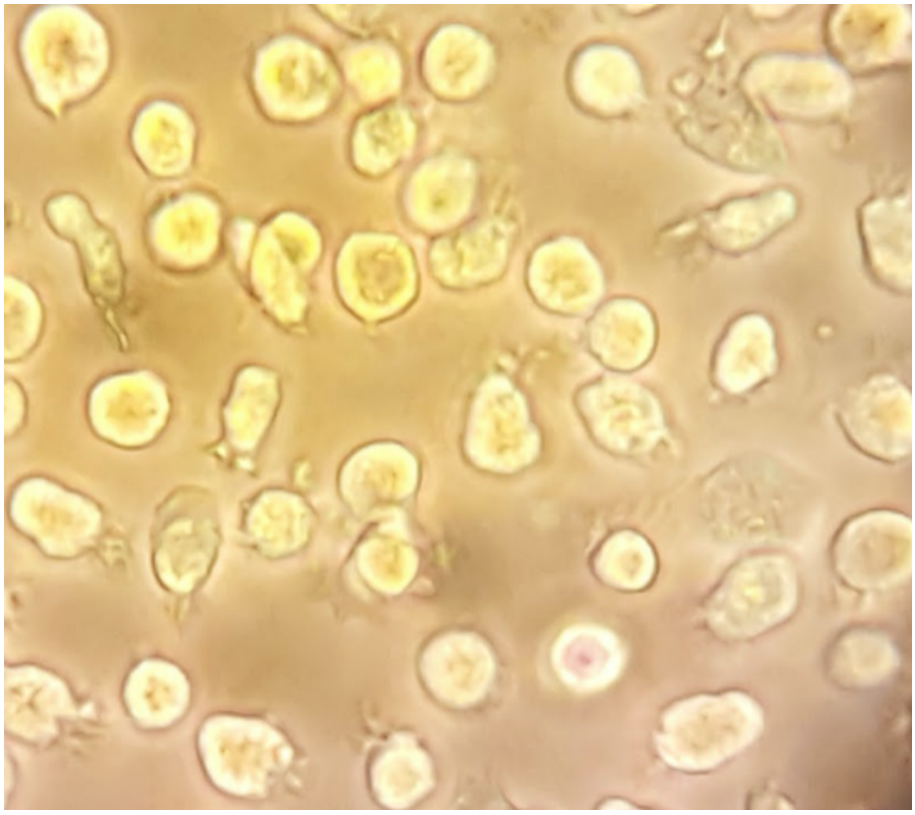
AALBORG UNIVERSITY

STUDENT REPORT

SCHOOL OF MEDICINE AND HEALTH

Characterization of IgE on human effector cells



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AALBORG UNIVERSITY
STUDENT REPORT

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Resume

Baggrund: Antallet af diagnosticeret allergiske tilfælde har været stødt stigende i de seneste 60 år med en stadig voksende tendens. Udviklingen medfører, at samfundet kommer til at stå over for en stor sundhedsmæssig udfordring i forhold til de socioøkonomiske omkostninger ved tab af arbejdsstyrke og den nedsatte livskvalitet for de påvirkede individer. Med afsæt i den immunoglobulin E (IgE) medieret reaktion, som skyldes en unormal immunreaktion, bliver der udviklet specifikke IgE antistoffer mod harmløst stimuli. Hovedaktørerne i den allergiske reaktion består af mastceller og de basofile granulocytter, som frigiver biologisk aktive mediatorer, der kan føre til udviklingen af rødme, hævelse, kløe og vejrtrækningsbesvær. Da diagnosen delvis baseres på en påvisning af IgE sensitisering, der er en forudsætning for at individet kan udvikle en allergisk reaktion, så påviser testen ikke nødvendigvis klinisk relevans, derfor er specificiteten af et antistof ikke tilstrækkelig som selvstændig diagnostisk markør, hvorved mere risikable provokationstests bliver udført. Med vægt på personaliseret medicin i allergien er diagnostikken essentiel, men det skaber også et behov for at differentiere imellem patienter for at sikre en højere behandlingseffekt og effektivisere sundhedsvæsenets ressourcer. Disse udfordringer leder til formålet af dette specialeprojekt.
Formål: Studiet har to primære hovedformål. Det første er at undersøge om en forskel i mastcellereaktivitet og sensitivitet kan findes mellem patienter med svær astma og raske kontroller ved forskellige IgE affiniteter ved brug af mastcelle aktiveringstest (MAT), og om denne påvirker frigivelsen af mediatorer i senfasereaktionen. Det andet hovedformål er at undersøge, om MAT og basofil aktiveringstest (BAT) kan anvendes til at skelne imellem patienter med høj og lav behandlingsrespons på sublingual immunterapi (SLIT).
Metode: For at undersøge mastcelleresponsen, blev humane mastceller isoleret fra $CD133^+$ stamceller fra enten GINA 4-5 astmatikere eller raske kontroller. Mastceller var modnet mindst seks uger med cytokinberiget medie. I det første hovedforsøg, blev mastcelle respons med forskellig husstøvmide-specifik rIgE affinitet målt ved at sensitisere mastceller i to uger med høj, mellem og lav-affinitet IgE. Mastcellerne blev aktiveret med rekombinant husstøvmideallergen og målt ved $CD63^+$ opregulering med flowcytometri. I det andet hovedforsøg, blev mastcellerespons med præallergen immunterapi (AIT) sera fra GT-08 studiet eller sera og plasma fra birkeallergikere fra eget regi målt ved at sensitiserere mastceller i to døgn. Mastceller og fuldblods basofile celler blev aktiveret med en relevant sublingual immunterapi (SLIT) tablet og målt ved $CD63^+$ opregulering med flowcytometri.
Resultater: Affinitetsbetinget forskel i mastcellereaktivitet og sensitivitet kunne ikke findes mellem patienter og kontroller. Dog kunne der observeres en højere gennemsnitlig reaktivitet($\pm$SE) hos de lav affine patienter på 68.84($\pm$17.11)% end kontrolgruppen på 40.68($\pm$30.77)%. Effektorcellers præ-AIT reaktivitet og sensitivitet udviste ingen tydelig gruppefordeling i sammenhold med global evaluation scores fra GT-08 studiet. Sammenhæng mellem præ-AIT reaktivitet og sensitivitet ved mastceller og basofile for birkeallergikere og behandlingseffekt af SLIT fandt ingen korrelation. MAT og BAT indikerede lav sammenlignelighed med lav korrelation, men MAT viste høj reproducerbarhed med $< \pm 10\%$ variation i CD63 opregulering og en variation på $\pm 207(\mu$DDE/ml) i sensitivitet.
Konklusion: Mastcelle degranulering viser en affinitets-afhængig respons med rIgE ved sensitivitet i hver gruppe, men ingen forskel kunne påvises i mastcelleaktiveringen mellem patienter og raske kontroller. Dog kunne det tyde på, at astmatikere reagerer kraftigere under reduceret stimulus end kontroller, men dette bør udforskes yderligere. MAT lavet med buffy-coats og passiv sensitisering med serum og plasma er reproducerbar. Mastcellerespons fra præ-AIT IgE havde ingen prædiktiv værdi som biomarker for effekten af AIT. BAT respons og rollen som prædiktiv biomarkør for effekten af AIT er inkonklusiv og bør efterforskes mere.

Abstract

Background: The number of individuals diagnosed with allergic diseases have been steadily increasing throughout the last 60 years - with a still rising trend. The high amount of allergic cases will ultimately lead to huge socioeconomic costs for the public due to loss of productivity and decreased life quality of the affected individuals. With focus on the immunoglobulin E (IgE)-mediated reaction, which is caused by an abnormal immune response to harmless substances in the environment, specific IgE (sIgE) is developed. The interaction of sIgE with the main effector cells of allergy, mast cells and basophils, lead to the release of several biological active mediators, resulting in the development erythema, edema, pruritus and dyspnea. As the diagnosis is partially based on the detection of sIgE sensitization, which is a prerequisite for the development of an allergic reaction, it is not always clinically relevant. Thus, sIgE is not a sufficient diagnostic marker, in which the need for more hazardous provocation challenges is performed. In a field with focus on personalized medicine, accuracy is important for diagnostics, however the need to discriminate between patients is also a cornerstone to obtain higher treatment effects and to streamline the resources of the health care system. The challenges described above have led to the objectives of this master thesis.
Aim: This project has two main objectives. The first aim is to investigate whether a difference in mast cell reactivity and sensitivity can be established between patients with severe allergic asthma and healthy controls at different affinities of IgE using mast cell activation test (MAT), and whether this affects the release of mediators during the late phase response. The second aim is to investigate, whether MAT and basophil activation test (BAT) can be used to characterize and discriminate high from low sublingual immunotherapy (SLIT) treatment responders.
Methods: To investigate mast cell response, human mast cells were isolated from $CD133^+$ progenitor cells from either GINA 4-5 asthmatics or healthy controls. Mast cells were matured at least six weeks with cytokine-enriched media. Mast cell response with different house dust mite-specific rIgE affinity was measured by $CD63^+$ upregulation with flow cytometry. Mast cell response with pre-AIT sera from the GT-08 study or sera and plasma from in-house birch allergic individuals was measured by sensitising mast cells over two days. Mast cells and full-blood basophils were activated with a SLIT tablet and measured by $CD63^+$ upregulation with flow cytometry.
Results: An affinity dependent difference in mast cell reactivity and sensitivity could not be established between the patient- and control group. However, an increased mean reactivity($\pm$SE), in the mast cells sensitized with low affinity IgE, was observed for the patient group 68.84($\pm$17.11)% compared to the control group 40.68($\pm$30.77)%. Neither the baseline reactivity nor sensitivity showed any clear groupings with the global evaluation scores from the GT-08 study. Correlation was found between baseline reactivity and sensitivity for individuals suffering from birch pollen allergy, whilst no correlation was found for treatment effect. MAT and BAT indicated low comparability and poor correlation, but MAT indicated high reproducibility with <10%CD63 expression variation and a variation of 207(μDDE/ml) in sensitivity.
Conclusion: Mast cell degranulation demonstrated an affinity dependent response with rIgE in sensitivity within groups, but no difference in CD63 upregulation could be established between patient- and control derived mast cells. The low affinity sensitized mast cells might indicate that asthmatics respond stronger to a reduced stimulus, but further research is needed. MAT performed with buffy coats, which have been sensitized with either serum or plasma, has proven to be reproducible. Mast cell response from pre-AIT treatment could not be established as a predictive biomarker for AIT treatment. The BAT response and its role as a predictive biomarker for AIT was inconclusive and require further research.

Preface and Acknowledgements

This master thesis was conducted by Madeleine Emilie Salzer and Marlene Quach during the 9th and 10th semester of Medicine with Industrial Specialization, Aalborg University, DK in collaboration with the Department of Respiratory Diseases and Allergy, Aarhus University Hospital (AUH), DK and Department of Clinical Medicine, Aarhus University, DK. The project spanned from September 1st, 2019 to May 29th, 2020. Our external supervisor, Professor Hans Jürgen Hoffmann[1,2] and internal supervisor, Associate Professor Emil Kofod-Olsen[3], provided supervision throughout this master thesis.
This project used mast cell activation test (MAT) and basophil activation test (BAT) to explore the role of IgE on effector cells in allergy diagnostics and treatment perspectives. The experimental setups were inspired by previous studies performed at AUH, while using the professional guidance and knowledge from the Department of Respiratory Diseases and Allergy. All experimental work has been conducted in the laboratory of The Department of Respiratory Diseases and The Department of Paediatrics and Adolescent Medicine by Madeleine Emilie Salzer and Marlene Quach with assistance from laboratory technician Line Nielsen. Mast cells and blood samples from birch dependent atopic patients were obtained from donor material at AUH. rIgE was provided by Lars H. Christensen[4] and patient sera from grass-dependent atopic patients were provided by Peter A. Würtzen[4]. External analyses on mast cell responses and allergen immunotherapy treatment response were performed at ALK-Abelló A/S, Hørsholm, Denmark by Jens Holm[4] and Peter A. Würtzen.

It has been highly interesting and educative to work in depth with mast cells and basophils in a dynamic department and contribute to the understanding of effector cells, allergens and allergic diseases. This master thesis provides new insight on the fundamental characteristics of IgE, the potential biomarkers and use in allergy diagnostics and offers a novel base for further experimental setups in elucidating the role of IgE on effector cells.
Our time as master students has been very rewarding, therefore we would like to give our most sincere appreciation to our main supervisor, Professor Hans Jürgen Hoffmann for invaluable professional and personal guidance throughout this project. We would also like to appreciate our internal supervisor, Emil Kofod-Olsen for valuable feedback. Furthermore, we thank the very supportive and highly competent staff and students at the Allergy Clinic and Department of Respiratory Diseases and Allergy at AUH. We would also like to thank laboratory technician Line Nielsen and clinical laboratory scientist Anne-Marie Toft for advice and training in the experimental work with mast cells and basophils, respectively. We must also express our deep gratitude towards Jens Holm, Peter A. Würtzen and Lars H. Christensen for further invaluable professional advice and support in this master thesis.

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Acronyms

2NP 2-nitrophenyl-caproate-Fab. 51

4PL four parameter logistic. 23, 25, 26, 32, 38, 40, 43, 53, 55

7-AAD 7-Aminoactinomycin D. 52

AIT allergen immunotherapy. 1, 2, 8, 13–17, 24, 28, 38–40, 42, 43, 47, 54–57

ANOVA analysis of variance. 25

APAAP alkaline phosphatase-anti alkaline phosphatase. 20

AR allergic rhinitis. 11–13, 54, 56

ARIA Allergic Rhinitis and its Impact on Asthma. 13

AUC area under the curve. 56

AUH Aarhus University Hospital. 3, 17, 22, 25, 28, 29, 31, 49, 52, 53

BAT basophil activation test. 1–3, 7, 12, 13, 15–17, 25–27, 42–46, 53, 55–57

Breg B-regulatory cell. 14

BV Brilliant Violet. 20, 24, 26

CCL2 chemokine ligand 2. 51

CCL3 chemokine ligand 3. 51

CCL4 chemokine ligand 4. 51

CI confidence interval. 43

DC dendritic cells. 13, 14, 55

DDE daily dose equivalent. 21, 23–26, 35–40, 43–45, 52, 53, 55, 56

Der p 2 Dermatophagoides pteronyssinus. 21–23, 32, 50

DNP dinitrophenyl-caproate-Fab. 51

EC50 half maximal effective concentration. 12

ELISA enzyme linked immunosorbent assay. 19, 29

EPJ electronic medical chart. 17, 25

ERK extracellular-signal-regulated kinase. 9

FAB facilitated antigen binding. 55

FBS fetal bovine serum. 18

FEV forced expiratory volume. 10

FITC fluorescein isothiocyanate. 19, 22–24, 26

GC MS gas chromatography–mass spectrometry. 23

GINA Global Initiative of Asthma. 1, 2, 10, 13, 17, 21, 28, 49

HDM house dust mites. 10, 11, 42

HR-test histamine release test. 19, 29

ICC intraclass correlation. 56

IFN-γ interferon-γ. 14

IgA immunoglobulin A. 14

IgE immunoglobulin E. 1–3, 6–15, 17, 19, 21–26, 28, 30–33, 38, 39, 42, 43, 47–51, 54, 56, 57

IgG immunoglobulin G. 13, 14

IL interleukin. 8–10, 13–15, 18, 22–24, 26, 47, 51, 55

IQR inter quartile range. 25, 38, 39, 43, 54

ITAM immunoreceptor tyrosine based activation motif. 10, 14

iTreg induced Treg. 14

JNK Jun amino-terminal kinase. 9

LAT linkers for activation of T cells. 9

LAT1 linkers for activation of T cells 1. 10, 11, 51

LAT2 linkers for activation of T cells 2. 10, 11, 51

LTRA leukotrine receptor antagonist. 13

Lyn tyrosine-protein kinase Lyn. 51

MACS magnetic activated cell sorting. 18

MAP p38 mitogen-activated kinase. 9

MAT mast cell activation test. 1–3, 7, 12, 13, 15–17, 24–27, 34–39, 41, 43–46, 51–57

MC mast cell. 28

MFI median fluorescence intensity. 20, 23, 29, 34, 35, 51, 52

NR non-reactive. 24, 25, 38–42, 53, 54

nTreg natural Treg. 14

PBMC peripheral blood mononuclear cells. 18

PBS phosphate buffered saline. 18–24, 26, 38, 43

PE phycoerythrin. 26

pen/strep penicillin/streptomycin. 18

PGD2 prostaglandin D2. 51

pipes piperazine-N,N-bis(2-ethanesulfonic acid. 19

PLC-γ phospholipase Cγ. 9

PP polypropylene. 21–24, 30

RAST radioallergosorbent test. 11, 42, 49

reg regulatory. 14, 55

rIgE recombinant IgE. 1–3, 12, 21, 22, 33

RPM rounds per minute. 18–20, 22, 23, 26

RQLQ rhinitis quality of life questionnaire. 7, 25, 27, 42, 44, 45, 56

SCF stem cell factor. 18, 47

SCIT subcutaneous immunotherapy. 13, 15, 55

SD standard deviation. 19, 28, 29, 31, 35, 37, 38, 43, 44, 55, 56

SE standard error. 1, 2, 22, 23, 31, 33

sIgE specific IgE. 2, 11, 12, 15, 25, 28, 40–42, 49, 54, 55, 57

sIgG specific IgG. 14, 15, 25, 40–42, 54, 55, 57

SLIT sublingual immunotherapy. 2, 13, 21, 25, 34, 42, 45, 52, 56, 57

SLP-76 lymphocyte cytosolic protein 2. 9

SNR signal-to-noise ratio. 23, 34, 35, 51, 52

SPT skin prick test. 11, 12, 15, 28, 42, 54

SQ specific quality. 21, 25, 39, 41, 52

SS stem span. 18

SSS stem span with serum. 18, 22–24, 26

Syk tyrosine-protein kinase Syk. 51

TBS tris-buffered saline. 20

TGF-β transforming growth factor-β. 14

Th0 T helper 0 cell. 14

TH1 T helper 1 cells. 13–15

TH2 T helper 2 cells. 8–10, 13–15

TNF-α tumor necrosis factor α. 51

TNSS total nasal symptom score. 25, 42

Treg T-regulatory cell. 14, 15

Vav proto-oncogene vav 1. 9

Contents

1 Background

This introductory part will provide an overview of allergy in general and in perspectives of allergic asthma and rhinitis. In the following sections, the epidemiology of the conditions and biological mechanisms will be described. Followed by a section about the current diagnostics methods and treatment options with focus in allergen immunotherapy (AIT). Lastly, the problem statement will be presented.

1.1 Allergy

Allergy is a hypersensitive reaction with an unfavorable adaptive immune response developed against harmless components. During the last 60 years, a rise in the numbers of allergic diseases has soared and it is estimated about one billion people is affected worldwide. This trend is expected to continue to rise and reach approximately four billion people in 2050 [1, 2].

Biologically, allergy can be divided into four distinct types of hypersensitivity reactions; 1) type I, the immunoglobulin E (IgE)-mediated reaction, 2) type II, the tissue specific reaction, 3) type III, the immune complex mediated reaction and 4) type IV, the cell mediated reaction. As the four mechanisms are closely interconnected with one another, the classification is not always clear cut, as several mechanisms can occur simultaneously or sequentially [3]. This master thesis will however only focus on the IgE-mediated hypersensitivity reaction.

1.1.1 Pathophysiology of IgE-mediated Allergy

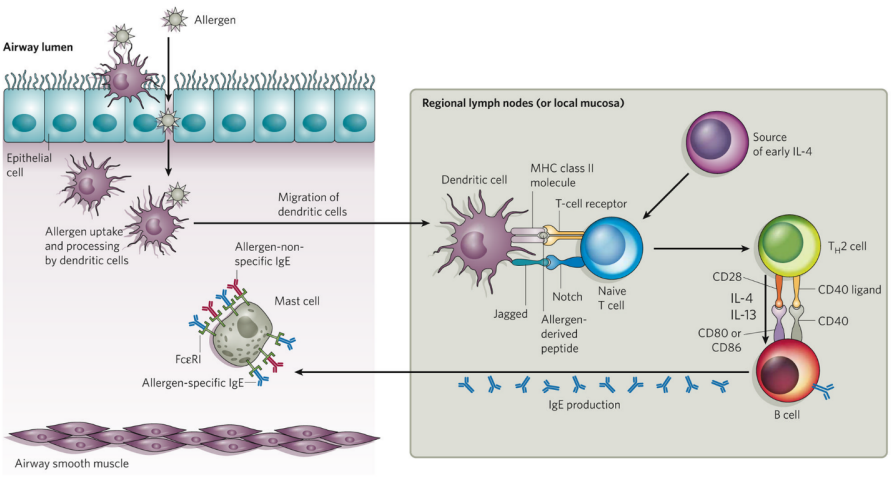


Figure 1: **Sensitization to Allergens in the Airway**

Illustration from Nature, 454: 445-454 by Galli et al. 2018. Allergen is captured by antigen presenting cells (dendritic cells) and processed. The activated dendritic cells migrate to the regional lymph node and presents the peptide from the allergen via the major histocompatibility complex class II to a naive T-cells, which develops into TH2 that produce IL-4 and IL-13. The presence of cytokines and the signals transmitted by CD40/CD40L and CD80/CD86/CD28 induces class switching in the plasma cells. IgE antibodies are subsequently distributed systemically through the blood. T helper 2 cells (TH2); interleukin (IL);immunoglobulin E (IgE).

The underlying mechanism of the type I reaction consists of two phases. The first phase is a sensitization phase, in which T helper 2 cells (TH2) are activated by the antigen presenting dendritic cells after exposure to an antigen. This leads to the production and release of several cytokines

such as interleukin (IL)-3, IL-4, IL-5 and IL-13, as illustrated in Figure 1 [2, 3]. Both IL-4 and IL-13 induce class switching to IgE in plasma cells, whilst IL-3 and IL-5 promotes the survival of eosinophils, which amplifies the TH2 reaction by generating cytokines. The second phase is initiated when a new encounter with the antigen occurs and is primarily driven by the effector cells (basophils and mast cells), which degranulate and causes the hypersensitivity reaction, defining the acute phase of the allergic response, while the late phase develops 2-6 hours after allergen exposure and peaks after 6-9 hours [1, 2]

1.1.2 Effector Cells in an IgE Mediated Hypersensitivity Reaction

The most prominent effector cells in allergy are the mast cells. These cells are found in all tissues and often close to epithelial barriers and contain a plethora of granules of preformed mediators [3]. Another effector cell type are the basophils, which in contrast to mast cells are mainly found in the bloodstream. These cells have a much shorter life span of two to three days, whilst mast cells are long lived tissue resident cells, which persists for months [4]. Basophils and mast cells share the same FcεRI degranulation pathway [5, 6].

