul 3.000 + 180 ul BSB	100	50		50	20
9	A-1.000	n Bet v1 (1.000 pg/ml)	20 ul 10.000 + 180 ul BSB	100	50		50	20
10	A-3.000	n Bet v1 (3.000 pg/ml)	60 ul 10.000 + 140 ul BSB	100	50		50	20
11	A-10.000	n Bet v1 (10.000 pg/ml)	Werkoplossing 10.000	100	50		50	20

Run naam:

Buis nr	Buis code	Allergeen/controle	Flowrate H=High	Events	Measure time	Q2 (% stimulated basophils)
1	PBa	Neg Contr. 1	H	550		
2	PBb	Neg Contr. 2	H	550		
3	PC1a	a-FcεRI 1a	H	550		
4	PC1b	a-FcεRI 1b	H	550		
5	A-3	n Bet v1 (3 pg/ml)	H	550		
6	A-10	n Bet v1 (10 pg/ml)	H	550		
7	A-100	n Bet v1 (100 pg/ml)	H	550		
8	A-300	n Bet v1 (300 pg/ml)	H	550		
9	A-1,000	n Bet v1 (1,000 pg/ml)	H	550		
10	A-3,000	n Bet v1 (3,000 pg/ml)	H	550		
11	A-10,000	n Bet v1 (10,000 pg/ml)	H	550		