Figure 2: **FcεRI Mediated Signal Tranduction Pathway**

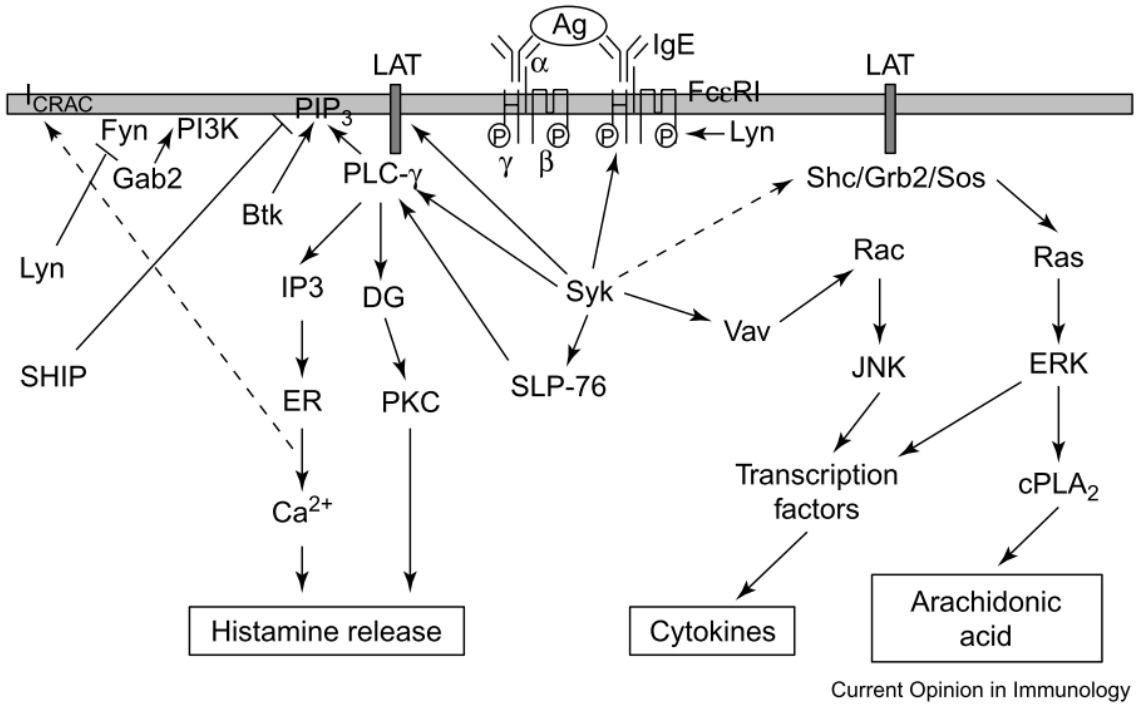


Illustration from Current Opinion in Immunology, 15: 639-646 by Siraganian et al. 2003. Mast cells and basophils share the same FcεRI mediated pathway. Cross-linking of IgE-antigen complex with the high affinity receptor, FcεRI, initiates a kinase induced cascade of phosphorylations of several proteins including LAT, PLC-γ, SLP-76 and Vav. GTPases are subsequently activated, which initiates the signaling pathways of extracellular-signal-regulated kinase (ERK), Jun amino-terminal kinase (JNK) and p38 mitogen-activated kinase (MAP). This leads to the degranulation of effector cells, in which phospholipid mediators and histamine are released. Immunoglobulin E (IgE); linkers for activation of T cells (LAT); phospholipase Cγ (PLC-γ), lymphocyte cytosolic protein 2 (SLP-76); proto-oncogene vav 1 (Vav), extracellular-signal-regulated kinase (ERK); Jun amino-terminal kinase (JNK); p38 mitogen-activated kinase (MAP).

On a molecular level, the antigen, IgE-antibody and high affinity receptor (FcϵRI), interacts and forms a complex on the cell surface, as depicted in Figure 2. When cross-linking occur, signaling kinase molecules are activated. The catalytic activity transphosphorylates the tyrosine residues of the neighbouring immunoreceptor tyrosine based activation motif (ITAM), which then provides a docking site for the binding of additional signaling molecules. [2, 7–9]. These molecules contribute to the stimulation of GTPases and activates the signaling pathways, which modulate transcription factors that regulate the *de novo* synthesis of cytokines, chemokines and the release of arachidonic metabolites [8]. Second messengers are generated, calcium influx occur, which ultimately lead to the degranulation of effector cells [2, 7, 9]. Upon degranulation, histamine, heparin and proteases are released in conjunction with newly synthesized mediators, which direct the inflammatory process and the subsequent recruitment of inflammatory cells in the late phase response. These events lead to vasodilation, increased vascular permeability and smooth muscle contraction, which characterizes the clinical manifestations such as tissue swelling, airflow obstruction and wheezing [2, 3].

1.1.3 Allergic Asthma

One of the most prevalent phenotypes in asthma is allergic asthma. Asthma is a chronic inflammatory disorder of the airways affecting over 300 million people worldwide [10]. Asthma is associated with 250.000 annual deaths worldwide, which leads to a significant socio-economic burden [11]. The symptoms include coughing, wheezing and dyspnea with a reversible and variable airflow limitation often starting in childhood [12]. Asthma diagnosis is based on clinical history and reversibility test with bronchodilators with >12% increase in FEV_1 [13]. Allergic asthma, and other asthma phenotypes are categorized as intermittent, mild, moderate or severe asthma based on the Global Initiative of Asthma (GINA) classications (Table 1) [13, 14]. The GINA classification is an evidence-based guideline for the diagnosis, management and prevention of asthma.

Table 1: **GINA Classification of Asthma Severity based on Clinical Features**

	Daily Symptoms	Nightly Symptoms	FEV1	FEV1 Variablity
Step 1 (Intermittent)	Symptoms less than once a week Brief exacerbations	2 times a month	80%	<20%
Step 2 (Mild Persistent)	Symptoms more than once a week but less than once a day Exacerbations may affect activity and sleep	>2 times a month	80%	20 - 30%
Step 3 (Moderate Persistent)	Symptoms daily Exacerbations may affect activity and sleep	>1 time a week	60% - 80%	>30%
Step 4 (Severe Persistent)	Symptoms daily Frequent exacerbations Limitation of physical activities	Frequent	60%	>30%

From The European Respiratory Journal, 31; 143-178 by Bateman et al. 2008. Using GINA, asthma is classified in four steps based on clinical features, such as daily and nightly symptoms and forced expiratory volume in one second (FEV1). Global Initiative of Astma (GINA).

The symptoms are a result of a hyper-responsive airway with reversible obstruction, remodelling of the tissue, and an infiltration of inflammatory cells [15]. Attacks or exacerbations in allergic asthma is caused by mediator release when the effector cells are re-exposed to the aeroallergen (e.g. house dust mites (HDM) or pollen) as described in section 1.1.1. Cytokines (IL-4 and IL-13) released from the TH2 cells are responsible for the destruction of epithelial cells and induce goblet cell metaplasia, which can later lead to a remodelling of the tissue with hyperplasia in the goblet cells, sub-epithelial fibrosis and increased deposition of collagen in the smooth muscles [12, 15]. Interestingly, studies have suggested that the severity of asthma may be dependent on a shift in the LAT1/LAT2 intracellular signaling pathway. The direction of the immunological response appeared to be modulated by the affinity of the IgE antibody on murine models [16]. As the strength of the signal is believed to be dependent on the duration of receptor engagement, low affinity would entail a reduced response, as it would dissociate more rapidly than high affinity, but differential use of adaptor proteins was observed [16]. LAT1 and LAT2 are both part of the FcϵRI signaling pathway,

in which higher affinity was associated with a stronger pro-inflammatory response through LAT1 with a higher degranulation, whilst low affinity was found to activate through LAT2 and alter the release of mediators to a more chemokine driven direction [16, 17]. The shift in the immunological response is thought to be associated with asthma severity, as higher levels of cytokines were linked with patients, who had a severe chronic asthmatic disease [18, 19]. However, more research is needed to characterize the IgE/effector cell interaction to understand the subtle biochemical changes that appear to regulate the disease outcome.

1.1.4 Allergic Rhinitis and the Impact on Asthma (ARIA)

Allergic rhinitis (AR), commonly referred to as allergic rhinoconjunctivitis or hay fever, is an allergic inflammation of the upper airways, specifically in the mucous membrane lining of the nose. It is a chronic inflammatory respiratory disease as allergic asthma, which induces symptoms of sneezing, itching, rhinorrhea and nasal obstruction after exposure to aeroallergens. Symptoms can be accompanied by conjunctival symptoms as itching, redness of the eyes and lacrimation [10]. AR is, as asthma, a global health problem affecting all ages and ethnicities with a peak onset in adolescence. AR is estimated to effect between 500 million and up to 1.4 billion people worldwide, mainly in the western countries, where 1/4 is affected [10, 20, 21]. Though AR is not associated with mortality, both medical costs and loss of productivity are a substantial economic burden to the society and the individual with AR [10]. Besides the organ-specific symptoms, AR also affects sleep quality, cognitive function, decreased school and work performance, which all leads to a decreased quality of life, especially during the peak pollen season [22, 23]. AR are distinguished by a seasonal or perennial type that are based on the type of offending allergen. Pollen-dependent AR is responsible for the seasonal symptoms, whereas the perennial is caused by the ubiquitous house dust mites (HDM) allergens and animal danders [22]. The diagnostic classification is based on the intensity and duration of symptoms, and the impact on quality of life, where it may be classified as intermittent or persistent with a degree of mild or moderate-severe [22]. The pathophysiology of AR involves mediator releases by cells which are implicated in both allergic inflammation and nonspecific hyperreactivity, which leads to remodelling of the upper airway [10, 24].

AR and allergic asthma are often co-morbidities and 10-40% of patients with AR have asthma, where AR is a predisposing factor for asthma and affects asthma, if uncontrolled. Nasal symptoms are also present in over 80% of patients with asthma [10]. These findings have given rise to the concept of "one airway one disease" as the nasal and bronchial mucosas share some similarities. AR are similar to allergic asthma in the IgE-mediated pathophysiology with mucosal involvement and IgE-mediated interactions with dendritic cells and mast cells and eosinophilic inflammation [24].

1.2 Allergy Diagnostics

As allergic conditions are one of the most prevalent diseases worldwide, it is important to obtain an accurate diagnosis in order to ensure optimal treatment. The diagnostic algorithm of clinically relevant allergy consists of an in-depth clinical history, detection of sIgE and allergen challenge, respectively. A reliable and cost effective first in line tool is the skin prick test (SPT), as this test have demonstrated good correlation with the outcomes of nose, skin, eye, bronchial and also food provocation challenges. The SPT is an *in vivo* test performed to evaluate the presence of IgE sensitization by introducing allergen into the skin of suspected allergic individuals. If the individual is sensitized, cross-linking of allergen-sIgE will bind to the membrane receptors on dermal mast cells. Degranulation will subsequently occur resulting in the characterizing wheals and erythema [25]. Another method for evaluating the presence of sIgE is the radioallergosorbent test (RAST). This is a solid phase sandwich immunoassay, which measures both the presence of allergen-sIgE and the total IgE. High total IgE is not considered a reliable marker for allergic status, as similar values are observed in other conditions, such as parasitic infestations and in some types of primary immunodeficiencies. In addition, low or normal values of total IgE does not exclude an IgE mediated disease. In contrast, the sIgE has greater diagnostic value, and it can be used to discriminate between

allergic- and non-allergic individuals as well as provide identification of relevant allergens [25]. As sIgE is a prerequisite for the development of an IgE-mediated hypersensitivity reaction, it should be noted that approximately 20% of individuals with allergen-specific serum IgE are asymptomatic, therefore it is not a sufficient diagnostic marker on its own, and should always be used in conjunction with the medical history and other tests, such as the basophil activation test (BAT) or a provocation challenge [1, 25]. The latter remains the golden standard for allergy diagnostics, as it can be used when the clinical history, SPT or sIgE are inconclusive. However, the provocation test is the most precarious and cumbersome test of the available diagnostic options, as it has a greater risk of inducing a systemic reaction and requires highly trained personnel [25]. A way to accommodate this challenge is by utilizing cellular tests as the BAT.

1.2.1 Basophil Activation Test

A highly complementary cellular *in vitro* test in allergy diagnostics is the basophil activation test (BAT), which is applied when discordance is found between the medical history of the patient and the results obtained from the SPT and sIgE [26]. This flow cytometry-based assay utilizes a series of allergen dilutions, often on whole blood, to activate the basophils and thereby determining the half maximal effective concentration (EC50) of the allergen. The process of the BAT consists of a functional flow cytometry analysis with basophils, which may be identified as $CD123^+/CD193^+/CD63^+$. The former two are cell surface markers that identify the cells, whilst the upregulation marker CD63 is measure of the degranulation response and correlates with histamine release [27]. CD63 is membrane protein, which is located in the same secretory lyzosomal vesicles as histamine on the effector cells, and has been shown to be a reliable measure for either basophil or mast cell activation [1, 26]. When compared to the sIgE test, the BAT provides greater clinical insight in the functional response of the cells as the reaction is induced by the cross-linking of the high affinity receptor and therefore thought to represent an *in vitro* surrogate of an *in vivo* allergic reaction. The sensitivity of basophils measured by CD63 upregulation has been demonstrated to correlate with the sensitivity in nasal provocation test in AR and bronchial provocation in asthma [27]. In addition, compared to the provocation test, the BAT provides a more reproducible allergen sensitivity without putting the patient at risk [1, 26]. The diagnostic quality of BAT has been demonstrated by Santos et al. 2018 to have high specificity and sensitivity when used to diagnose food and venom allergies, but BATs have also shown to be sensitive in diagnosing AR even at low levels sIgE [28]. BAT has also been shown to be useful tool to monitor the effect of immunomodulatory therapies [26, 28–31]. Despite being a reliable complementary diagnostic test, cohort studies have also indicated approximately 10-15% basophils to be unresponsive towards the tested allergen for some individuals, defined as non-responders, when they do not respond to IgE-mediated signalling [26, 32]. A cross sectional study, which included 476 individuals showed basophil anergy in approximately 10% of the cohort, illustrating that BAT is not an effective test for all patients [20].

1.2.2 Mast Cell Activation Test

A novel *in vitro* diagnostic approach to identify IgE mediated hypersensitivity is the mast cell activation test (MAT). This is another functional flow cytometry-based assay, which consists of mast cells being passively sensitized with IgE derived from atopic patients or recombinant IgE (rIgE). There are different applications for the use of mast cells in the MAT, and besides the possible novel diagnostic approach, MAT can also be used for kinetic studies [5]. Mast cells can be cultured from peripheral blood-derived progenitor cells (e.g. $CD133^+$) or LAD2 mast cells [4, 32, 33]. Similar to the BAT, CD63 upregulation is also measured as the marker for cell degranulation, but there are no non-responders in MAT and it does not require fresh blood [32]. The MAT has shown to be able to discriminate between peanut sensitivity and peanut allergy [4, 32]. The effectiveness of this test was explored in peanut allergy diagnostics. MAT compared to other current diagnostic tests such as the SPT, sIgE and BAT found a favorable diagnostic accuracy in identifying those with clinical reactivity to peanut when compared to SPT, sIgE and the BAT [4]. One potential challenge in this

assay may be the huge heterogeneity reported in mast cells, as the microenvironment of the cells have been suggested to play a major role on the functional level of mast cells. Thus, concern has been expressed regarding the reliability of the test when using blood precursor as the source for mast cells [4, 34]. The use of MAT are still very much a topic for further research and the application in kinetic studies to further elucidate the role of IgE, and how it may impact the clinical aspect of allergy is interesting, as well as the novel diagnostic features and a possible implication as a screening tool to be used alongside of BAT in guided allergen-specific treatments.

1.3 Current Treatment Options in Allergic Respiratory Diseases

The current conventional treatment strategies for allergies are based on the avoidance of the allergen and to use pharmacotherapy to reduce symptoms and, in the case of asthma, the risk of acute exacerbation. The treatment for asthma is determined by the Global Initiative of Asthma (GINA) guidelines. The GINA scores for guided treatment consists of five steps, GINA 1-5, which describes recommended drugs with add-on therapies for each increasing step. It consists of inhalation- or oral corticosteroids, long and short-acting β_2-agonists, leukotrine receptor antagonist (LTRA) and biological therapies [13]. Mild asthma can be controlled with step 1 and 2, moderate asthma is controlled by step 3 and severe asthma by step 4-5. For some patients, who are diagnosed with severe refractory asthma, the use of high doses of corticosteroids have proven unsuccessful in controlling their condition, in which significant respiratory symptoms persists with reduced lung function. This has led to the use of biologics for asthma management. This treatment option consists of targeting antibodies, inflammatory molecules and cell receptors to disrupt the pathways that lead to inflammation and causes the asthmatic symptoms [35]. One of the antibody therapies are the anti-IgE treatment, which are recommended for both asthma and AR [22]. Other antibody therapies are aimed against the inflammatory molecule IL-5 or the IL-5 receptor, if the asthma are accompanied by an eosinophilic phenotype. In allergic rhinitis pharmacotherapy consist of oral or intranasal H_1-antihistamines, intranasal corticosteroids or LTRA based on the Allergic Rhinitis and its Impact on Asthma (ARIA)-guidelines [22]. Though these treatment options have shown to be effective in relieving symptoms, none are curative, and symptoms recurs after treatment withdrawal. A possible long-lasting option is AIT, which is indicated for treatment in AR with moderate-severe symptoms that are not well controlled with conventional drugs, as well as for allergic asthma in GINA step 3. However, it demands precise diagnosis to identify the relevant allergic component [13, 22, 36].

1.3.1 Allergen Immunotherapy

AIT (also known as specific immunotherapy, SIT) is the only therapy that exerts immuno-modulating effects by targeting the relevant immune cells and thereby alleviates the allergic disease. Compared to other therapies, AIT has shown the best treatment effect in AR [36]. The therapy is based on high allergen exposure from extracts to desensitise the patient and ultimately induce tolerance by immune-modulations [36–38]. Though the desired effect of AIT takes longer to achieve than the conventional drugs, it has a long-lasting effect after discontinuation [37, 38].

Despite the versatile use of AIT, this paragraph will focus on its use in the aero-dependent allergies and to only include the allergic respiratory diseases, asthma and rhinitis. The most commonly used form of AIT for inhalant allergens exists in forms of subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) [36]. AIT is administered over three to five years, where SCIT consist of an up-dosing phase followed by maintenance dose at four- or eight weeks intervals [36]. Both administration forms are effective after two- to four months after treatment initiation [39].

The immunological mechanisms behind AIT induces tolerance by switching from a TH2 response to a TH1 response, when regulatory DCs are exposed to high levels of allergens (Figure 3) [37–39]. Secreted tolerogenic cytokines, especially IL-10 leads to class-switching from IgE to mainly IgG in plasma cells, which inhibits the allergen-specific TH2 response and blocks IgE-mediated effector cell activation [38–40].

Figure 3: **Mechanism of Allergen Immunotherapy-induced Tolerance**

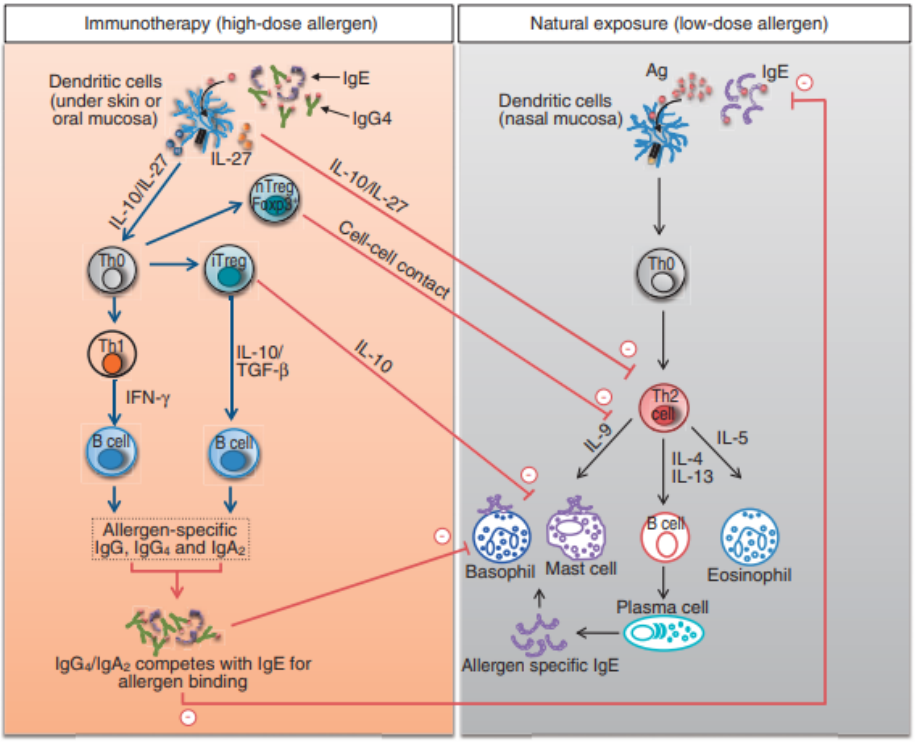


Illustration from Allergology International. Allergen Immunotherapy and Tolerance 2013; 62:403-413 by Matsuoka et al. 2013. Natural low allergen exposure drives an adaptive TH2 response leading to allergic inflammation (right illustration). The high dose allergen exposure achived in AIT to regulatory DC produces a deviation in the T-cell response (left illustration). The DC starts secreting IL-10, IL-12 and IL-27, which stimulates the naive Th0. Th0 cells become Treg (iTreg and nTreg) and TH1. The nTreg directly inhibits the TH2 cells by cell-cell contact. The production of the anti-inflammatory IL-10 by iTreg induces B-cells to switch into Breg, which in turn suppresses B-cells, T-cells and DC in the "normal" allergic inflammation. The iTreg further stimulates B-cells with IL-10 and TGF-β. Both tolerogenic cytokines induces class-switching in the antigen producing B-cells. IL-10 induces class-switching from IgE to IgG, mainly IgG4, whereas TGF-β lead to the production of IgA and may inhibit the allergen-specific TH2 response. The TH1 produces IFN-γ, which also stimulates the antigen-producing B-cell to synthesize IgG. T helper 2 cells (TH2); allergen immunotherapy (AIT); dendritic cells (DC); IL; T helper 0 cell (Th0); induced Treg (iTreg); natural Treg (nTreg); T helper 1 cells (TH1); B-regulatory cell (Breg); interferon-γ (IFN-γ); T-regulatory cell (Treg); transforming growth factor-β (TGF-β); immunoglobulin G (IgG); immunoglobulin A (IgA).

The clinical benefit is thought to be explained by this induction of blocking antibodies, which reduces the mediator release of mast cells and basophils, as part of an early phase immune tolerant response. Allergen-sIgG binds to the FcγRIIb receptor on both mast cells and basophils, blocking the signal transduction of IgE by simultaneously sending inhibitory signals to block ITAM. It may also be that IgG physically blocks the allergen from binding to IgE [1, p. 294]. A change in the late phase-response would reduce the number of effector cells found at the mucosal sites [40, 41]. The clinical effects are observed in the reduction of anti-allergic drug use, reduced nasal, ocular and/or bronchial symptoms and increased quality of life [38–40].

There is still variations in the clinical practice and different national guidelines in the performance of AIT [38, 41]. Furthermore, difficulties in choosing the relevant allergens in poly-sensitized

subjects, determining the molecular sensitization profiles, the general poor adherence to the long term treatment and inconsistency in applying challenge tests can contribute to decreased efficacy in AIT [1, 31, 37], as efficacy is approximately 30-50% [38, 42–44]. There is a need for a better stratification of patients and selections of responders to increase efficacy. AIT is both time consuming, costly and with the risk of substantial side effects including mild-moderate skin or respiratory reactions and even anaphylaxis in some patients [36, 38]. A way to stratify the patient population is to use biomarker guided selections. Biomarkers for monitoring and detection of tolerance during AIT have been described for immunological and clinical changes. Immunological biomarkers are total IgE and sIgE, sIgG4, changes in T-cell proliferation; allergen-induced TH1, TH2, Treg expansion and the cytokines produced (especially IL-10) and changes in basophil sensitivity *in vitro*. *In vivo* surrogate biomarkers are changes in sensitivity in allergen challenges in SPT, nasal, conjunctival or bronchial provocation [38, 40]. The decrease in basophil sensitivity has been widely demonstrated [30, 42, 45]. For instance the study Schmid et al. 2014, found a decrease in basophil sensitivity after only three weeks of grass allergen SCIT treatment and could be used as an indicator of clinical effect [45]. Those used today are based on efficacy and can only be assessed after treatment initiation. A call for a predictive biomarker will accommodate the cost-effectiveness challenge. Besides the obvious personal gain, there is a need in allergology to fully understand the mechanism behind why some people develop tolerance in AIT and others do not and why some naturally develop remission in their allergic disease. Furthermore, AIT is expected to receive more attention and gain better footing in the clinics as the safety profiles have been improving and there will be a need to treat the rising number of people with allergic disorders worldwide [36, 41]. This further substantiates the need for a predictive biomarker. A possible predictive biomarker has been explored by Schmid et al. (unpublished). In their study, 24 patients participated and treated for grass-dependent allergy with SCIT. The patients were included based on a BAT reactivity $\geq$40% $CD63^+$. The mean treatment effect was very high with 67% based on medication scores. This inclusion criterion may have a possible role in the search for a predictive marker in efficacy.

1.4 Problem Statement

In this master thesis we want to substantiate the knowledge in IgE-mediated responses in the effector cells and how it may contribute to clinical diagnostics and treatment. The objectives can be divided into two main parts The first objective was to characterize the role of IgE affinities on human derived mast cells using MAT and determine whether a change in cellular reactivity, sensitivity and subsequent production of cellular mediators could be detected based on affinity, as it may reflect clinical features. Mast cells derived from patients with severe asthma or healthy control subjects were sensitized with either high, intermediate or low affinity IgE specific for house dust mites, stimulated with allergen and assessed with flow cytometry to evaluate the degranulation response through the upregulation of CD63 for either patient- or control derived mast cells. Sensitized mast cells were also challenged with allergen over a 24-hour period to monitor the release of mediators in order to characterize the late phase response induced by different affinities. To summarize, the hypotheses are the following:

- Whether a difference in mast cell reactivity or sensitivity could be established between patients with severe asthma and healthy controls at different affinities of IgE.
- The mediator release is dependent on the IgE affinity. Higher affinity is presumed to shift the signal transduction pathway to an increased production of cytokines, whilst lower affinity is associated with a chemokine driven response.

The second main objective was to evaluate the predictive value of BAT and MAT, as methods to determine the clinical effect by classifying which patients may gain the most benefit of AIT. For the MAT, human derived mast cells were passively sensitized with baseline patient sera obtained from the GT-08 study, a comprehensive AIT for grass pollen treated patients with known treatment responders [43]. Patient sera were randomized, blinded and activated with grass pollen allergen.

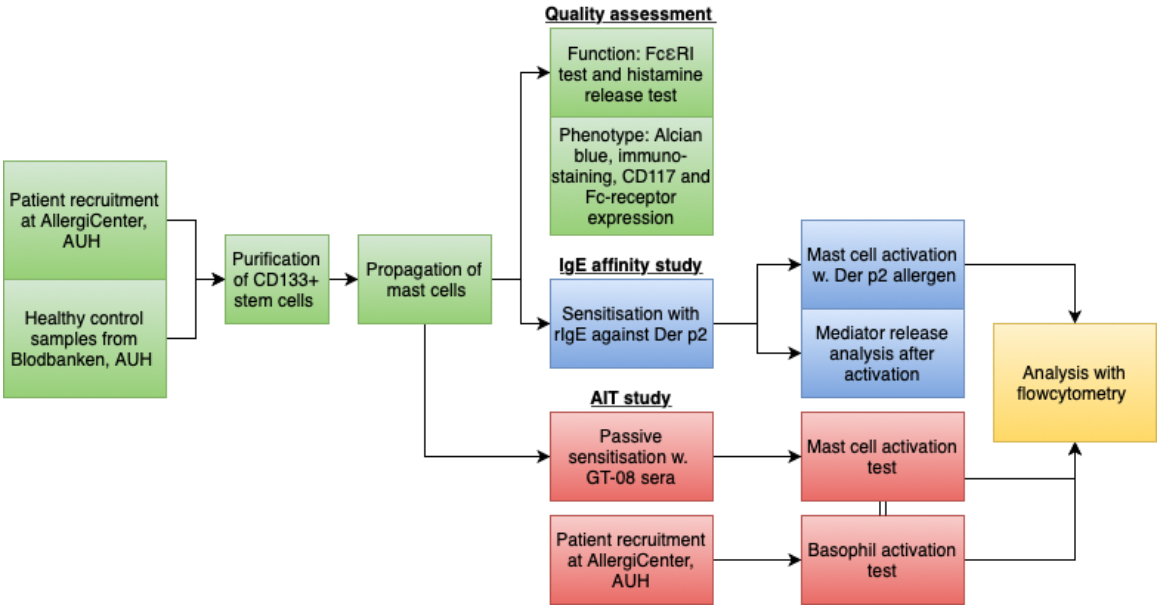
Activated mast cells were analysed with flow cytometry using CD63 upregulation, in which cell reactivity and sensitivity was used to stratify groups. Results were validated using the treatment outcomes from the GT-08 study. For the BAT, patients treated with AIT for birch pollen-dependent allergy were recruited. The baseline basophils from the patients were activated with birch pollen allergen and analysed with flow cytometry using CD63 upregulation, in which cell reactivity and sensitivity was used to stratify groups and compared to change in symptoms before and after four months of treatment. The hypotheses are:

- A high reactivity and/or low sensitivity of mast cells and basophils can be used to characterize and discriminate good treatment responders from non-reactive treatment responders.
- The MAT and BAT responses correlate in regard to reactivity and sensitivity.

2 Methods

In this master thesis project, two main studies were conducted to 1) investigate the effect of IgE affinity on mast cell reactivity and sensitivity, and explore the cell mediators released during a mast cell degranulation response 2) investigate the applicability to use activation tests with basophils and mast cells with patient specific IgE to predict the treatment effect of AIT, whilst comparing the cell activation methods. Prior to the main experiments, quality assessment studies were conducted to investigate mast cell functionality and quality of purified mast cell cultures. The study design of this project can be assessed in Figure 4. In the following sections, a brief overview of subject recruitment will be summarized, the methods of isolation, propagation and quality assessment of mast cell cultures elaborated, and lastly the analytical methods and statistical approach to the main studies will be described.

Figure 4: **Workflow**



Workflow of the study design. The study design shows cell distribution to the various research objectives; quality assessment studies, IgE affinity studies, and allergen immunotherapy (AIT)-studies. Results were obtained with Attune Acustic Focusing Cytometer (Applied Biosystems, Thermo Fischer). Aarhus University Hospital (AUH); immunoglobulin E (IgE); mast cell activation test (MAT); basophil activation test (BAT)

2.1 Subject Recruitment for Mast Cell Isolation

Prior to patient recruitment, an application to the National Committee on Health Research Ethics was submitted (project ID: 1-10-72-209-19). Patients attending the Allergy Center at AUH were screened for inclusion- and exclusion criteria through their electronic medical chart (EPJ) and by a brief patient consultation meeting (see Appendix II). Samples from healthy controls were obtained from the staff or 50 ml buffy coats were acquired from the blood bank at AUH. Written informed consent was obtained in compliance with the Declaration of Helsinki. A total of 15 subjects participated in the project, in which eight were patients diagnosed with severe asthma (GINA>2) and seven were controls. Data disclosed from patients and controls were recorded anonymously in the clinical database REDCap.

2.2 Isolation of Mast Cells

450 ml whole blood or 50 ml buffy coats were obtained in a polyvinyl chloride blood bag with 63 ml CPDA-1 (Macopharma, France). Whole blood was diluted 1:1 in phosphate buffered saline (PBS), whilst buffy coats were diluted 1:2. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient with Ficoll-Paque (GE Healthcare, Sweden) via centrifugation at 1 500 rounds per minute (RPM), 20 °C, acceleration and brake set on 1. Plasma was removed and PBMC were harvested and resuspended in 4 °C PBS, and centrifuged at 1 500 RPM, acceleration and brake at 9, at 4 °C for 5 minutes. Supernatant was removed and cells were resuspended in 10 ml PBS and centrifuged. Supernatant was discarded, and the PBMC were resuspended in 10 ml magnetic activated cell sorting (MACS)-buffer (see Appendix IV.A for MACS-buffer components). The PBMC were counted with trypan blue staining, in a hemocytometer, using a light microscopy (Leitz Wetzlar, DE). The PBMC were centrifuged and resuspended in blocking reagent (CD133 MicroBead Kit, Miltenyi Biotec) and human lyophilized CD133 microBeads (Miltenyi Biotec) according to cell count. The PBMC were incubated on a laboratory tilt shaker at 4 °C for 30 minutes. PBMC were washed with 10 ml of MACS-buffer and centrifuged. The supernatant was discarded and resuspended in MACS-buffer. MidiMACs (MidiMACS^{TM} Separator, Miltenyi Biotec) and a LS-separation column (mil-130-042-401, Miltenyi Biotec) was used to elute isolated CD133^{+} cells. The CD133^{+} cells were centrifuged, the supernatant discarded and resuspended in 2 ml cell culture medium containing StemSpan^{TM} (Stemcell Technologies, cat# 09650, CA), 100 ng/μl IL-3 (RD Systems, cat# 203-IL-xx), SCF (0.25 mg/ml, cat# 255-SC-xx, RD Systems), IL-6 (0.25 mg/ml, cat# 206-IL-xx, RD Systems) and penicillin/streptomycin (pen/strep) (cat# 15140122, Gibco^{TM}). Cells were cultured on a 24 well-plate (Costar®, ThermoFisher) at a density of 500 000 cells/ml and incubated (ThermoScientific, DK) in 5% CO_2 at 37 °C.

2.3 Propagation of Mast Cells

CD133^{+} progenitor cells were propagated to mature mast cells for six to nine weeks. Three different culture media were used, Table 2.

Table 2: **Overview of culture media for mast cells**

Culture medium	Week	Composition
SS with IL-3	0-2	SS with 1% Pen/strep IL-6 (0.25 mg/ml) SCF (0.25 mg/ml) IL-3 (100 ng/μl)
SS	3-5	SS with 1% Pen/strep IL-6 (0.25 mg/ml) SCF (0.25 mg/ml)
SSS with IL-4	6-8	SS with 1% Pen/strep IL-6 (0.25 mg/ml) SCF (0.25 mg/ml) IL-4 (10 μg/ml) FBS

Overview of culture media composition for mast cell propagation. All three culture media for mast cells consisted of the base SS medium. Culture medium was changed weekly. Stem span (SS); stem span with serum (SSS); interleukin (IL); stem cell factor (SCF); fetal bovine serum (FBS).

2.4 Quality Assessment of Mast Cell Cultures

Mast cell cultures were tested for cell reactivity by evaluating their ability to respond through the FcϵRI receptor and degranulate. An anti-FcϵRI antibody test, histamine release test (HR-test) and immunohistochemistry on mast cell phenotype were executed when cell cultures were seven weeks of age. Furthermore, trypan blue staining was performed weekly to monitor cell viability and assess total cell count. The cells were also stained with alcian blue, when cultures reached six weeks of age to assess the purity of the cell composition in the culture. During the culture period, mast cell biomarkers were monitored over a nine-week period. Cell cultures, which could not be activated $\leq$50% with highest concentration of anti-FcϵRI antibody, did not homogeneously express FcϵRI or were $\leq$75 % metachromatic were excluded from this project.

2.4.1 FcϵRI Activation Test

100 μl cell suspension containing 50 000 mast cells/ ml, were transferred to a Falcon® 5 ml round bottom polystyrene tube (Corning Science) for a negative control and for a test tube, respectively. 10 μl of 0.5 mg/ml purified anti-human FcϵRI antibody (cat# 334602, Biolegend, USA) diluted 1:10 in PBS were added to the cells in the test tube and incubated for 30 minutes at 37 °C in a water bath. Both samples were subsequently stained with 4 μl of 0.5 mg/ml fluorescein isothiocyanate (FITC) conjugated anti-human CD63 antibody (Biolegend, USA), diluted 1:25 in PBS, and incubated for 20 minutes at 4 °C in the dark. Samples were diluted in PBS and read on an Attune Acustic Focusing Cytometer (Applied Biosystems, Thermo Fischer) at 421/530 nm for FITC. Results were recorded with Attune Cytometric Software V2.1 and analysed with FlowJo^{TM} v10.6.1 (Becton Dickinson Company). Activated mast cells were identified as CD63$^+$ cells with high side- and forward scatter in a cytogram with doublets excluded. Results were stated as percentage of activated CD63$^+$ mast cells ($\pm$SD) with a threshold of 2% of the negative sample. Test sampling was repeated on three cultures on the following days.

2.4.2 Histamine Release Test (HR-Test)

150 000 mast cells in a density of 500 000 cells/ml were seeded in a cryotube (Cryotube Vials, ThermoFisher, DK). 2 μg/ml myeloma IgE was added and incubated in a 5% CO_2 incubator at 37 °C overnight. Cells were washed in 1 ml of piperazine-N,N-bis(2-ethanesulfonic acid (pipes)-solution and centrifuged at 1 500 RPM at 4 °C for 5 minutes (see Appendix IV.B for pipes-solution components). Supernatant was discarded and the pellet was resuspended in 400 μl pipes. Samples were either activated with 100 μl of 6.8 g/ml anti-IgE (Agilent, DK) or 100 μl pipes (negative controls) and incubated for 30 minutes at 37 °C. Activation was blocked with 4 °C pipes-stam (see Appendix IV.B for pipes-stam components). Mast cells were centrifuged for 5 minutes at 1500 RPM at 4 °C and pellet and supernatant was separated. Samples were stored at -20 °C.
Frozen supernatant and pellet samples were thawed at 100 °C for 10 minutes, vortexed and centrifuged at 13 500 g for 10 minutes. Pellet samples were diluted 1:10 in pipes-stam. 20 μl samples containing either pellet or supernatant was added to a 180 μl pipes-stam and transferred to a histamine coated microtiter plate (Reflab, DK, lot 200K24A17). Four histamine standards were prepared using 98+% histamine dihydrochloride (cas# 6928, Alfa Aesar) diluted in pipes-stam. Concentrations ranged from 20 ng/ml to 80 ng/ml, and pipes-stam was used as the negative control. The plate was incubated for 1 hour at 37 °C and washed with demineralized water for 90 seconds. The plate was subsequently air dried and shipped to RefLAB (DK) for enzyme linked immunosorbent assay (ELISA) analysis. Results were stated as the percentage of the total cellular histamine release in cell lysate and determined by anti-IgE activated cells in the pellet and supernatant samples.

$$HistamineRelease[\%] = 100 \times \frac{supernatant[ng/ml] \times 2}{pellet[ng/ml] - supernatant[ng/ml]}$$

$$supernatant = 0.15 \times meanvalue, pellet = 0.15 \times meanvalue \times dilutionfactor$$

A correlation analysis was performed on the results obtained from the histamine release test and the FcεRI test. Data analysis was done with Prism v.8.4.1 software (GraphPad) and the results were stated as a R^2-value and a Pearson's correlation coefficient, r (p-value).

2.4.3 Alcian Blue Staining

In the sixth week, purity of the mast cell cultures was evaluated with an alcian blue stain (Ampliqon, DK). Mast cells were stained in a ratio of 1:2 and counted in a hemocytometer using a light microscopy and a 170/0.17 NPI 25/0.50 objective. The results were reported in percentage of metachromatic cells visible of total cell count.

2.4.4 Immunostaining

To evaluate the mast cell tryptase$^+$ and chymase$^+$ phenotype, the mast cells were identified by immuno-based assay, alkaline phosphatase-anti alkaline phosphatase (APAAP) (Dako Real^{TM} Detection System APAAP, DK, lot 20003619). 90 μL cell suspension with a concentration of 200 000 mast cells/ml was transferred to a TPX chamber with cytoslides (Shandon, ThermoFisher, USA) dried in 99% ethanol. Three cytoslides were made for each cell culture to assess isotype, chymase or tryptase staining. The cytospin cytocentrifuge (Cytospin 3, Bie Berntsen, DK) was set at 600 RPM, 5 minutes at medium acceleration. The slides were fixed with acetone and methanol in a 1:1 mixture and dried for a minimum of 5 hours. The fixed cell samples were circumscribed by a delimiting pen, Dako Pen (Agilent, DK) and air dried for 30 minutes. The slides were subsequently stained with either primary anti-tryptase (1 μg/ml) antibody or primary anti-chymase (1 μg/ml) antibody and incubated overnight in a humid chamber at 4°C. The samples were washed in tris-buffered saline (TBS) (Ampliqon, DK Q410211000) for 15 minutes before adding secondary rabbit anti-mouse antibody and chromogen detection mix to the cytoslides according to manufacturer's protocol by Dako, Dako Real^{TM} Detection System APAAP, DK, lot 20003619 (see Appendix V). The samples were stained with the Dako chromogen mix and incubated for 20 minutes in a humid chamber before washing in distilled water for 15 minutes and air dried. The samples were assessed by light microscopy at 40X objective (Plan 40X/0.65, Japan), in which the percentage of tryptase$^+$ and chymase$^+$ mast cells were identified.

2.4.5 Pilot study - Identification of Isolated Mast Cell Cultures through Surface Markers

Some mast cell cultures were monitored weekly from week 0-9 for the expression of specific mast cell surface markers, CD117 and FcεRI. BV605-conjugated anti-human CD117 antibody (cat# 313218, Biolegend) and BV421-conjugated anti-human FcεRI antibody (cat# 334624, Biolegend) were titrated to optimize resolution of the quality assessment study. In addition, preliminary studies were conducted to test the performance of antibodies incubated separately or in conjunction with each other. 100 μl containing 50 000 mast cell/ml culture media were stained with 2 μl anti-human CD117 (80μg/ml) and 10 μl anti-human FcεRI and incubated for 30 minutes at 37 °C. Samples were diluted in PBS and analysed on an Attune Acustic Focusing Cytometer and read at 405/421 for BV421 and 405/603 nm for BV605, respectively. Data was recorded with Attune Cytometric Software and analysed with FlowJo^{TM}. Mast cells were identified by high forward- and side scatter in a cytogram with doublets excluded. Results were stated in relative median fluorescence intensity (MFI).

2.5 Optimization Study of Plastic Ware for Activation Assays

Cell activation methods were tested in a Nunc^{TM} 96-Well polypropylene (PP) DeepWell Storage Plates (cat#260252, Thermo Fischer) and Falcon^{TM} 5 ml round-bottom polystyrene tubes (Fischer Scientific, UK). This experiment was performed twice with different incubation and activation times. Nine weeks old mature mast cells were seeded at concentration of 100 000 cells/ml and sensitized with 10% serum from a donor with grass pollen dependent asthma for one or three days in 5% CO_2 at 37 °C. 100 μl cell suspension was transferred to 20 wells strategically selected throughout the 96-well PP plate (cat#260252, Thermo Fischer) and two Falcon tubes consisting of one positive and one negative control. 10 wells were used for activation with allergen, whilst the remaining 10 wells were negative controls. Mast cells were either activated with freeze dried tablet of 75 000 specific quality (SQ)-T Grazax, diluted in PBS to a final concentration of 60610.00 μdaily dose equivalent (DDE)/ml, or with PBS in the negative samples. Allergen concentrations was calculated by the dilutions of one SLIT tablet (Grazax), which equals to one daily dose dissolved in 1.5 ml PBS. The 96-well plate was incubated for 1 hour or 1.5 hours at 37 °C and subsequently stained with anti-CD63 antibody and analysed with flow cytometer as described previously in section 2.4.1. The 96-well plate was recorded with a Attune NxT Autosampler (Applied Biosystems, Thermo Fischer) integrated with the Attune Acustic Focusing Cytometer. Mast cells were gated according to section 2.4.1 and results were stated as percentage of activated CD63^{+} mast cells in the 96-well PP plate and Falcon polystyrene tube.

2.6 Characterization of IgE Affinity on Human Mast Cells

Seven subjects with severe asthma (GINA 4-5) and five healthy subjects or buffy coats were included. Mast cells were cultured from isolated CD133^{+} progenitor cells, propagated for at least six weeks, and sensitized with rIgE pairs of either high, intermediate or low affinity for Der p 2. For this study, the recombinant IgE-clones were carefully chosen based on 1) the epitope placement on the allergen, 2) the distribution of allergen specific affinity. The ranking of IgE affinity for each clone was predefined by ALK-Abelló (Hoersholm, Denmark).

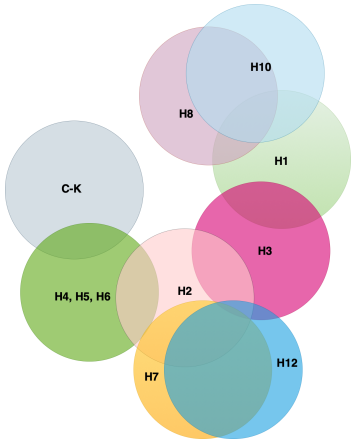


Figure 5: Overview of Epitope Placement of IgE-Clones

Illustration based on Christensen et al, 2008, Journal of Allergy and Clinical Immunology;122:298-304. Overview of the epitope placement across available IgE-clones from ALK Abelló, Hoersholm, Denmark. The IgE-clones H10 and H12, H8 and H12:H7, and H10:H12 and H7:H12 were used for high, intermediate and low affinity sensitization, respectively. Immunoglobulin E (IgE).

The parameters of the study were set to 1) ensure no overlap of epitopes between the antibodies (Figure 5), 2) maintain the epitope placements across all three conditions, 3) establish a notable

different distribution of allergen specific affinity. The distribution of the affinities was calculated as the square root of the products of two paired IgE-clones. An estimated 130-fold difference of affinity was found between the highest to lowest sensitization condition. Recombinant antibodies and Der p 2 allergen used in this study were all kindly provided by Lars Christensen (ALK-Abelló A/S, Hoersholm, Denmark).

2.6.1 Sensitization of Mast Cells

The experimental setup was based on a similar study performed at the Department of Respiratory Diseases and Allergy in AUH [5]. Isolated mast cell cultures from either severe asthmatics or healthy controls, were divided in three equal subcultures containing $1x10^6$ cells and seeded in a density of 500 000 cells/ml. Each sample were sensitized with 80 kU/l of total recombinant IgE-antibodies containing 7% for each Der p 2 specific IgE clone and 86% non-specific IgE. The IgE-clones H10 and H12, H8 and H12:H7, and H10:H12 and H7:H12 were used for high, intermediate and low affinity sensitization, respectively (Table 3).

Table 3: **Overview of IgE-Clones for Sensitization**

Condition	IgE-clone pair (K_D)		$\sqrt{Product of K_D}$
High	H10 (0.45)	H12 (1.12)	0.71
Intermediate	H12:H7 (5.10)	H8 (13.10)	8.17
Low	H7:H12 (30.00)	H10:H12 (284.00)	92.30

The rIgE are denoted as Hx:Hy, which represent the heavy and light chain of the antibody, respectively. Each cell culture was divided, and subsequently exposed to three conditions of different binding affinities to Der p 2; high, intermediate and low. The disassociation constant (K_D) was measured by surface plasmon resonance and stated in nM. Higher K_D indicates lower affinity and vice versa. A 130-fold difference was observed between the highest and lowest antibody clone pairs. Recombinant immunoglobulin E (rIgE), Dermatophagoides pteronyssinus (Der p 2).

Mast cell subcultures were propagated with sensitization medium for two weeks in an incubator with 5% CO_2 at 37 °C. Throughout the sensitization phase, each cell culture medium was changed weekly and monitored biweekly with light microscopy (Leitz Wetzlar, DE).

2.6.2 Mast Cell Activation Test to Evaluate Effect of IgE affinity

Sensitized mast cells were diluted 1:5 in culture medium with SSS+IL-4 in concentration of approximately 83 000 cells/ml. Cell subcultures were distributed in 12 wells in a 96-well PP plate for each condition. Recombinant Der p 2 was serial diluted in PBS in 11 half-logarithmic and logarithmic concentrations. The twelfth concentration was a negative control with PBS. Allergen concentrations ranged from 0.001 ng/ml to 1 000 ng/ml. Mast cells were activated in the PP plate by mixing a 100 μl cell suspension with 100 μl allergen solution of varying concentrations and incubated for one hour in a 37 °C water bath. Mast cells were stained with anti-CD63 antibody, as described in section 2.4.1. Plates were incubated for 20 minutes at 4 °C and diluted in PBS for analysis on the Attune NxT Autosampler and read at 421/530 nm for FITC. Data analysis was performed with FlowJo™ and mast cells were gated as described in section 2.4.1. Mast cell reactivity was measured as the fraction of $CD63^+$ mast cells±SE.

2.6.3 Characterization of the Late-Phase Response in Mast Cell Degranulation

Sensitized mast cell subcultures were activated at three different time points to obtain the progression of release of various cytokines, chemokines and eicosanoids that characterize the late-phase response of an allergic reaction. Mast cells were centrifuged at 2 000 RPM for 10 minutes at 4 °C, in which 100 μl supernatant was collected as the negative baseline sample and stored at -80 °C. Mast cell cultures were subsequently activated with the optimal allergen concentration of Der p 2, subdivided

in three samples and incubated for 3, 6 and 24 hours, respectively at 37 °C in a water bath. Following each time point, samples were centrifuged at 2 000 RPM for 10 minutes at 4 °C. Supernatant and pellets were separated and stored at -80 °C. Samples were stored for future external analyses using inflammation multiplex panel and gas chromatography–mass spectrometry (GC MS), which are not available in this master thesis.

2.6.4 Statistical Analysis of IgE Affinity Differences on Mast Cells Reactivity and Sensitivity

Dose-response curves were generated using Prism v.6 software (GraphPad), in which the fraction of $CD63^+$ mast cells was plotted against the $\log_{10}$ allergen concentrations of Der p 2. For each culture and conditions, the maximal mast cell reactivity was obtained. The sensitivity, defined as the concentration of allergen to elicit half maximal response (EC50), were estimated based on nonlinear curve fitting and four parameter logistic (4PL) using Prism. Higher values of EC50 indicate lower mast cell sensitivity. All data was analysed for normality distribution with a Shapiro-Wilks test and histogram appearance. The results were stated in mean(±SE). Cell growth was plotted with logistic regression on Prism and results were stated as median (minimum and maximum) A Mann Whitney U test was conducted to compare the three respective IgE affinities between the asthmatic- and control group on either the mast cell reactivity or -sensitivity. The same analysis was also performed between the two groups (controls and asthmatics) on stem cell yield and on mast cell growth. A within analysis was carried out in order to examine the effect of IgE affinity on either the patient or the control group using the Friedman test. All statistical analyses were performed with SPSS (IBM ® SPSS Statistics ®, v.26), and p-values ≤ 0.05 were considered significant. Power analysis was conducted in G*Power, v. 3.1.9.4.

2.7 The Predictive Value of Mast- and Basophil Activation Tests for Immunotherapeutic Treatment

2.7.1 Optimization Experiment of Mast Cell Quantity and Serum Concentration for Mast Cell Activation

Eight to nine weeks mature mast cells were seeded in cryotubes with a punctured lid in concentrations of 1 250, 2 500, 5 000 or 10 000 mast cells/100 μl SSS culture medium with IL-4. Each sample was sensitized with either 5%, 10% or 20% human serum from a donor with grass pollen dependent asthma. The samples were incubated for 2 days at 37 °C in a 5%CO_2 incubator. Mast cells were transferred to a 96-well PP plate in duplicates and 10 μl of Grazax® solution was added, which was serially diluted 1:10 in PBS in three final concentrations. These ranged from 0.606 mDDE/ml to 60.61 mDDE/ml for two cultures, and 0.606 nDDE/ml to 60.61 nDDE/ml for one culture. 10 μl PBS was added to a negative control for each condition and the samples were incubated for 1 hour in a 37 °C water bath. The samples were subsequently stained with 4 μl anti-CD63 antibody as described in section 2.4.1. The samples were diluted in 1 ml PBS and recorded with Attune NxT Autosampler. Results were analysed with Attune Cytometric Software and FlowJo^{TM} and stated as percentage of activated $CD63^+$ mast cells, signal-to-noise ratio (SNR) [MFI_{pos}/MFI_{neg}] and MFI of negative samples. The SNR was calculated as a fraction of MFI in the positive sample of mast cell gated specimen and MFI in the negative sample in the mast cell gate measured at 530 nm for FITC. The experiment was repeated on three mast cell cultures, one buffy coat derived culture and two allergic asthmatic donor derived cultures for validation of the results.

To assess whether serum affects cell membrane integrity in different serum fractions, a live/dead exclusion method using trypan blue was performed. Two mast cell cultures seeded in cryotubes at a density of 350 000 cells/ml and were incubated with 0%, 5%, 10% or 20% serum. Cultures were incubated for 2 days with 5% CO_2 at 37 °C. Mast cells were centrifuged at 1500 RPM, 4 °C for 5 minutes and resuspended in 200 μl SSS culture medium. 10 μl mast cells were stained with trypan

blue and live and dead cells were counted with light microscopy (Leitz Wetzlar, DE). Results were reported as fractions of live cells.

2.8 Predictive Value of Mast Cell Activation Test in Allergen Immunotherapy

Sera from patients who have completed the GT-08 study with Grazax were selected. The GT-08 study was a randomized, double-blinded, placebo-controlled, multinational phase III trial. The study included adults with a history of moderate-to-severe grass pollen–induced allergic rhinoconjunctivitis, with or without asthma, and were inadequately controlled by symptomatic medications [43]. 102 patient sera samples were carefully selected and kindly supplied by ALK-Abelló A/S, Hoersholm, DK. The patients were defined by a global evaluation score, which were based on the subject's yearly self-evaluation after one, two and three years of treatment. The scores were "much worse", "worse", "the same", "better" and "much better". Based on these scores, ALK-Abelló defined the following groups as "non-reactive (NR)", "medium", "high" and "late" responders. These groups were made specifically for this project and was not a part of the original trial (Table 4).

Table 4: **Groups by Global Evaluations Scores**

Group	Score parameters
High (n=33)	Much better year 1 and the same, better, or much better year 2 and 3
Medium (n=36)	Better year 1 and the same, better, or much better year 2 and 3
NR (n=25)	Much worse, worse, or the same year 1, 2, and 3
Late (n=9)	Better in year 2 or 3

Groups were defined by a global evaluation score, which were based on the subject's yearly self-evaluation after one, two and three years of treatment. Subjects were classified in four categories depending on their AIT-response. Allergen immunotherapy (AIT); non-reactive (NR).

For the MAT, patient samples were randomized and blinded throughout the experiment to the analytical site. An anonymised sample ID was provided by ALK-Abelló to identify samples. The global evaluation scores were disclosed after experimental completion and were linked to the sample ID. 10 patient samples were provided with additional sera to test the reproducibility of MAT.

2.8.1 Sensitization and Activation of Mast Cells with Patient Sera

MAT were conducted in four different mast cell cultures to explore 1) the overall reactivity and sensitivity of mast cells with patient specific IgE (n=96 patient samples), 2) reproducibility in three cultures (n=10 patient samples) and 3) reactivity and sensitivity from patient-derived mast cells (n=13 patient samples). Seven-nine weeks old mature buffy coat-derived mast cells were divided into either 96 or 10 samples and seeded in 24-well plates with 5 000- 7 000 mast cells/100 μl SSS+IL-4 media solution. Each sample was sensitized with 5% patient serum and incubated in a CO_2 incubator at 37 °C for two days. Mast cells were activated in a 96-well PP plate with 10 μl of dissolved Grazax®, as described in section 2.7.1, in eight concentrations, which were serially diluted 1:4 in PBS to final concentration range of 0.092 to 1515.15 μDDE/ml. Allergen concentrations were optimized through preliminary experiments. A negative mast cell control consisted of 10 μl PBS and a positive control of 10 μl BV421 conjugated anti-human FcϵRI antibody diluted 1:10 in PBS. The samples were incubated for 1 hour in a 37 °C water bath. The samples were subsequently stained with FITC conjugated anti-human CD63 antibody, as previously described in section 2.4.1. The samples were diluted in PBS, recorded with Attune NxT Autosampler and read at 405/421 nm for BV421 and 421/530 nm for FITC. Results were analysed with Attune Cytometric Software and FlowJo^{TM} and stated as percentage of activated $CD63^+$ mast cells with a threshold of 2% of the negative control. Percentage of $CD63^+$ mast cells were plotted against $\log_{10}$ of allergen concentration. A

4PL regression with curve fitting was used to calculate sensitivity [EC50, μDDE/ml], defined by half maximal effective concentration, with Prism software.

Subanalysis was performed externally by ALK-Abelló, with other markers obtained by ALK-Abelló. These readouts were total nasal symptom score (TNSS) based on nose symptoms over the entire grass season, baseline levels of sIgE, sIgG4 and sIgE/sIgG4 ratio and how they relate to reactivity, sensitivity or the effect groups.

2.8.2 Statistical Analysis of Mast Cell and Patient Response

All data was analysed for normal distribution with a Shapiro-Wilks test and histogram appearance. Differences in reactivity or sensitivity between groups of high, medium, NR and late treatment response was analysed with a Kruskal-Wallis test as a non-parametric test to a one-way ANOVA. To test reproducibility, a Wilcoxon signed rank test, correlation analysis (Spearman's ρ) and a Bland Altman plot were carried out in order to compare mast cell sensitivity and reactivity agreement and pairing between two mast cell cultures. Statistical analyses were performed with Prism v. 8.4.1 software (GraphPad) and p-values $\leq$0.05 were considered significant. Data was stated as medians with inter quartile range (IQR).

2.9 Predictive Value of Basophil Activation Test in Allergen Immunotherapy

2.9.1 Patient Recruitment

Prior to patient recruitment, an application to the National Committee on Health Research Ethics was submitted (project ID: 1-10-72-210-19). Patients $\geq$18 years who were offered SLIT treatment with Itulazax® at the Allergy Clinic at AUH were screened for inclusion and exclusion criteria through their EPJ and by a brief patient consultation meeting (see Appendix III.A). Inclusion criteria consisted of patients >18 years with a confirmed clinical diagnosis of allergic rhinitis and total serum IgE of $\geq$ 30 to $\leq$ 700 IU/ml. If patients were willing to participate, blood samples were collected at their baseline visit for treatment start up in the Allergy Clinic. Blood samples were drawn in a BD Vacutainer® Heparin Tube for BAT and BD Vacutainer® Plus Plastic Serum Tube (BD Bioscience, USA) for serum collection to be used in a MAT.

2.9.2 Symptom Score

The endpoints of treatment effect were recorded as the difference in self-evaluated symptoms and life quality based on a rhinitis quality of life questionnaire (RQLQ) (see Appendix III.B) at baseline and after 4 months of SLIT treatment. The RQLQ comprised of 28 questions within domains of activity limitation, sleep problems, nose symptoms, eye symptoms, non-nose/eye symptoms, practical problems and emotional function. The questions were ranked 0-6, where 6 is the worst and the maximum score was 168. The patients were instructed to rank their scores, retrospectively on the last tree and birch pollen season of 2019. Follow-up calls were made one month after treatment initiation to ensure compliance. Patients who continued their treatment with Itulazax after approximately four months, at the beginning of the tree and birch pollen season, were contacted and asked to complete a RQLQ over their current symptoms. The beginning of the tree pollen season was defined as days with one or more tree pollen counts of 10 $\geq$ grains/ $m3$ for alder (*Alnus*) and elm (*Ulmus*), 5 $\geq$ grains/ $m3$ for hassel (*Corylus*) and 30 $\geq$ grains/ $m3$ for birch (*Betula*) reported by Astma-Allergi Danmark locally for Aarhus. Data disclosed from patients were recorded anonymously in the clinical database REDCap.

2.9.3 Basophil Activation Test

Reactivity and sensitivity in basophils towards treatment preparations of Itulazax® freeze-dried tablet, 12 SQ-Bet (ALK-Abelló A/S, DK) were measured using fresh baseline whole blood from the

patients approved for Itulazax®. The allergen preparations were made by dissolving Itulazax® in 1.5 ml for a final concentration of 666.67 mDDE/ml. Itulazax was serial diluted 1:10 in PBS. 10 μL of allergen dilution were distributed into each of the nine polystyrene tubes for final concentrations of 0.0618 nDDE/ml to 6060.61 μDDE/ml/tube. 10 μL of 0.5 mg/ml purified anti-IgE (cat# 555894, BD Bioscience) diluted 1:5 in PBS was added one polystyrene tube as a positive control. Negative controls were performed in triplicates in which 10 μL PBS was added. A detection antibody mix was prepared with 1:20 BV421^{TM}-conjugated anti-human CD193 (cat# 310714, Biolegend), 1:10 PE-conjugated anti-human CD123 (cat# 306006, Biolegend) and 1:50 FITC-conjugated anti-human CD63 in 200 μL PBS. 100 μL fresh drawn heparinized blood from the patient was distributed to all tubes with 10 μL antibody mix. The samples were vortexed and incubated in a 37 °C water bath for 30 minutes. 2 ml BD FACS^{TM} Lysing Solution (cat# 349202, BD Bioscience, USA) diluted 1:10 in sterile water was added to each tube, vortexed and incubated in the dark for 15 minutes. The samples were centrifuged at 400 g, 20 °C, acceleration and brake set on 9 for 7 minutes. The supernatant was discarded, and the samples were resuspended in their remaining solution. The samples were read with a BD FACS Canto II Flow Cytometer (BD Bioscience, USA) at 585 nm for PE, 450 nm for BV421 and 530 nm for FITC. Data was recorded with BD FACSDiva^{TM} v.6.1.2 Software. The samples were gated and analysed with FlowJo^{TM}. Basophils were identified as CD123$^+$/CD193$^+$ cells with low side scatter and high forward scatter. Activated CD63$^+$ basophils were determined by setting a threshold of 2% in negative samples without allergen. Reactivity was determined from this fraction of activated CD63$^+$ basophils. Basophil sensitivity (EC50) [μDDE/ml] was calculated for each condition by fitting a 4PL curve to the fraction of activated basophils plotted against the $\log_{10}$ allergen concentration.

2.9.4 Mast Cell Activation Test versus Basophil Activation Test

To evaluate how BAT correlates with MAT, patient IgE were collected as described in section "2.9.1 Patient Recruitment" to test in a MAT. Plasma were collected from 11/12 patients and sera from 4/12 patients. Blood samples drawn in a BD Vacutainer Heparin Tube and a BD Vacutainer Plus Plastic Serum Tube (BD Bioscience, USA) were centrifuged at 20 °C, 3 000 RPM, acceleration and brake set on 9, 10 minutes to separate plasma and serum. The supernatant was collected from the tubes and frozen at -80 °C until all patient samples were collected. Two mast cell cultures used at seven or eight weeks old from buffy coats were seeded at a density of 55 000-70 000 mast cells/ml and sensitized with 5% serum or plasma for two days in a 5% CO_2 incubator. Plasma samples were added 5% heparin buffer consisting of SSS+IL-4 medium with 377.78 IU/ml heparin to prevent coagulation. To activate mast cells, a dissolved Itulazax tablet was serially diluted, as described in section 2.9.3 and 10 μl was added to the respective allergen tube. 10 μL 1:5 purified anti-IgE in PBS was added to one polystyrene tube as a positive control. 10 μL PBS was added to polystyrene tubes as negative control. 100 μl sensitized mast cells was distributed to all tubes. The samples were vortexed and incubated in a 37 °C water bath for 30 minutes. All samples were stained with FITC-conjugated anti-human CD63 antibody as previously described in section 2.4.1. The samples were centrifuged at 400 g, 20 °C, acceleration and brake set on 9 for 7 minutes. The supernatant was discarded, and the samples were resuspended in its remaining solution. The samples were read with a BD FACS Canto II Flow Cytometer at 530 nm and recorded with BD FACSDiva^{TM} Software. The samples were gated and analysed with FlowJo^{TM}. The negative samples was set at a threshold of $\sim 2-3\%CD63^+$ mast cells.

2.9.5 Statistical Analysis of Activation Tests and Patient Response

A four-parameter logistic regression with curve fitting was used to calculate sensitivity (EC50) with Prism. All data was analysed for normal distribution with a Shapiro-Wilks test and histogram appearance. To test reproducibility in BAT and MAT, a Wilcoxon signed rank test, correlation analysis (Spearman's ρ) and a Bland Altman plot were carried out in order to compare mast cell sensitivity and reactivity between the two tests. Treatment effect was calculated by the difference in

RQLQ-scores from baseline values to birch-pollen season follow-up values and stated as percentage of reduction. Reactivity or sensitivity from BAT and MAT were correlated to the reduction in RQLQ-scores using Spearman's ρ. Statistical analyses were performed with Prism, and p-values ≤ 0.05 were considered significant. Power analysis was conducted in G*Power, v. 3.1.9.2 with effect size based on correlation coefficients.

3 Results

In the following sections, the results will be presented in five main parts. The first part, section 3.1, contains the status of the mast cell donors and consists of a clinical overview of the patient population (Table 5) and a brief summary of the control population (Table 6). The second part, section 3.2, contains an overview of the quality of the isolated mast cell cultures followed by the results from the material comparison study in 3.3. Lastly, the results from the first (Affinity study) and second (AIT-study) main study will be presented in section 3.4 and section 3.5-3.6, respectively.

3.1 Sample Population

Eight patients with a mean age(±SD) of 48.75(±16.36) years were included in this project. Six patients were diagnosed with allergic asthma consisting of one male and five females with GINA-scores≥4. 2/8 patients were classified with non-allergic asthma, thus no specific IgE could be defined.

Table 5: **Clinical overview of Patient Population for Mast Cell Cultures**

Patient ID	Age/gender	Allergic status	SPT	Specific IgE (kU/l)	Total IgE (kU/l)	GINA	Culture ID	MC distribution
1	54/female	Allergic asthma	NA	d1 (0.43), e1 (1.25)	498	4	3419B	Affinity study
2	52/male	Eosinophilic asthma	+	NA	148	5	3619A	Affinity study
3	63/female	Allergic asthma	NA	d1 (0.39), e1 (0.48)	361	5	3619B	Affinity study
4	30/female	Allergic asthma	+	g6 (8.9), t3 (23.6) e1 (0.48)	64	4	3719A	Affinity study
5	22/female	Asthma	-	NA	59	4	3819A	Affinity study AIT-study
6	70/female	Allergic asthma	NA	fx5 (0.43), fx1 (1.25)	NA	5	4019A	Affinity study
7	57/female	Allergic asthma	NA	g6 (0.97)	175	4	4219B	AIT-study
8	42/male	Allergic asthma	NA	t3 (17.9), w6 (0.66) e1 (0.38), d2 (0.38) g6 (8.1)	305	4	0220A	Affinity study AIT-study

Isolated mast cell cultures were obtained from patients attending the Allergy Clinic, AUH. Patients were screened for inclusion- and exclusion criteria prior to participation. Patient ID, age/gender, allergic status, SPT, sIgE, total IgE, GINA-score, culture ID and distribution of mast cell cultures have been described. House dust mite (d1); cat (e1); grass (g6); birch (t3); food antibody (fx5); egg white (fx1); mugwort (w6); mast cell (MC); allergen immunotherapy (AIT); Aarhus University Hospital (AUH); skin prick test (SPT); specific IgE (sIgE); immunoglobulin E (IgE); Global Initiative of Asthma (GINA); mast cell (MC); not available (NA).

Table 6: **Overview of Control Population for Mast Cell Cultures**

Control ID	Age/gender	Donor origin	Culture ID	MC distribution
1	58/male	Healthy donor	3419A	Affinity study, optimization study
2	NA	Buffycoat	3519A	Affinity study, AIT-study
3	NA	Buffycoat	4719B	Affinity study, AIT-study
4	NA	Buffycoat	4819A	Affinity study, AIT-study
5	NA	Buffycoat	5119A	AIT-study
6	NA	Buffycoat	0420A	Affinity study, AIT-study
7	NA	Buffycoat	0520A	AIT-study

Control subjects were obtained from staff or the blood bank at AUH. Seven controls were included in this master thesis. Control ID, age/gender, origin of samples (healthy donor or buffy coat), culture ID and distribution of mast cells to the respective experiments have been reported. Mast cell (MC); Aarhus University Hospital (AUH); not available (NA).

3.2 Quality Assessment of Mast Cell Cultures

The mast cell cultures chosen for experimental use were based on several phenotypic and functional parameters. Phenotypically, a mean(±SD) of 90.38(±8.28)%, 90.72(±15.52)% and 4.55(±12.13)% were observed for the metachromatic, tryptase and chymase staining, respectively. Functionally, the mast cell cultures had a mean reactivity(±SD) score of 75.08(±19.19)% using the FcϵRI-activation test. Additional sampling for three mast cell cultures (3719A, 4819A and 0520A) showed a mean difference of 30.33(±14.50)% of $CD63^+$ cells when the test was repeated. The mean release of histamine was 58.13(±33.33)%. Correlation analysis showed non-significant moderate correlation between the histamine results and the activation values with R^2=0.32, r=0.57 (p-value=0.07).

The trial monitoring of surface markers on some of the mast cell cultures showed no stable development for FcϵRI expression nor $CD117^+$ expression. For culture 4819A, which was followed through nine weeks, the MFI ranged from 370 to 4712 for the FcϵRI expression, whilst the $CD117^+$ expression ranged from -108 to 5764 MFI. The fluctuating development for both surface markers was observed in the few cultures tested and the test was discontinued.

Table 7: **Overview of Quality Assessment Test on Mast Cell Cultures**

Quality test		Phenotype		Activation	
Culture ID	A. blue	Tryptase	Chymase	CD63+	HR
3419A	85	100	0	95.5	100
3419B	90	50	0	86.6	75.86
3519A	80	100	0	44.1	86.61
3619A	85	80	0	65.9	45.95
3619B	90	80	0	80.8	100
3719A	NA	95	0	52.5	14.98
3819A	75	100	40	54.3	41.72
4019A	85	100	10	55.5	7.41
4219B	NA	95	10	52.2	36.04
4719B	90	100	0	92.6	88.10
4819A	100	98	0	89.6	42.75
5119A	100	NA	NA	92.8	NA
0220A	100	NA	NA	95.6	NA
0420A	100	NA	NA	95.6	NA
0520A	95	NA	NA	72.6	NA

The performance from the quality assessment tests in percentage for each individual mast cell culture. The phenotype of the mast cell cultures have been stated in percent of positive cells for the alcian blue, tryptase and chymase staining, respectively. Activation through the high affinity receptor, FcϵRI, was reported as the fraction of %$CD63^+$ mast cells. The results from the histamine release test obtained through ELISA was stated in percent. Alcian blue (A. blue); histamine release test (HR-test); enzyme linked immunosorbent assay (ELISA); not available (NA).

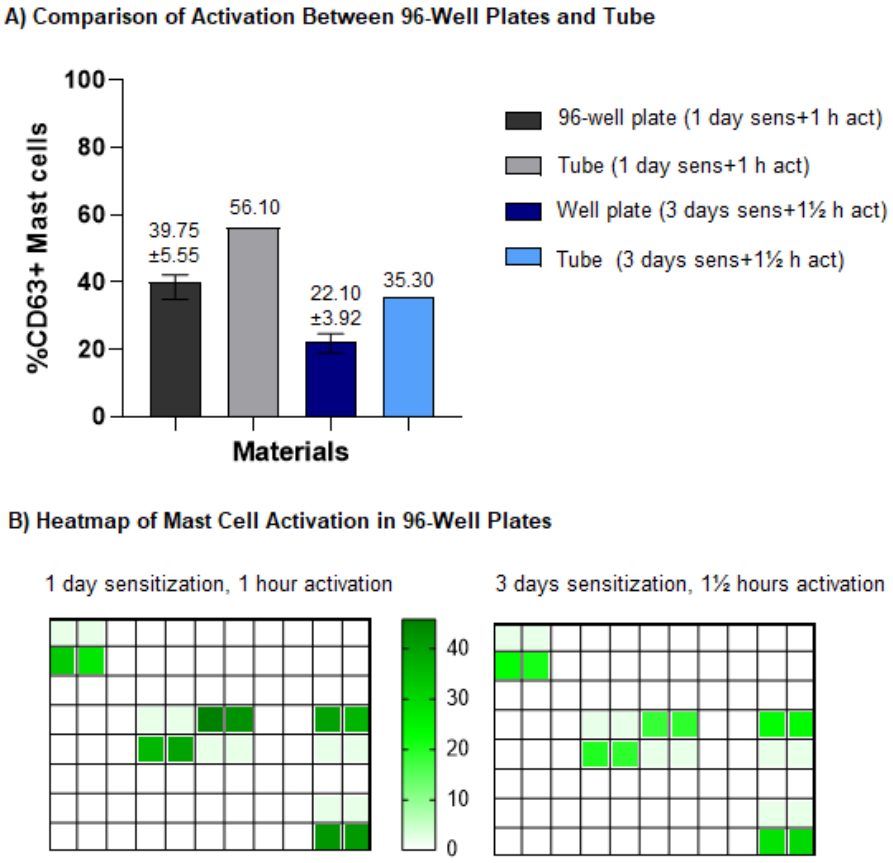
3.3 Optimization - Material Comparison of Activation Assays

The mast cell activation assay has been standardized, at the Department of Respiratory Diseases and Allergy AUH, to be performed in Falcon round-bottom polystyrene tubes. To obtain a more automated procedure for the processing of several samples, the optimization experiment aimed to investigate mast cell activation in a 96-well plate to be analyzed with a Attune NxT Autosampler. Mast cells were commonly activated with allergen and incubated in falcon tubes for 30 minutes at 37 °C. Plates were tested similarly but with altered incubation time, according to experiences from

ALK-Abelló, in order to examine the heat distribution in the PP plate and determine whether cell activation was feasible.

Results of mast cell activation in plates compared to tubes can be assessed in Figure 6A, whilst a heatmap of the plate activation is illustrated in Figure 6B. Mast cells in the 96-well plate, which had been sensitized for one day and activated for 1 hour were considered to be fairly low activated. The second measurement with three days of sensitization and 1.5 hours of activation showed even lower mast cell reactivity. The mean difference of activation between plates and tubes was 15.35% $CD63^+$ mast cells.

Figure 6: **Mast Cell Activation Test in Well Plates and Tubes**



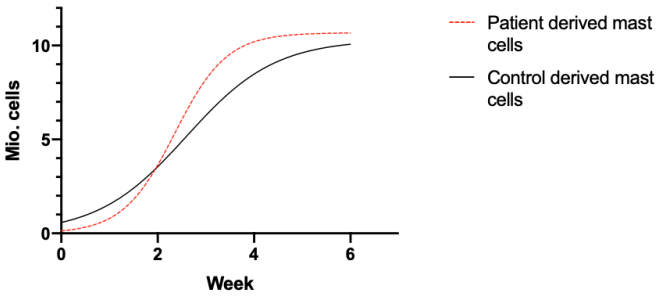
Assessment of mast cell activation in 96-well polystyrene plates or polypropylene tubes. A) Mast cells were sensitized with 10% serum derived IgE for either one day (gray columns) or for three days (blue columns). Activation with grass allergen (Grazax) were achieved with either 1 hour or 1.5-hour incubation in the well plates (n=10 wells). Tubes were activated for 30 minutes as standard incubation time (n=1 tube). B) Heatmap of mast cell activation in the well plate with 1 hour activation (left plate) and 1.5 hours activation (right plate) Colours represent the level of reactivity expressed as $CD63^+$ mast cells. Sensitization (sens); activation (act).

3.4 IgE Affinity Dependent Mast Cell Response in Severe Asthmatics and Healthy Control Subjects

3.4.1 Affinity Dependent Mast Cell Reactivity and Sensitivity

To investigate whether a difference in mast cell reactivity and sensitivity could be established between patients with severe asthma and healthy subjects, mast cells were incubated with either high, intermediate or low affinity IgE for two weeks. Seven patient samples and five control samples were processed in total in this study (Table 5 and 6). The mean age(± SD) of the asthmatic group was 48.75(±16.36) and consisted of two males and five females. No mean age or gender ratio could be reported for the control group, as the majority of the healthy controls originated from anonymous donors from the blood bank, AUH. Stem cell yields at week 0 were similar between the asthmatic group and the control group with median values (minimum and maximum) of 1.10×10^6 (0.75×10^6-1.40×10^6) and 1.14×10^6 (0.73×10^6-3.00×10^6), respectively (p-value=0.32). In addition, the median cell count throughout the cell culturing for the asthmatic group was 8.22×10^6 mast cells (0.6×10^6-20.9×10^6), whilst the control group was 6.72×10^6 mast cells (0.6×10^6-15.5×10^6). Mann Whitney U test indicated no statistical difference in mast cell growth between the groups, p-value=0.80 (Figure 7).

Figure 7: **Mast Cell Growth (0-6 weeks)**

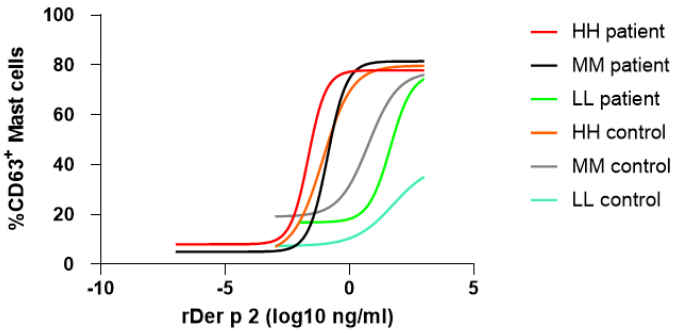


The figure depicts the stem cell yield (week 0) and the subsequent development and growth of mast cells (week 6) for the asthmatic (red curve, n=7) and control group (black curve, n=5) fitted with a four parameter logistic regression. The growth curves are based on the weekly cell count using the trypan blue exclusion method and light microscopy (Leitz Wetzlar, DE). The age of the mast cells was defined as weeks and plotted on the x-axis while the y-axis represents the mast cells stated in millions.

To obtain a dose-response relationship, the $\log_{10}$ of allergen (ng/ml) was plotted against the fraction of $CD63^+$ mast cells (Figure 8). For 1/7 patient samples, and 3/5 control samples, the sensitivity (EC50), could not be established with the maximum allergen concentration of a 1 000 ng/ml for the low affinity IgE condition. Therefore, these were defined as double of the highest allergen concentration and fixed to a EC50 of 2 000 ng/ml.

The Mann Whitney U-test indicated p-values of 0.94, 0.37 and 0.37 for the high, intermediate and low affinity condition between the asthmatic patients and the healthy controls. Thus, no statistical difference was observed in mast cell reactivity when comparing the respective IgE affinities (Table 8). However, the mast cells still appeared to be highly reactive in the asthmatics at the lowest IgE affinity with a mean reactivity(±SE) of 68.84(±17.11)%, whilst the reactivity was slightly lower in the mast cells cultured from the healthy controls with a mean(±SE) of 40.68(±30.77)%. Within-group analysis showed that mast cell sensitivity increased significantly for both the asthmatic and control group with decreasing IgE affinity with p-values of 0.00 and 0.02, respectively. The same was observed for the reactivity of the control group, p-value=0.04 (Table 8 and Figure 9). A 4-, 30- and 1.3-fold difference was observed between the patient- and control groups in sensitivity for

Figure 8: **Dose Response for Asthmatic and Control Groups**



Mast cells were activated with Der p 2 and stained with anti-CD63 antibody. The cells were recorded with flow cytometer (Attune Acoustic Focusing Cytometer, Thermo Fischer) and identified as $CD63^+$ mast cells. The allergen concentration was plotted against the mast cell reactivity and fitted with a 4PL curve for either the asthmatic (n=7) or control (n=5) group. The x-axis was defined as log_{10} allergen concentration of Der p 2, while the y-axis was defined as the percentage of $CD63^+$ mast cells. The red, black and green curve denoted HH patient, MM patient and LL patient represents the mast cells cultured from the asthmatic patients for the high, intermediate and low affinity IgE, respectively. Similarly, the orange, grey and turquoise curves represent the healthy control group. Mast cell reactivity is defined as [max%$CD63^+$], whilst the sensitivity (EC50) is defined as the allergen concentration, which induces 50% of the maximum response. Graphs and data were analysed with Prism v8.4.1 (GraphPad) statistic software. HH was defined as two high affinity IgE-clones (H10 and H12), MM defined as two intermediate IgE-clones (H12:H7 and H8) and LL defined as two low affinity IgE-clones (H7:H12 and H10:H12). Dermatophagoides pteronyssinus (Der p 2); immunoglobulin E (IgE); four parameter logistic (4PL).

the high, intermediate and low affinity condition. However, no statistical difference was observed in sensitivity between the groups with p-values of 0.08, 0.17 and 0.14, respectively (Table 8). Within-groups, the mast cell sensitivity increased approximately 3 790-fold for the asthmatics and 700-fold for the controls from the low to high affinity IgE condition.

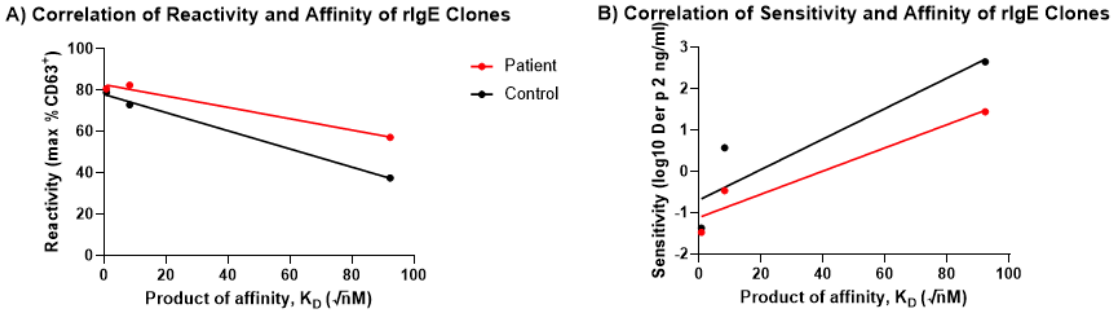
The EC50 was arbitrarily set at 2 000 ng/ml for some of the low affinity IgE cultures, as the half maximum inflection point was indeterminable through the dilution range. Thus, the Mann Whitney U test was repeated with values of 500 ng/ml and 1 000 ng/ml and both showed p-values>0.05. To obtain a 95% power, the required total sample sizes were estimated to be 682, 14 202 and 126 subjects for the high, intermediate and low affinity condition. If affinity was disregarded and patients and controls were compared, the required total sample population was 284 subjects, in which neither are obtainable sample sizes.

Table 8: **IgE Affinity Dependent Mast Cell Reactivity and Sensitivity**

Affinity	Mast cell reactivity [% of CD63+ cells]			Mast cell sensitivity [ng/ml]		
	Patient (n=7)	Control (n=5)	P-value	Patient (n=7)	Control (n=5)	P-value
High (HH)	77.43 (±1.61)	79.77 (±4.01)	0.94	0.02 (±0.08)	0.08 (±0.39)	0.08
Intermediate (MM)	77.39 (±2.76)	77.38 (±9.61)	0.37	0.19 (± 0.14)	5.75 (±0.37)	0.17
Low (LL)	68.84 (±17.11)	40.68 (±30.77)	0.37	75.83* (±0.45)	56.68* (± 1.66)	0.14
P-value	0.25	0.04		0.00	0.02	

For the three IgE affinities investigated, mast cell reactivity has been stated as the mean of the CD63$^+$ mast cells(±SE) for either the seven cultures from the asthmatic group or the five cultures from the healthy control group. The sensitivity of the mast cell cultures has been stated in ng/ml(±SE) for each group. Fields with a mark indicate that sensitivity has been defined as double of the highest concentration (2 000 ng/ml) for one out of seven patient samples and three out of five control samples. Immunoglobulin E (IgE); standard error (SE).

Figure 9: **Correlation between Affinity of rIgE Clones and Reactivity and Sensitivity**



Correlation of the product of IgE affinities (high, intermediate and low) in nM for the asthmatic (red) or control group (black) with mast cell reactivity (A) and sensitivity (EC50) (B). The patient and control population both indicate that an increased IgE affinity was associated with an increased mast cell reactivity and decreased sensitivity. Immunoglobulin E (IgE).

3.4.2 Evaluation of the Late Phase Response

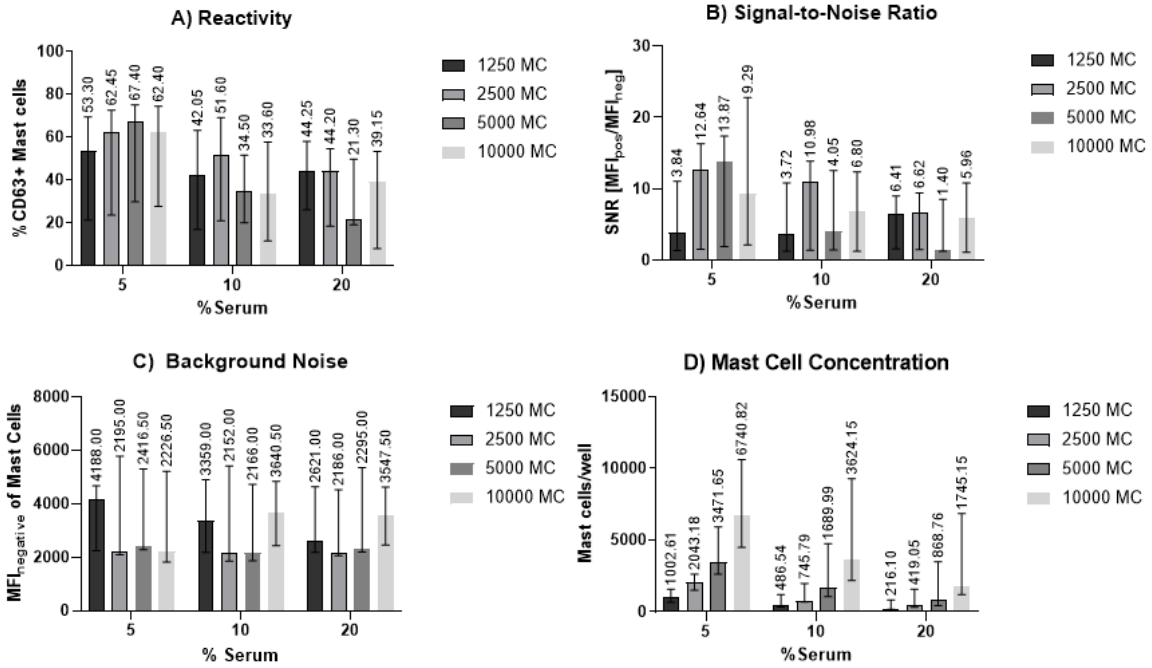
To investigate whether IgE affinity or donor origin changed the cellular mediators released during mast cell degranulation, mast cell cultures were activated for 3, 6 and 24 hours and compared to their baseline sample to monitor the release of cytokines and eicosanoids. However, these external analyses have yet to be performed, thus no results can yet be reported.

3.5 The Predictive Value of Mast- and Basophil Activation Tests in Allergen Immunotherapy

3.5.1 Optimization of Passive Sensitization and Activation in Human Mast Cells with Patient Sera

A pilot study was executed to obtain the most favourable conditions for mast cell sensitization with human sera as no standardized method was described at the laboratory. The concentration of mast cells and the serum fraction needed to elicit a reliable cellular response detected by flow cytometry required further investigation, as these elements are two key components in a MAT. Furthermore, the duration of human serum exposure was explored on mast cells to minimize possible toxicity and background noise. Our previous data indicated that mast cells sensitized for two days showed higher reactivity when activated with anti-FcεRI and a higher SNR than those sensitized for one or three days. Thus, the experimental setup in this pilot study was based on these data.

Figure 10: **Mast Cells in Different Cell Concentrations and Serum Fractions**



Mast cells (MC) from three cultures were incubated with 5%, 10% or 20% human serum with 1 250, 2 500, 5 000 or 10 000 mast cells/100 µl for two days. Mast cells were activated with diluted concentrations of grass allergen (dissolved Grazax tablet) in duplicates and stained with anti-CD63 antibody for recording with flow cytometry (Attune NxT AutoSampler, Thermo Fischer). Mast cells were gated based on high granularity and size in a FSC/SSC cytogram. The bar charts depict the median of three cultures and error bars as range. A) Reactivity was measured as maximum %CD63^{+} mast cells. B) SNR. C) Background noise defined as MFI in the negative samples [MFI$_{negative}$] of gated mast cell. D) Mast cell count per well (610 µl), which is calculated by event counts in the mast cell gate. Graphs and data were analysed with Prism v.8.4.1 (GraphPad). Median fluorescence intensity (MFI); signal-to-noise ratio (SNR).

Mast cells from three different cultures (3519A, 3819A and 4219B) were incubated according to section 2.7.1 with various serum fractions (5%, 10% or 20%) and cell concentrations of 1 250, 2 500, 5 000 or 10 000 mast cells/100 µl. Allergen concentrations were based on our previous data or a dilution curve specific for the culture 4219B using the dissolved SLIT tablet for grass allergy,

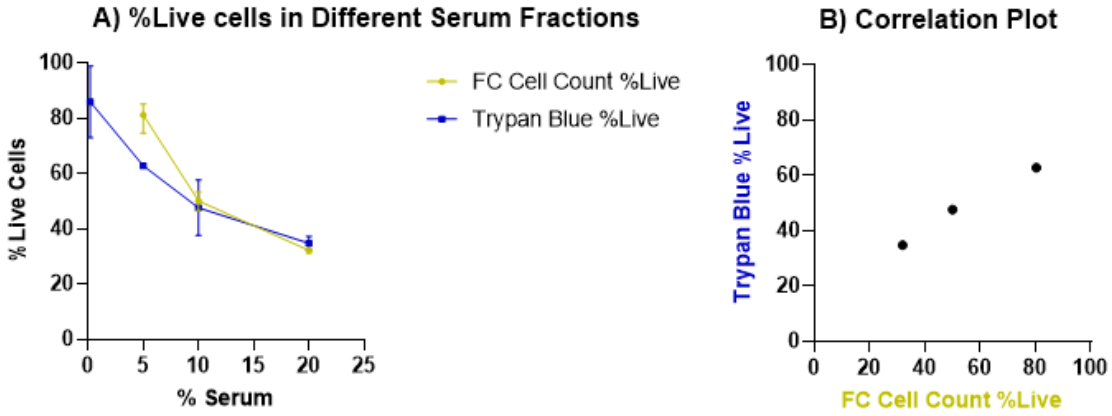
Grazax. Reactivity was measured according to section 2.4.1. The maximum cell reactivity for grass was measured at the highest allergen concentration at 60.61 mDDE/ml in 3519A and 3819A and at 0.061 μDDE/ml in 4219B. Mast cell concentrations calculated by flow cytometer event count revealed a mean(±SD) of 80.37(±5.63)% of original seeded mast cells.

Reactivity was highest at 5% serum across all cell concentrations in all three cultures, Figure 10A). SNR [MFI_{pos}/MFI_{neg}] was highest at 5% serum across all cell concentrations in the three cultures. In 2/3 cultures, the samples containing "10 000" and "5 000" mast cells had highest SNR (Figure 10B). Background noise [MFI_{neg}] was similar across serum concentrations and did not seem to follow any tendencies based on cell concentrations (Figure 10C). Conditions containing 5 000-10 000 mast cells/100μl at 5% sera were the most favourable and can be used in a MAT with human serum sensitized mast cells.

3.5.2 Viability of Mast Cells Incubated with Human Serum

Event counts of mast cells recorded with flow cytometry seemed to fall drastically when incubated with higher concentrations of human serum (10% or 20% serum) as seen in Figure 10D. Mast cell counts (n=3 cultures) were reduced with a mean(±SD) of 42.08(±17.14)% from 5% to 10% serum and 65.03(±18.18)% from 5% to 20% serum.

Figure 11: **Mast Cells Viability in Different Serum Fractions**



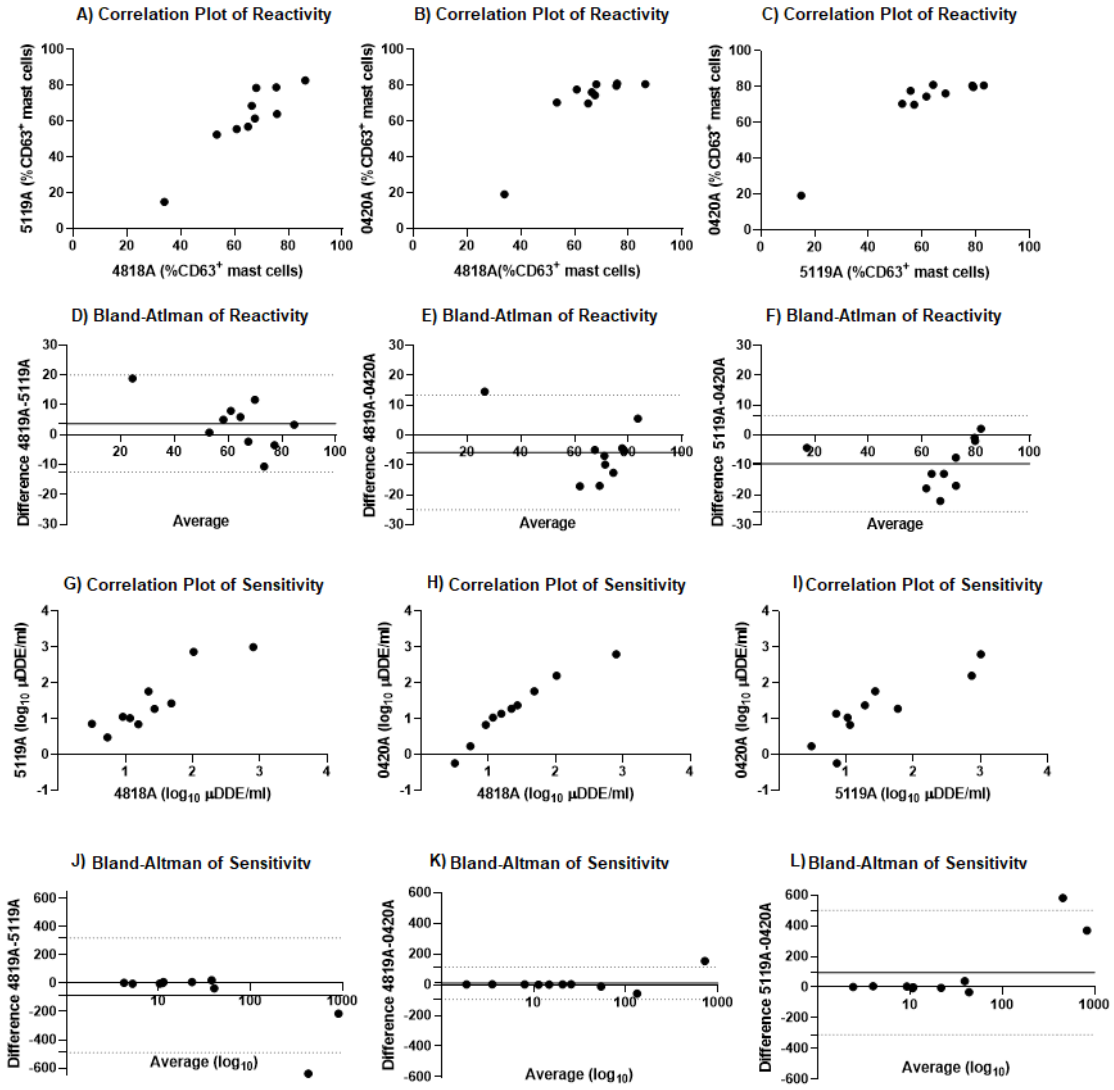
A) Blue graph depicts mast cells from two cultures were incubated with 0%, 5%, 10% or 20% human serum at a concentration of approximately 300 000 cells/ml for two days. Dead and live mast cells were counted in a hemocytometer using the trypan blue exclusion method with a light microscope (Leitz Wetzlar, DE). The percentage of live cells was calculated from total cell count. Yellow graph depicts the mast cells from three different cultures incubated with 5%, 10% or 20% human serum at for two days. They were counted with a flow cytometer (FC) (Attune Acoustic Focusing Cytometer) and % live cells were calculated by recorded cell count. The graph shows the mean value of the original seeded. The graphs represent the mean values and standard deviation as error bars. B) Correlation plot of % live cells with the trypan blue and flow cytometer count at three measurements (5%, 10% or 20% human serum).

Viability of mast cells incubated with 0%, 5%, 10% and 20% human serum for two days was then evaluated in two cultures (3819A and 4719B) with the trypan blue exclusion method. A clear tendency was observed, in which data indicated that the higher concentrations of serum used, the lower the fraction of live cells was observed (Figure 11). The mast cell count from flow cytometer analysis was compared to the trypan blue viability measurements to see whether they correlated. Cell counts from the previous experiment (section 3.5.1) with 5%, 10% and 20% human serum was accumulated and converted to [%live cells] based on the fraction of original seeded mast cells. Data

showed a non-significant correlation of ρ=1.000, p=0.167. The data suggest a clear tendency with perfect correlation, but the samples size may be too small to be significant.

3.5.3 Reproducibility of Mast Cell Activation Assay

Figure 12: **Reproducibility of MAT with Grass Allergen**

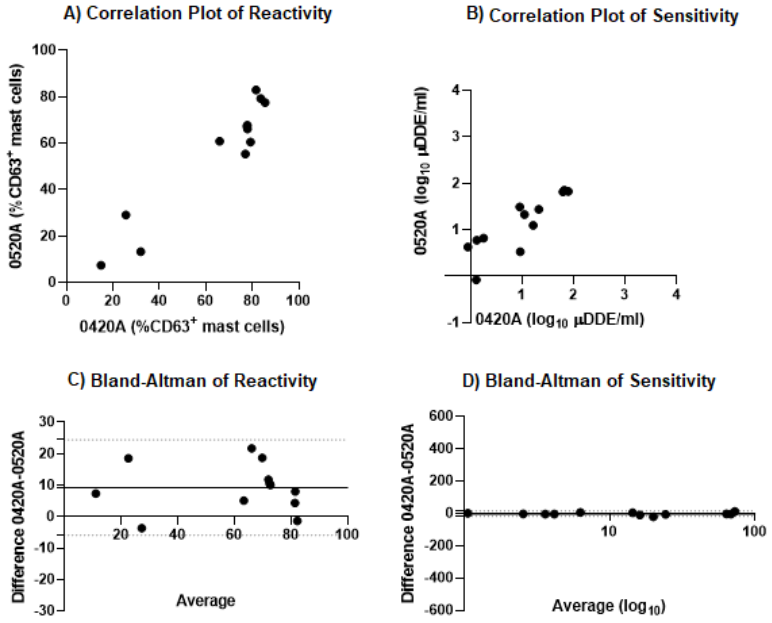


MAT performed for three different buffy coat-derived mast cell cultures sensitized with sera from grass-dependent allergic subjects (n=10). Mast cells were activated with grass allergen (dissolved Grazax tablet) in polypropylene 96-well plates for 1 hour. The cells were stained with anti-CD63 antibody and recorded with flow cytometry. A-C) Correlation plot for reactivity [max%CD63$^+$]. D-F) Bland Altman plots for reactivity. G-I) Correlation plot for sensitivity (EC50) [log$_{10}$ µDDE/ml]. J-L) Bland Altman plots for sensitivity. Bland Altman plots were made as difference vs average. Dotted grid lines indicate upper and lower 95% limits of agreement and the solid grid line represents the mean difference (bias). Graphs and data were analysed with Prism v8.4.1(GraphPad) statistical software. Mast cell activation test (MAT).

The MAT was tested for reproducibility in five mast cell cultures. The assay was validated with sera (n=10) from grass allergic patients in three cultures and with plasma or sera of birch-allergic patients (n=12) in two cultures.

Reactivity of the three cultures activated with grass allergen (n=10) correlated, ranging from R^2=0.825, ρ=0.758 to R^2=0.856, ρ=0.903 ($p \leq 0.015$). There was significant difference between the measurements with a paired Wilcoxon signed rank test, but reactivity varies slightly from day to day, which could be confirmed in positive controls for mast cell cultures, as described in section 3.2. There was no trend in a Bland-Altman analysis and <10% in difference and data was spread around the mean difference (bias) line. The median value from three cultures had a mean difference(±SD) of -5.82(±9.80)% $CD63^+$ for reactivity (Figure 12D-F). The mean difference was considered low without clinical relevance.

Figure 13: **Reproducibility of MAT with Birch Allergen**



MAT performed from two different buffy coat-derived mast cells sensitized with plasma or sera from birch-dependent allergic subjects (n=12). Mast cells were activated with birch allergen (dissolved Itulazax tablet) in polystyrene tubes for 30 minutes. The cells were stained with anti-CD63 antibody and recorded with flow cytometry. A-B) Correlation plot for reactivity [max%$CD63^+$] (A) and sensitivity (EC50) [log_{10} μDDE/ml] (B). C-D) Bland Altman plots for reactivity (C) and sensitivity (D) (n=10). Bland Altman plots were made as difference vs average. Dotted grid lines indicate upper and lower 95% limits of agreement and the solid grid line represents the mean difference (bias). Graphs and data was analysed with Prism v8.4.1(GraphPad) statistical software. Mast cell activation test (MAT).

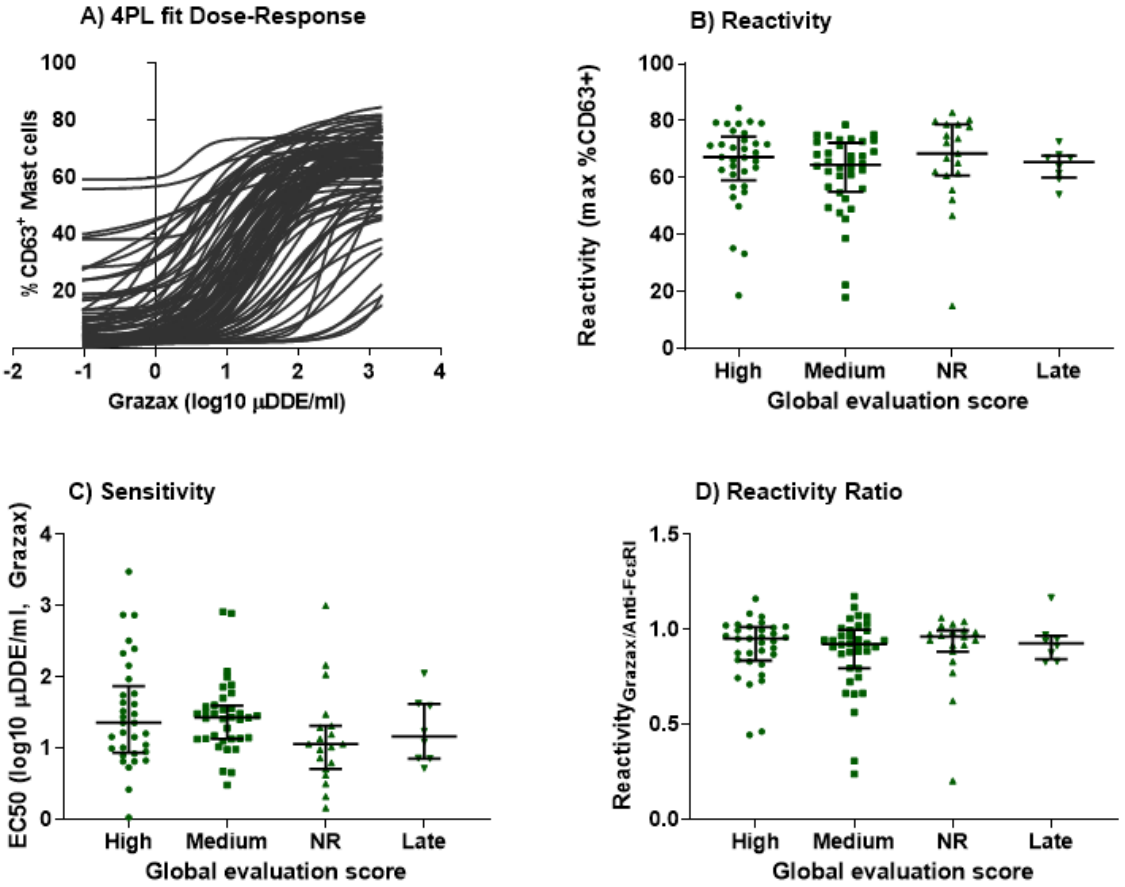
Sensitivity was strongly correlated, R^2=0.757, ρ=0.855 to R^2=0.940, ρ=1.000 ($p \leq 0.001$) and there was no significant difference between the measurements (Wilcoxon paired signed rank test) ($p>0.3$). There was very good agreement with no obvious trends in the Bland Altman analysis for sensitivity. Correlation and Bland Altman plots can be assessed in Figure 12G-L. The median value from three cultures had a mean difference(±SD) of -86(±207) μDDE/ml. The mean difference was low with no clinical meaning, but the limits of agreement were wide for two of the tests due to two extreme values (Figure12J and L). This may indicate a weakness in the MAT, where sensitivity was more difficult to determine at higher allergen concentrations.

Similar tendency in data was found in the two mast cell cultures sensitised with birch pollen

plasma (n=9) and sera (n=3). The data was very strongly correlated of R^2=0.908, ρ=0.840 ($p \leq 0.001$) for reactivity and R^2=0.912, ρ=0.860 ($p \leq 0.001$) for sensitivity. The mean difference ($\pm$SD) of reactivity was 9.28% ($\pm$7.76) and -3.00 ($\pm$9.00) μDDE/ml for sensitivity. The difference in reactivity and sensitivity were not of clinical relevance. This shows that the data was very well reproduced and represented in a MAT with passive sensitization. Correlation and Bland-Altman plots can be assessed in Figure 13.

3.5.4 Predictive Value of Mast Cell Activation Test in Grazax Treatment

Figure 14: **GT-08 Analysis**



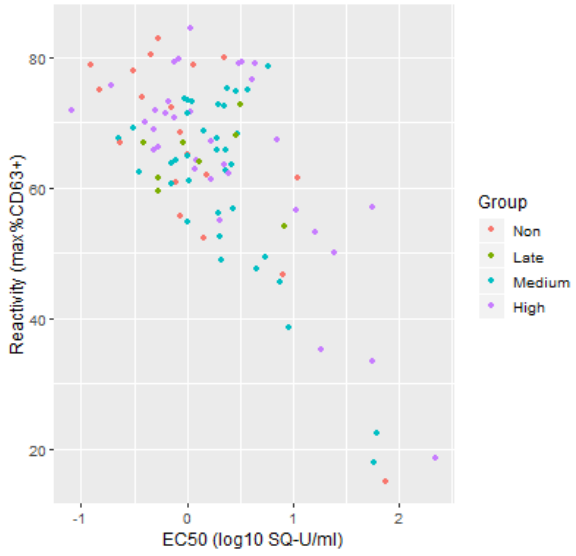
A) Dose response curve fitted with a 4PL curve for MAT with pre-AIT serum derived IgE (n=96). Mast cells were sensitized with 5% serum for two days. Mast cells were activated with Grazax dissolved in PBS and serially diluted 1:4 and stained with anti-CD63 antibody. The cells were recorded with flow cytometer (Attune Acoustic Focusing Cytometer, Thermo Fischer) and identified as %CD63$^+$ mast cells. A) Dose response of patient responses, B-D) Reactivity [max %CD63$^+$] (B), sensitivity [log_{10} μDDE/ml] (C) and reactivity ratio of activation with Grazax/anti-FcϵRI (D) in the four global evaluation groups (high, medium, NR and late). (n=96); n_{high}=33, n_{medium}=36, n_{NR}=19, n_{late}=8. Crossbar and error bars contain median and IQR. No significant difference was found between groups in B-D. Graphs were created in GraphPad Prism v8.4.1 statistic software. Four parameters logistic (4PL); MAT; allergen immunotherapy (AIT); phosphate buffered saline (PBS); daily dose equivalent (DDE); non-reactive (NR); inter quartile range (IQR).

The MAT was evaluated for the ability to screen serum from patients within indication for AIT and predict possible response. Mast cells were sensitized with pre-AIT sera-derived IgE with response groups made for this project based on the GT-08 study with "high", "medium", "NR" and "late" responders. Reactivity and sensitivity in mast cells towards treatment preparations of Grazax® freeze-dried tablet were measured using the baseline sera.

Reactivity and sensitivity in mast cells were calculated as described in section 2.8.1. Patient samples (n=96) were analysed on one buffy coat derived mast cell culture (5119A) at seven weeks old. Dose-response curve can be seen in Figure 14A displaying a wide variation in the patient responses. Median (IQR) reactivity and sensitivity for all samples were 66.60 (57.65-73.10)% $CD63^+$ and 19.34 (9.63-42.44) μDDE/ml, respectively.

Reactivity and sensitivity were correlated, R^2=0.486, ρ=-0.449 (p<0.0001) and there was a tendency of sensitivity decreasing as reactivity increases, which can be observed in Figure 15.

Figure 15: **Reactivity with Sensitivity Measurements in Groups**



Reactivity [max %$CD63^+$] and sensitivity [EC50 log_{10} SQ-U/ml] plotted with colour coded groups of global evaluation scores (high, medium, non-reactive (non) and late) to identify clusters. Plot was created with BlueSky Statistics v6.30. Specific quality-Units (SQ-U).

The plot also identified a cluster of data points in the upper left quadrant, not reflecting a correlating relationship between reactivity and sensitivity. This can be explained by dose-response curves having the same reactivity but different sensitivity, as other factors as antibody affinity also affects the response [6], as demonstrated in Figure 9.

Median (IQR) reactivity [max %$CD63^+$] for "high"; "medium"; "NR"; "late" group were 67.30 (59.10-74.45)%; 64.50 (55.13-72.30)%; 68.50 (60.80-78.80)%; 65.55 (60.10-67.83)%, respectively. Results can be assessed in Figure 14B. Reactivity presented with similar values across the groups and a Kruskal-Wallis analysis confirmed that no statistical significance could be observed (p=0.325).

Median (IQR) sensitivity for "high"; "medium"; "NR"; "late" group were 22.77 (8.69-76.20); 27.02 (13.58-39.79); 11.45 (5.10-20.73); 14,82 (7.17-41.75) μDDE/ml, respectively. Results can be assessed in Figure 14C. A Kruskal-Wallis analysis showed no statistical significance in difference between sensitivity in any group (p=0.125).

To examine further aspects, the maximum reactivity potential was compared to the global evaluation groups. The maximum reactivity potential was measured as the ratio of reactivity from Grazax activated mast cells and reactivity of positive controls (patient specific samples activated

with anti-FcϵRI antibody)) in Figure 14D. No significant difference was found in a Kruskal-Wallis analysis (p=0.832).

The data sets for reactivity and sensitivity were plotted against each other in colour coding for the global evaluation groups to visualize any clustering tendencies (Figure 15). Results showed no obvious clustering with the different groups being highly dispersed.

3.5.5 Extended Analyses

The 4PL dose-response curves in Figure 14A were overall well fitted based on R^2 and 95% confidence interval values. However, some of the patient responses exhibited curves without a plateau at higher concentrations, in which max reactivity was not representative (1) or were highly reactive with >10% $CD63^+$ at lowest allergen concentration (2). The rest had full dose-response curves with plateaus. To explore if the reactivity and sensitivity from these responses were contributing to noise in the data, the curves were excluded based on feature (1) and (2). Exclusion was done prior to pairing with the global evaluation groups to maintain blinded data. From exclusion criteria (1) n=10 were identified, leaving n=86 for analysis (group A1). From exclusion criteria (2) n=20 were identified, leaving n=76 for analysis (group B1). The data was paired with the global evaluation groups. A Kruskal-Wallis test was carried out with reactivity and sensitivity. No significant difference was found in reactivity in the global evaluation groups. Sensitivity was significantly different. In order to identify where the difference was, a Dunn's multiple comparison analysis was used as non-parametric post hoc test for Kruskal-Wallis. The difference was found between medium and NR group in data group A1 (p=0.008), indicating that the NR was more sensitive.

It was also evident in Figure 14A that most of the data was centred around the middle part. Another approach on data analysis was to include data previously excluded above and use weighted data to have a more evenly distribution of dose-responses. Data within sensitivity values of 0.72-1.97 $\log_{10}$ μDDE/ml, which dominated the center, were identified (n=61) blinded from the global evaluation groups. These were randomized and divided into three groups with the rest of the data, creating three new data groups of A2 (n=56), B2 (n=55) and C2 (n=55). Kruskal-Wallis analysis did not show any significant difference between the global evaluation groups in reactivity or sensitivity in any of the weighted groups (A2, B2, C2).

To investigate whether patient derived mast cells were better at discriminating between global evaluation groups, than buffy coat derived mast cells, reactivity and sensitivity from n=13 patient samples were measured against groups from the 0220A mast cell culture (Table 5). Mast cells with allergic asthma phenotype were not able to discriminate significantly between groups (p=0.274 for reactivity and p=0.821 for sensitivity) with a Kruskal-Wallis analysis.

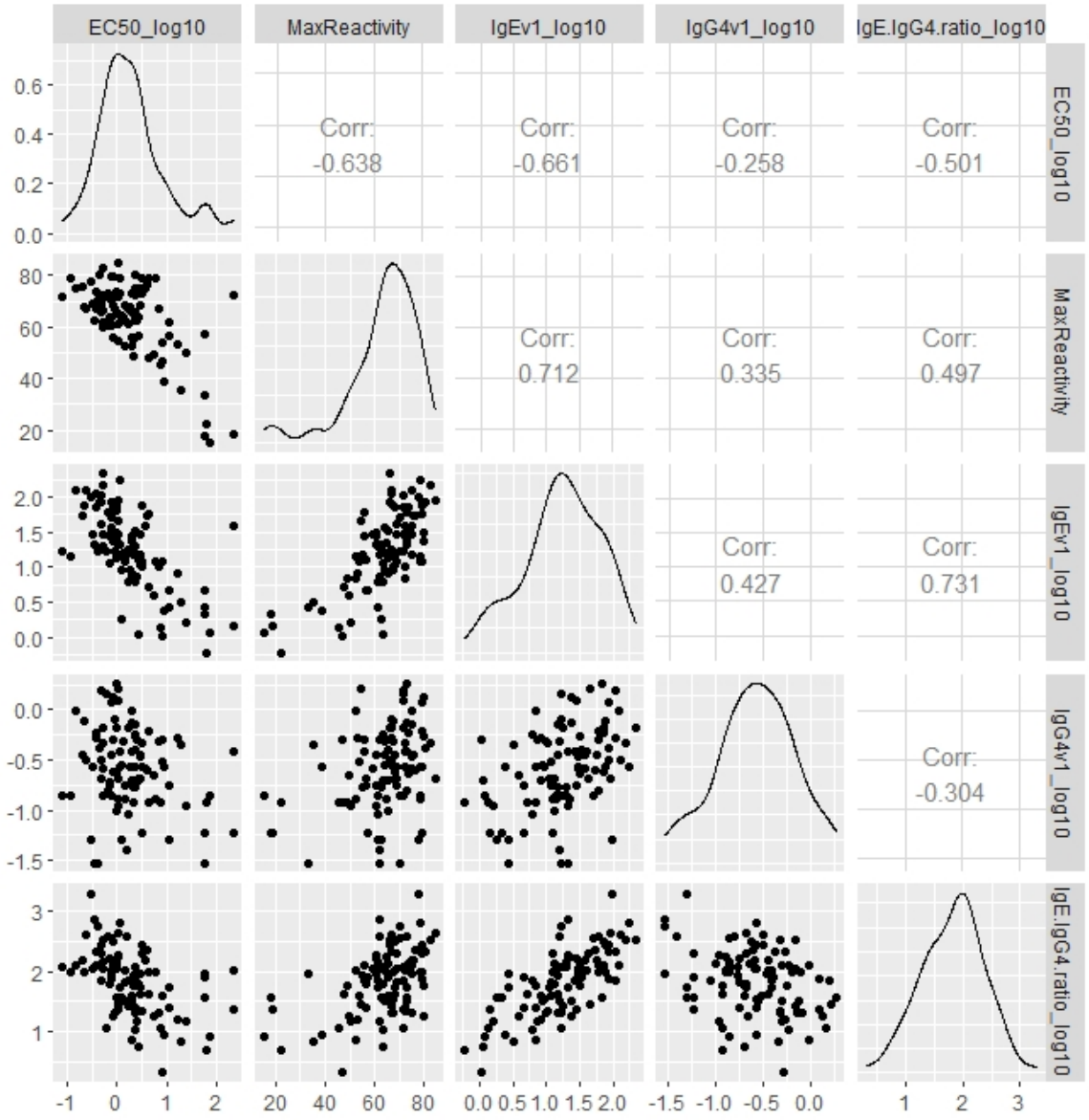
3.5.6 External Analysis by ALK-Abelló on Humoral Markers in GT-08

External analysis was carried out by ALK-Abelló to investigate whether humoral markers used in AIT, such as sIgE, sIgG4 and sIgE/sIgG4 ratio at baseline levels were correlated with reactivity and sensitivity from the mast cell data. Further analysis was made to examine the value of sIgE and sIgG4 as a predictive marker for AIT effect. All data was disclosed by ALK-Abelló to this study as screen prints from statistical software outputs as the original data contained sensitive information.

Reactivity and sensitivity were both moderately correlated to baseline sIgE, r=0.712 (p<0.001) and r=-0.661 (p<0.001), respectively. Baseline sIgG4 also showed a weaker correlation to reactivity and sensitivity, r=0.335 (p<0.001) and r=-0.258 (p=0.011) respectively. sIgE/ sIgG4 ratio was correlated to reactivity and sensitivity of r=0.497 (p<0.001) and r=-0.501 (p<0.001), respectively. Based on the correlation plots and values, it did seem that the sIgE/ sIgG4 ratio correlation to mast cell responses, was mainly affected by the sIgE. Correlation plots with Pearson's correlation coefficient can be assessed in Figure 16.

The analysis on data from group A1, section 3.5.5, was also interpreted and analysed with a linear model. A significant difference in sensitivity in the medium group compared to NR was

Figure 16: **Correlation Analysis of MAT Data and sIgE/sIgG4**



Data analysis from ALK-Abelló, DK. Correlations analyses and distribution of data over reactivity [max %CD63$^+$], sensitivity (EC50) [log_{10}SQ-U/ml, Grazax], baseline sIgE [log_{10}kU/l], sIgG4 [log_{10}mgA/l] levels and sIgE/sIgG4 [log_{10}kU/l] ratio. sIgEv1 and sIgG4v1 indicate baseline levels (=visit 1). Curves from top left and down to right corner show distribution of data. On the left sides are 10 correlations plots with Pearson's correlation coefficient on the right. Analysis was made with R statistical software. Specific quality-units (SQ-U).

found, p=0.045. The multiple R^2 value for the whole model was R^2=0.064, and the low value indicate data may not be well fitted in the model.

Baseline sIgE and sIgG4 levels in the effect groups of global evaluation scores were statistically analysed using a linear model with log_{10} transformed values with R statistical software. The high, medium and late groups were compared to the NR group. Baseline sIgE was only significantly different from NR in the medium group (p=0.034), but with multiple R^2=0.049, meaning <5% of

the variation can be explained in the model. Baseline sIgG4 did not show any significant difference between groups ($p>0.05$), multiple $R^2=0.015$. This was in line with sIgG4 being induced after AIT treatment start [39].

TNSS were analysed to examine whether they were better in predicting effects of the global evaluation groups. The NR group had significantly higher TNSS than the medium ($p=0.04$) and high global evaluation group ($p=0.007$), though the model could only account for 8.4% in the variation, multiple $R^2=0.084$. Beside the poor statistical fit, TNSS was better defined between NR group from the higher effect groups among other possible end points as eye symptoms, medication scores and combined scores. TNSS were furthermore investigated to see if it could explain the baseline parameters as reactivity, sensitivity ($\log_{10}$ transformed EC50), sIgE and sIgG4. All results across the different parameters showed non-significant ($p>0.05$) differences between comparisons and the model could only explain <8% of the variation (multiple $R^2=0.079$).

3.6 Predictive Value of Basophil Activation Test in Itulazax Treatment

Basophils were investigated for the ability to screen patients within indication for AIT and predict possible treatment effect. To investigate this hypothesis, basophils from pre-AIT hemolyzed whole blood were used in a BAT. Reactivity and sensitivity in basophils towards treatment preparations of Itulazax freeze-dried tablet were measured using the whole blood from baseline according to section 2.9.3. As Itulazax is the newest SLIT tablet from ALK-Abelló A/S (DK) and was launched in the fall 2019, the treatment effect was also recorded and evaluated.

Table 9: **Clinical overview of Patient Population for SLIT Therapy with Itulazax**

Patient ID	Age/gender	SPT	sIgE (kU/l)	Total IgE (kU/l)	BAT-responder	RQLQ-score (Reduction %)
1	29/male	NA	t3 (47.9), d1 (6.9), g6 (42.5) w6 (2.45), e5 (2.07)	196	+	53.49
2	73/male	NA	t3 (1.51), d1 (10.2), g6 (5.3)	1808	+	52.38
3	38/female	+	t3 (63.1), g6 (21.4), e1 (2.23)	209	+	-11.25
4	24/male	+	t3 (50.2), d1 (1.12), e1 (2.02)	53	+	51.82
7	32/male	NA	t3 (20.5)	373	+	77.23
10	48/female	+	t3 (14.9), g6 (14.7)	63	+	76.12
12	71/male	+	t3 (2.01), d1 (2.00), g6 (0.80)	139	-	6.45

A total of 12 patients participated in this experiment, in which seven subjects completed the study. SPT for birch allergen was confirmed for four patients. sIgE was only specified for RAST class ≥2 (≥0.71). Validity of BAT was denoted with plus sign for a valid BAT result, in which $CD63^+$ >20% with anti-IgE or negative sign if $CD63^+$ <20%. RQLQ-score was measured by reduction in symptoms based on RQLQ-symptom scores measured at baseline visit and at least four month later in the beginning of the birch pollen season of 2020. Positive values represent a reduction in symptoms and negative values denotes a worsening in symptoms. Basophil activation test (BAT); skin prick test (SPT); specific IgE (sIgE); radioallergosorbent test (RAST); House dust mite (d1); cat (e1); cat (e5); grass (g6); birch (t3); not available (NA).

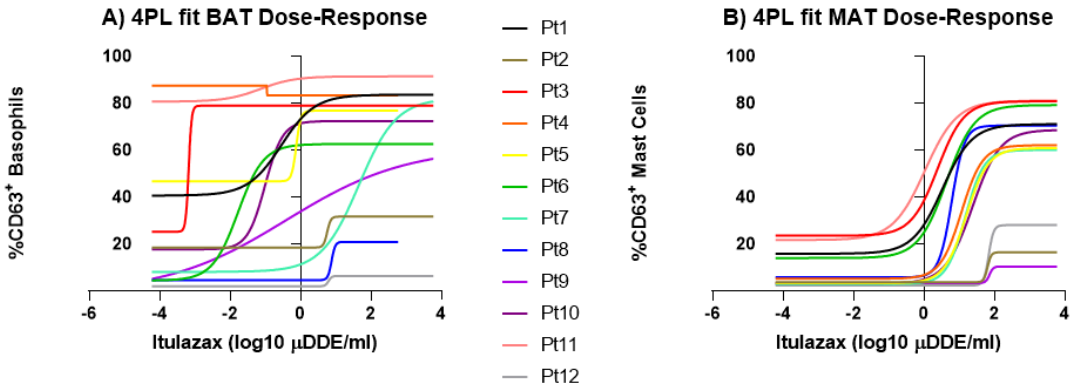
A total of 12 patients with birch dependent allergic rhinitis were recruited. The sample population consisted of 2/3 male and 1/3 female with a mean age of 42.41 years. Patient 2 started SLIT therapy for grass dependent allergy after enrolling this study. Patient 3 received SLIT therapy for grass dependent allergy and patient 12 received SLIT therapy for HDM dependent allergy.

Three patients were excluded due to incomplete treatment after the follow-up period. Two of the patients experienced heavy side-effects and were taken of the SLIT therapy. One patient had other medical complications, which interfered with the SLIT therapy and was also excluded. Two of the patients could not be reached, leaving seven respondents (Table 9). Of these 71.43% were male and the average age was 45.29 years.

3.6.1 BAT and MAT

Patient 8 and 12 were categorized as non-responders or had an inconclusive result as their positive controls with anti-IgE were <20% $CD63^+$. For patient 4 and 11, sensitivity could not be established with the lowest allergen concentration of 0.0606 nDDE/ml. Therefore, these were defined as half as half of the lowest concentration and fixed to a (EC50) of 0.0303 nDDE/ml.

Figure 17: **Dose Response for Itulazax with a BAT and MAT**



A) Dose response curves for BAT fitted with a 4PL curve with pre-AIT whole blood (n=12). Samples were activated with dissolved Itulazax freeze dried tablet serially diluted 1:10 in PBS. Basophils were stained with anti-CD123, anti-CD193 and anti-CD63 antibody in hemolyzed whole blood and recorded with flow cytometer (BD FACS CantoII, BD Bioscience). Cells were identified as $CD123^+/CD193^+$ $\%CD63^+$ basophils. B) Dose response curves, mean values of two buffy coat derived mast cells sensitized with 5% pre-AIT plasma (n=10) or serum (n=2) for two days, were fitted with a 4PL curve. Serum samples were used for patient 11 and 12. Samples were activated with dissolved Itulazax freeze dried tablet serially diluted 1:10 in PBS and cells were stained with anti-CD63 antibody. Cells were recorded with flow cytometer and identified as $\%CD63^+$ mast cells. The x-axes were defined as the log_{10} allergen concentration (μDDE/ml). Allergen immunotherapy (AIT); daily dose equivalent (DDE).

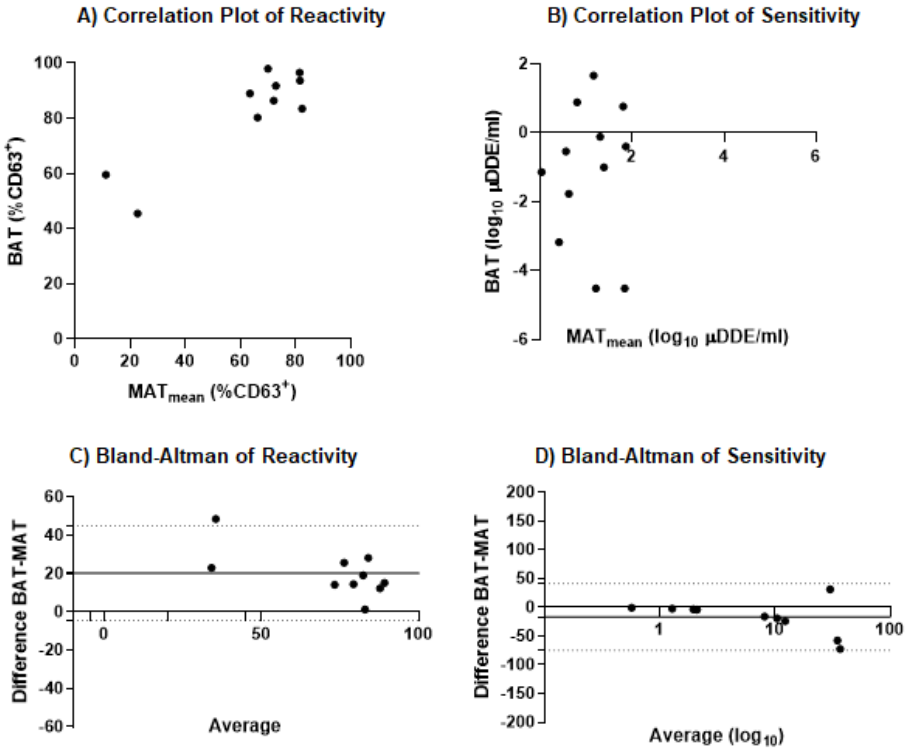
A dose response curve can be seen in Figure 17A. Patient 2 and 5 had an ambiguous 4PL slope and 95%CI could not be completely calculated in patient 2-6, 9-11. These values should be interpreted carefully. Median (IQR) reactivity and sensitivity in the valid sample population (n=10) were 84.97 (75.13-94.43) 0.19 (0.01-2.00) μDDE/ml respectively. Reactivity and sensitivity [$\log_{10}$ μDDE/ml] had a low non-significant correlation, R^2=0.002, ρ=-0.403 (p=0.194).

To investigate the performance of BAT (n=10) and MAT (n=10), the reactivity and sensitivity of either cell types were compared against one another (Figure 17). Data from two patient samples were excluded as they were categorized as non-responders in the BAT. MAT were performed in two mast cell cultures (0420A and 0520A). The mean results from the two cultures were used in the statistical analysis. Mast cell $reactivity_{mean}$ and $sensitivity_{mean}$ (n=12) had a median (IQR) of 70.93 (36.31-79.24)% $CD63^+$ and 15.32 (3.77-53.83) μDDE/ml, respectively. Reactivity and sensitivity [$\log_{10}$ μDDE/ml] were strongly correlated, R^2=0.725, ρ-0.895 (p=0.0002).

MAT in patient 2, 9 and 12 had an ambiguous 4PL slope and a 95% CI could not be completely calculated. Reactivity (n=10) was compared between BAT and the mean value of two MATs performed in two different mast cell cultures (0420A and 0520A). A Wilcoxon signed rank test with matched pairs showed significant difference between reactivity in the BAT and the mean MAT reactivity, p=0.002. Pairing of reactivity were moderately correlated, R^2=0.789, ρ=0.539 (p=0.114), but there was a mean difference(±SD) in BAT and MAT of 20.15 (±12.56)% $CD63^+$. A Bland Altman plot showed no trend, but a high bias where the data points were scattered, especially patient 9 had a

very different test result with a different reactivity and dose-response curve. Sensitivity (n=10) was compared in the same way as described above. There was significant difference between BAT and the MAT measurements, p=0.049. There was no correlation between the assays, R^2=0.0003, ρ=0.119 (p=0.711). Mean difference(±SD) in BAT and MAT was -17.00(±29.70) μDDE/ml. Correlation and Bland-Altman plots can be assessed in Figure 18A-D.

Figure 18: **Correlation and Bland Altman Plots for BAT and MAT**



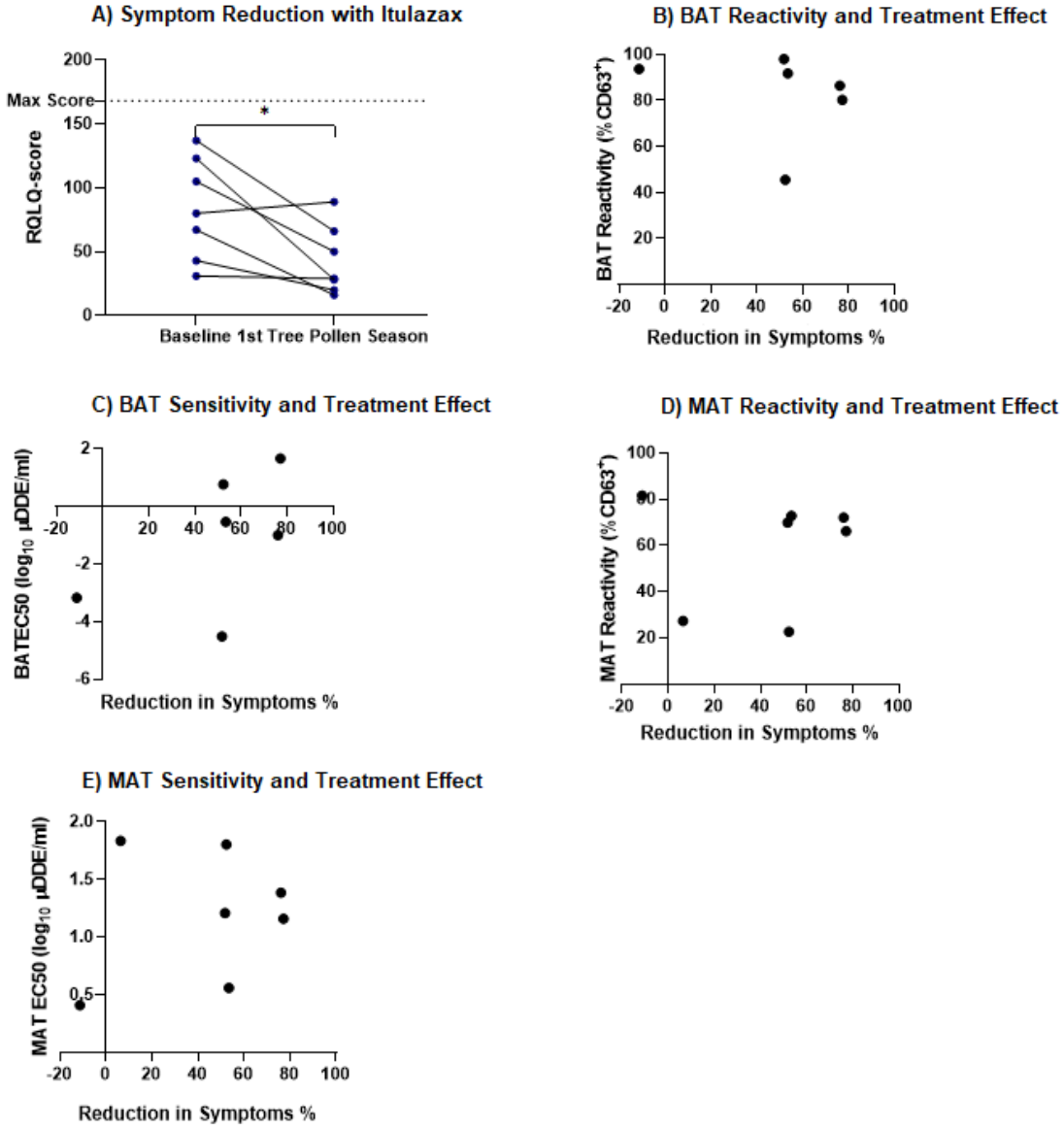
A-B) Correlation plot for reactivity [max%CD63$^+$] (A) and sensitivity (EC50) [$\log_{10}$ μDDE/ml] (B) in BAT and MAT measures (n=10). C-D) Bland Altman plots of reactivity (C) and sensitivity (D) (n=10). Bland Altman plots were made as difference (BAT-MAT) vs average (BAT+MAT/2) with either reactivity or sensitivity. Dotted grid lines indicate upper and lower 95% limits of agreement and the solid grid line the mean difference (bias). It should be noted that data was not normally distributed. Graphs and data were analysed with Prism v8.4.1(GraphPad) statistical software. Basophil activation test (BAT); mast cell activation test (MAT).

3.6.2 Predictive Value of Activation Assay-Score and RQLQ-Score

The change in RQLQ-score from baseline to the first tree pollen season (n=7) was significantly reduced with a mean(±SD) of -49.14%(±32.40), p=0.027. The mean difference(±SD) of the RQLQ was -41.14(±37.54) (Figure 19A). One patient did not change, and another had a worsening in symptoms. BAT reactivity and sensitivity at baseline levels (n=6) did not correlate significantly with treatment effect, ρ=-0.600 (p=0.242) and ρ=0.714 (p=0.136).

1/7 of the final participants was considered a non-responder. As MAT has the advantage of eliminating non-responders, the treatment effect was further evaluated with baseline MAT-values. MAT reactivity and sensitivity at baseline levels (n=7) was neither successful in identifying any trends. There was no significant correlation with treatment effect, ρ=-0.00 (p>0.999) and ρ=-0.00 (p>0.999), respectively. Cut-off values for predictive effect were not possible to define with

Figure 19: **Correlation Analysis of Activation Assays and Treatment Effect**



Seven patients with birch-pollen dependent allergic rhinitis received SLIT treatment with Itulazax. A) Change in RQLQ-symptom scores at baseline visit outside pollen season and the beginning of tree-pollen season of 2020 at least four months after baseline (n=7). Tree-pollen season was defined for birch as ≥30 grains/m^3. B) Correlation of baseline BAT reactivity [max %CD63$^+$] and treatment effect (n=6). C) Correlation of baseline BAT sensitivity (EC50), log$_{10}$ µDDE/ml] and treatment effect (n=6). D) Correlation of baseline MAT reactivity [max %CD63$^+$] and treatment effect (n=7). E) Correlation of baseline MAT sensitivity (EC50) [log$_{10}$ µDDE/ml] and treatment effect (n=7). Treatment effect was measured by reduction in symptoms based on RQLQ-symptom scores where positive values are a reduction in symptoms and negative values are a worsening in symptoms. Analysis was made with Prism Graphpad v. 8.4.1 statistical software. Basophil activation test (BAT); daily dose equivalent (DDE); mast cell activation test (MAT); rhinitis quality of life questionnaire (RQLQ); sublingual immunotherapy (SLIT).

a small sample size and high variation. To obtain a 95% power, the required total sample sizes were estimated to be: BAT reactivity=266; BAT sensitivity=46; MAT reactivity=1 124; MAT sensitivity=284. The sample size in MAT did not seem obtainable, but BAT may provide better alternative. Sensitivity values were better fitted for further investigation in general. In order to accommodate the non-responders in BATs 15% should be added to the samples size, equaling to a total of 305 participants. Furthermore, there was a substantial dropout due to side effects and loss to follow-up, why more patients should be included to investigate BAT as a predictive biomarker. If only BAT-sensitivity was investigated, fewer can be included.

4 Discussion

In this study, we investigated mast cell and basophil responses in activation tests with different IgE compositions and sources in regard to the effector cell granulation and AIT baseline perspectives. In the following paragraphs, the quality assessment of the mast cell cultures and the optimization study with plastic ware will be discussed in section 4.1 and 4.2, respectively. This will be followed by the Affinity (4.3) and AIT studies (section 4.4 and 4.5), in which the results will be represented and discussed in relation to current literature. The discussion will include the expectations of the experiments, the limitations of the studies and future perspectives.

4.1 Quality Assessment of Mast Cell Cultures

From Table 7, the mast cells were phenotypically fairly similar to each other. The alcian blue staining showed high metachromatic values of 90.38±8.28%, indicating the $CD133^+$ progenitor cell cultures had differentiated into mast cells [46]. It should be noted that the alcian blue test appeared to stain some of the cell cultures poorly. Low scores of approximately 20% metachromatic cells were observed. This implied low quality cell cultures, in which the cell potential for experiments may be minimal. These observations may be due to the alcian blue being expired, as the staining appeared to partially cluster and crystallize. Therefore, it is possible that the early cell cultures (culture 3419A-4019A) have underperformed and that the results obtained does not reflect the true staining value, as subsequent staining with new alcian blue showed cultures approaching scores close or equal to 100%. However, high values in activation tests with anti-FcεRI antibody supports the functional quality of the cell cultures.

Other major mast cell characteristics are the expression of tryptase and/or chymase. In this study, the mast cell phenotype was predominantly $tryptase^+$, which was consistent with a previous study, who also reported peripheral blood derived mast cells to express tryptase [47].

There has been some discordance in literature about whether the expression of proteases in mast cells are reversible and whether the microenvironment or culture conditions favour the development and survival of a particular phenotype. In a study by Shimizu et al. 2002, they found that human derived $CD34^+$ progenitor cells, from the bone marrow or cord blood, expressed both tryptase and chymase, when the cells were in the presence of IL-6 and SCF [48]. Maaninka et al. from 2013, also demonstrated $CD34^+$ progenitor cells to express both proteases, when cells were incubated with only SCF. The authors concluded that circulating mast cells originate from a common mast cell progenitor that have to potential to express the entire panel of granule proteases [49]. Interestingly, IL-4 was suggested to induce expression of tryptase and chymase, however this current study only observed chymase expression for a few of the cultures [48]. It should be emphasized that the chymase expression observed in the cultures were generally low, and this may have impacted the subjective evaluation of the mast cell cultures as the presence was debatable.

The decisive factors for including the mast cell cultures for experimental work were primarily based on the results obtained from the alcian blue staining and FcεRI activation test, as these were performed when cells reached six and seven weeks of age, respectively. Thus, the immunostaining (tryptase and chymase expression) and the histamine release tests were performed as retrospective quality tests. The degranulation capacity was measured using two separate methods, activation test and histamine release test. Both functional tests measure the degranulation of FcεRI activation. The results indicated that the response through the high affinity receptor and the histamine release was very similar for some cultures but varied greatly for others. One culture (4019A) showed a moderate activation response of 55.5% through the FcεRI-receptor, but only 7.41% release of histamine. The histamine value for this culture was far below the threshold of use, as described in section 2.4. This was very puzzling, as CD63 upregulation on activated mast cells was supposed to correlate with histamine release, as culture 4719B for instance indicate [33]. A partial explanation may be that high daily variation in activation through the high affinity receptor was observed. Across three of the mast cell cultures, in which the FcεRI test was repeated, a mean difference of 30.33±14.50% could

be reported. To improve the accuracy of the results, it could be suggested that the FcεRI activation test should have been repeated with more replicates. This applies for the histamine release samples as well, as this would have attributed to more reliable test results, but further testing is needed.

The fluctuating results obtained from the surface expression study were also very confounding, as results varied from high one week to low in the next. This was in stark contrast with a previous study by Dahl et al. 2004, which demonstrated a gradual increase in the FcεRI expression from week 0-12, while the CD117 expression increased and reached a plateau from week 4-12 [50]. The unstable development was thought to be caused by technical issues with the Attune flow cytometer. Furthermore, the performance of the fluorochrome used for anti-FcεRI, where verified on mast cells on a different flow cytometer instrument. Subsequent tests performed on a BD FACS Canto II flow cytometer showed higher receptor expression of FcεRI, but the values were still low compared to the literature [50]. As this was a trial study, no further testing was performed. In summary, the cell cultures used in this study were of relatively high quality with a few cultures underperforming in the histamine release test.

4.2 Plastic Ware in Activation Tests

When comparing the results from the material dependent activation methods, a 15.35% mean difference was observed in favour of tube activation in the two independent experimental setups. This suggests that plate activation may not have achieved the same optimal temperature distribution as the tubes. By incubating the plates for 1 hour to 1.5 hours, an increase of mast cell reactivity was expected. However, the experiment showed a decrease of mast cell reactivity from 38.24($\pm$5.55)% to 22.46($\pm$3.92)% in the plates for the two incubation times, respectively. The tube also indicated reduced reactivity with an increased serum incubation time, but still higher than the plates. The differences in performances in the plates could be due to the difference of serum exposure or the activation time. To decrease the variables with serum incubation time and activation time, the procedure could have been streamlined further for the plate setup. Here, the anti-FcεRI-antibody could have been used in conjunction with the $CD63^+$-antibody to demonstrate the ability of the cells to respond through the high affinity receptor and degranulate, whilst the factor of serum is absent. It is important to note that the sole purpose of this optimization study was to demonstrate the feasibility of mast cell activation in plates and not to determine the best method, therefore despite the low reactivity observed the main objective of the optimization study was achieved. However, further experiments using plates to perform allergen dilution curves showed high reactivity up to 94.8% with one-hour activation time and a dependable dose-dependent response indicating plate activation to be effective.

4.3 IgE Affinity Assessment on Human Mast cells

4.3.1 IgE Affinity Dependent Mast Cell Reactivity and Sensitivity

Mast cells were sensitized for two weeks with 80 kU/l of either high, intermediate or low affinity allergen specific IgE clones and activated with allergen to evaluate mast cell reactivity and sensitivity, as described in section 2.6.1. The results obtained from this study demonstrated that IgE affinity was able to affect the degranulation response of the mast cells, however no statistical difference in reactivity or sensitivity was observed across the three IgE affinities tested when comparing the asthmatic group with the healthy control group (Table 8). This corresponds well with the study by Krohn et al. [51], which investigated the influence of donor status on mast cell sensitivity and stem cell yield. In their study, patient or control derived mast cells were cultured under identical conditions, stimulated with IgE and activated with allergen as performed in this study. No difference in sensitivity between asthmatics and controls was found. Furthermore, the fractions of $CD63^+$ mast cells were reported to be 54.4% (asthmatics) and 48.4% (controls), which appear similar and does not indicate a clear immediate difference between the two groups. As it was previously established that patients with atopic uncontrolled asthma, or mild atopic asthma, had an increased infiltration of

mast cells in the alveolar parenchyma and an upregulation of FcεRI expression compared to a healthy control group, one may hypothesize that a possible difference may be observed between the groups in reactivity, sensitivity and stem cell yield [52, 53]. However, the results from Krohn's study suggest that the mast cell response depend more on the culture conditions rather than the donor origin. Furthermore, their stem cell yield was not statistically significant between the groups indicating that the amount of stem cells acquired may be independent of donor origin as well. From these results, it was alluded that isolated mast cells derived from asthmatics or controls were indistinguishable from each other [51]. This implication has been further substantiated by this current study. Congruous with Krohn's study, no statistical difference was found between the asthmatic- and control group in stem yield, nor did the cell growth differ between groups. The greatest difference in mast cell reactivity between the asthmatic- and control group was observed for the lowest IgE condition with a net difference of approximately 28% $CD63^+$ mast cells (Table 8). This indicates that patient derived mast cells may have an increased degranulation response compared to the control group, however the same pattern was not observed in either the high or intermediate condition (Table 8). The ambiguity of the results for mast cell reactivity are inconclusive in discriminating between the asthmatic population from the controls. This may be due to the lack of power in the groups, as the total sample population only consisted of 12 subjects in total. Subsequent power analyses also indicated the need for very high sample sizes, which were not feasible.

One of the primary differences between Krohn's study and this current one consists of the recruitment population, in which their study included subjects with a milder GINA-classification. Thus, one may surmise that when increasing the severity of the asthmatic diagnosis, the likelihood of finding a change between the groups would be greater, but no data has yet been indicative of that. As no clear statistical difference could be established in either mast cell reactivity or sensitivity between the patient and control group, this study could not provide evidence to suggests the use of IgE affinity as a possible complementary marker to sIgE in diagnostics.

It should be stated that not all participating subjects in this study were diagnosed with allergic asthma and not all inclusion criteria were fulfilled. Furthermore, the majority of the control samples obtained, consisted of anonymous donors from the blood bank with limited information, in which screening could not be performed meaning that symptom-free asthma patients may be possible donors [54]. This could inadvertently have created variables, which may have affected the outcome of the study as the sample population becomes less clearly defined for both groups. As the quality assessment also indicate, a functional variation of the mast cell cultures was observed in both the asthmatic and control group (Table 7). To improve the screening process for patient recruitment, a more moderate level of specific IgE above 0.70 kU/l could have been included, as low RAST value may be due to potential laboratory uncertainties and errors.

The effect of IgE affinity on mast cell reactivity and sensitivity was also investigated by Hjort et al. 2017 [5]. In their study, the sample population consisted of a control group, in which mast cells were obtained from five buffy coats from the blood bank, AUH. Cells were cultured, sensitized with either high or low affinity IgE and activated. Mast cell reactivity were 65% and 20% for the high and low affinity clones, respectively, whilst the sensitivity increased almost 13 000-fold from 0.9 ng/ml to 0.07 pg/ml. From Table 8, the mast cell reactivity for the healthy control group were much higher in this study with $\%CD63^+$ cells being 79.77% and 40.68% for the high and low IgE pairs, respectively. The sensitivity decreased approximately 700-fold. The difference in mast cell response could be due to the IgE affinities used for sensitization in the two studies. In Hjort's study, the square root of the product of the IgE affinities were 1.25 nM and 287 nM (224-fold difference) for the high and low condition compared to 0.71 nM and 92.30 nM (130-fold difference) used in this current study (Table 3).

For some of the participants, the sensitivity could not be established with an allergen range of 0.01-1 000 ng/ml for the low affinity IgE condition and was fixed at 2 000 ng/ml. Expanding the allergen range would have increased accuracy and improved the quality of data. However, the baseline cell counts were a limiting factor for additional tests, as the majority of the mast cells were prepared for a cytokine and eicosanoid analysis. For two of the mast cell cultures, the dose response

experiment was able to be repeated, as the data indicated very fluctuating responses in reactivity, even at the lowest allergen concentrations. As the dose response experiments were repeated, the mast cells behaved in their usual pattern, in which lower concentration of allergen stimulated a response with the high affinity IgE, whilst the mast cells with the low affinity IgE degranulated at the highest allergen concentrations. The daily variations observed suggest that mast cells may be driven by some sort of circadian rhythm, as similar patterns of daily variation were also observed during the FcεRI quality assessment test. Interestingly, it was also reported that human basophils may be influenced by the circadian rhythm, as a variation in two clock genes, Per 1 and Per 2, was observed. However, a study by Lind et al. from 2018 could not confirm a variation of CD63 upregulation over a 24-hour period, and concluded it as being independent of diurnal variation [55]. The challenge of observing different mast cell responses in the same culture could be overcome by making triplicates in the experimental setup, as well as being consistent on the time point for experiments. This would increase the statistical validity of the data, but then it would require a greater cell number per condition. The need for triplicates was further substantiated in Figure 8. Here, one can note that the low affinity condition for the asthmatic group, and the intermediate condition for the control group had relatively high degranulation responses at the lowest allergen concentrations compared to the other conditions. A possible explanation could be that the dose response curves were much more sensitive to potential outliers, because the sample population was small with seven patients and five controls. Patient 2, who had eosinophilic asthma had a markedly increased response at the lowest concentrations with a fraction of $CD63^+$ mast cells of about 40% for the low affinity condition (culture 3619A). The same was observed for the intermediate condition of the control sample for culture 3519B.

It has previously been stated that properties such as IgE affinity, fraction of specific IgE, total IgE and the expression of the FcεRI receptor are factors that determine the degranulation response of basophils and mast cells when exposed to an allergen [6, 51, 56]. Increasing the concentration of total IgE might have imitated the allergic environment to a greater extent. However, this was shown to increase the variation in cell responses, which would not be optimal for this study, as one of the objectives was to compare mast cell cultures between groups [51, 56]. In addition, one can propose that the total IgE concentration used in this study was a reasonable value, as the IgE levels correspond to what is observed in allergic patients with moderate to high total IgE, and is similar to what is observed in the 75th and 90th percentile of the adult population [51, 57, 58].

Aside from the properties mentioned, a study by Gieras et al. 2016, also showed that the proximity of IgE epitopes on an allergen affected the allergenic activity [59]. When tested on a rat basophilic cell line (RBL-2H3), the study demonstrated that a closer proximity of epitopes induced a more potent degranulation response than when epitopes were distantly placed. Thus, the same epitope placement of IgE clones were used across all three conditions in this study to establish the same experimental settings for mast cell reactivity comparisons. As the Der p 2 allergen is a relatively small molecule of 14 kDa, the maximum amount of IgE clones that are able to bind simultaneously to the allergen is three antibodies [6]. Therefore, it could be speculated, whether increasing the amount of non-overlapping epitopes could induce the maximum potential of the mast cells. Christensen et al. 2008, demonstrated on basophils that this only affected the cells partially, where basophil sensitivity was increased with 5-20-fold more than IgE clone pairs, but the reactivity appeared unchanged [6]. Whether this also holds true for mast cells remains as a potential future perspective in this study.

4.3.2 The IgE Affinity Dependent Late Phase Response

The samples prepared for mediator release was stored for future analysis. The mediator release of cytokines, chemokines and eicosanoids from mast cells might have given a better understanding of the biochemical mechanisms that determines the outcome of an allergic reaction. The objective was to gain insight as to whether the cellular response would change based on the type/status (severe asthmatics or controls) and perhaps substantiate or contradict the findings from the Affinity study.

As the results have not been obtained, this discussion will primarily focus on previous studies and on the experimental expectations to the Affinity study.

The impact of the early response on the late phase reaction was first characterized on bone marrow derived mast cells from mice [16]. Mast cells were exposed to a high and low affinity antigen (1 000-fold difference), DNP and 2NP, to explore how the affinity for the IgE antibody modulates the effector response of mast cells. Their findings indicated that a shift in the molecular signaling occurs in the FcϵRIγ site of phosphorylation to a more Lyn-Syk-LAT1 dependent pathway for the high affinity DNP cells, whilst the low affinity 2NP phosphorylated the LAT2 dependent pathway [16, 17]. When the signaling pathway shifted to LAT1, an altered release of cellular mediators was observed. Results indicated that cytokine production was significantly increased compared to 2NP, which lead to an increased release of pro-inflammatory mediators such as TNF-α, IL-6, IL-13 and elicited a 20% stronger degranulation response than the 2NP cells. In contrast, 2NP induced a greater chemokine release of chemokine ligand 2 (CCL2), chemokine ligand 3 (CCL3) and chemokine ligand 4 (CCL4) compared to DNP [16]. In summary, a clear difference in the late phase response was established between low and high affinity for mice.

The same signaling shift in the allergy response could not be fully reproduced in humans [19]. When buffy coat derived mast cells were stimulated at three time points (3, 6 and 24 hours) and compared to a baseline sample (0 hour), the high affinity condition showed an increased secretion of cytokines after the first three hours, in which increased levels of IL-8 and IL-13 were observed. The expression of CCL2, CCL3 and CCL4 was found for both the high and low condition. This is in contrast with the murine study by Suzuki et al. [16], which showed that these particular chemokines were predominantly induced in the low affinity IgE condition. The results showed no decrease in expression levels even after 24 hours indicating that the effects were long lasting, and PGD2, which causes broncho- and vasoconstriction, was significantly elevated at the high IgE affinity condition [19]. The difference in expression levels between the two studies might have been due to the affinities used, as the murine study had a greater difference in affinity of a 1 000 fold, whilst a 224 fold difference was used in the study by Hjort [16, 19]. Based on these results, one could consider whether the affinity range of a 130-fold, used in this study, may induce a similar expression pattern or if the range is too low to create a detectable difference in expression levels of cytokines and chemokines. However, a high mast cell response was observed in reactivity for the cell cultures sensitized with high- and intermediate affinity IgE, whilst some of the low affinity cultures elicited no clear degranulation response when activated with allergen. Therefore, one would surmise it is possible to observe a difference using this range of affinity. In summary, the Affinity study of the late phase response was to expand the experimental setup by Hjort and add a patient group. The novelty of the study and the main objective was to provide insight as to whether the mediator release was affinity- or donor dependent. If the results could be reproduced from the murine study by Suzuki et al. [16], and a pattern of release could be defined, then it would perhaps be possible to target several cytokines, and thereby redirect and alter the allergic response. Ultimately changing how allergy is being treated from a symptomatic form of treatment to a causative one.

4.4 MAT with patient IgE-response to grass allergen

4.4.1 Optimization of sensitization and activation in human mast cells with patient sera

Mast cells in various concentrations were passively sensitized for two days with 5%, 10% or 20% serum and activated with grass allergen to evaluate reactivity, MFI_{neg} and SNR. Conditions containing 5% serum with a seeding density of 5 000-10 000 mast cells/100 μl were deemed most feasible for a MAT. These parameters were used as the framework for subsequent experiments using human serum for sensitising mast cells. However, as illustrated in Figure 10, the curve suggests reactivity may continue to increase at lower fractions of serum. More experiments using more serum concentrations, as 2%, 1% and 0.5% would be needed to confirm this. This could be further explored by measuring receptor density in the mast cells and IgE levels in the serum to ensure that the FcϵRI was saturated,

when using lower concentrations of serum.

The concentration of mast cells counted with a flow cytometer was 80.37% of the original seeded amount. It was therefore aimed to use a seeding density of approximately 7 000 mast cells/100 μl if the mast cell yield of the culture allowed it. Variation in mast cell concentrations after two days may be influenced by viability of mast cells and that some cells are lost during processing.

There is some challenges in defining allergen concentrations and sensitivity, when using dissolved SLIT tablet in experiments. The allergen content of the SLIT tablets used are defined by specific quality (SQ) units, which are an arbitrary unit. The Grazax tablet has a defined dose of 75 000 SQ-T and the Itulazax tablet is at 12 SQ-Bet, though they should have the same allergologic impact. The concentrations are often reported in SQ-U/ml, but it gives a blurred image, when comparing the allergens. The allergen content in ng/ml is not available for Itulazax and in order to compare and uniform the sensitivity measurements, there was a need for another unit to reflect how much the allergens were diluted. In this study we used daily dose equivalent (DDE)/ml, where 1 daily dose equals 1 tablet, as explained in section 2.5.

The allergen concentrations used in these experiments were based on preliminary protocols available from AUH. The standard solutions of Grazax at final concentration equivalent to 60.61-0.61 mDDE/ml were used in two of the cultures (3519A and 3819A). These concentrations were not able to produce a dose-dependent response but were presented with a linear curve. In the third culture (4219B), a 10-point allergen dilution curve was made to identify the optimal allergen concentrations. The mast cells responded with high reactivity, and the allergen concentration were adjusted based on the lowest concentration (0.0061 μDDE/ml), which produced a lower reactivity at the subsequent measurements. As the measurements for this culture were generally lower for all parameters including cell count, this accounts for some of the high variations seen in the results. However, the tendencies between the three cultures were still comparable as they exhibited the same trends in reactivity, SNR and MFI_{neg}. MFI_{neg} were lower in serum concentrations of 10% and 20%, which could be explained by a much lower mast cell count. It can therefore not be determined precisely if higher serum concentration would produce more background noise. However, flow cytometry assessment revealed FSC/SSC cytograms with a lot more debris around the mast cell populations the higher the serum fractions. Fluorescence in the histograms also presented a right skewed tail in the negative samples, which were more prominent, the higher the serum fraction.

Based on the lower concentrations of recorded mast cells in higher serum concentrations, mast cells were evaluated for viability in two independent experiments, as serum may be toxic for the cells [60]. Cytotoxic sera in *in vitro* cell cultures can be caused by engaging in the humoral defence of classical pathway complement activation (antibody-mediated complement cytotoxicity) or endotoxins, as the serum was not screened for endotoxins nor heat-inactivated [60]. The results indicate mast cells loose viability when incubated at higher concentrations of human serum over two days and supports the low cell counts from flow cytometry. But the data suggests that using 5% serum was enough to fully sensitize the mast cells based on the degranulation response. Flow cytometry analysis using viability dyes, as 7-AAD, would be able to assist in the viability evaluation. Another possibility was to use imaging flow cytometry to assess the cell membrane integrity and using more than one serum donor, as there may be a subject-subject variation in toxicity, depending on the individual composition of the sera.

4.4.2 Validation of MAT

When comparing MATs in test-retests, from buffy-coat CD133^+ derived mast cells, the result was that reactivity and sensitivity of the MAT with human serum and plasma are reproducible. Sensitivity was very well represented in the different statistical tests. Reactivity did however show some variations, but as discussed in section 4.1 day to day variations were observed, but not of clinical relevance.

Between three MATs with sera from grass-pollen induced allergy, there was more variation in sensitivity than those of the two MATs with plasma or sera from birch-pollen induced allergy. One

explanation could be that the there was difference in the methodology. The grass-pollen MAT were activated in well plates for 1 hour and recorded with Attune NxT Autosampler and the birch-pollen MAT were activated in tubes at 30 minutes and recorded with BD FACS Flowcytometer. The latter was performed in this way to match the BAT method, when comparing the results of these. Polystyrene tubes may be a more reliable material and there will always be a small difference when using two different instruments to measure samples. It should also be noted that the difference between the tests were stated in means(±SD), though data were not normal distributed, but this was done in order to accommodate the Bland Altman test, which is based on parametric values. The wide limits of agreement, in the MAT with grass-allergen (Figure 12J and L), is sensitive to the only two data points, which had a high difference. It should be noted that the other eight data points had a very low difference, showing higher reproducibility. However, this small variation reflects that the MAT does have some challenges in determining the true sensitivity at high allergen concentrations, which need to be addressed.

Serum and plasma have both been used in MATs. There was a concern that plasma samples will form clots if not treated properly. In this study the blood samples were drawn in heparin tubes and added extra heparin for precaution to the isolated thawed plasma samples. Though one could argue that it was unnecessary, as full blood BATs, containing coagulation factors, performed in at the laboratory at AUH, were fully functional when only using the heparin from the collection tubes. Santoz et al. 2015 and 2018 performed MATs with passive sensitization of LAD2 mast cells with plasma from peanut allergic patients but is is unknown how the plasma samples were prepared. [32, 61]. Bahri et al. 2018 performed a MAT similar to the procedures used in this study [4]. They used buffy coat-derived mast cells at eight to ten weeks of age. The mast cells were passively sensitized overnight with human 10% sera from peanut allergic patients. Activation was investigated with flow cytometry by CD63 expression in both studies and they could successfully activate the the mast cells. In this present study, similar results in response of reactivity and sensitivity were obtained when comparing heparinized plasma and serum (n=3). Thus, MAT was still very reproducible when performed with either serum or plasma.

4.4.3 Predictive Value of Mast Cell Activation Test in Grazax Treatment

Mast cells were sensitized over two days with patient sera from the GT-08 study and activated with diluted Grazax as allergen and evaluated for reactivity and sensitivity, where results are stated in section 3.5.4. The majority of the mast cells showed dose-dependent degranulation detected by $CD63^+$ by flow cytometry. The 4PL fit dose-response curves showed overall reliable sensitivity calculations with comparable 95% confidence intervals. Only one data set had an ambiguous slope and sensitivity could not be calculated by the software, why it was fixed at 3030.304 μDDE/ml. Some of the mast cells responses were very reactive and did not have a dose-dependent degranulation. The allergen concentrations used were verified and adjusted by preliminary experiments with a wide range of allergen dilutions in MATs with eight sera samples available from grass-dependent allergic patients. One could always include more allergen concentrations in a repeated experiment, but daily variation in reactivity could influence the outcome when comparing the data. When evaluating mast cell response from the MAT in regard to the global evaluation scores or nose symptoms, no statistical association could be confirmed. The use of a MAT to discriminate against treatment effect in allergen immunotherapy has not been done before, and the results cannot be validated in current literature. This study was based on unpublished data from Schmid et al., who proposed that the reactivity of the effector cells could be used to discriminate the low responders from the good responders in allergen immunotherapy. Baseline reactivity did not have the expected distribution in the groups of global evaluation scores. Ideally the reactivity would be high in the "high" group and decrease in values in "medium" and "non-reactive (NR)". The assumption that a high mast cell response would be a marker of how well the immune system reacted with the treatment preparation, leading to better induction of tolerance was not met. Santoz et al. 2018 did a diagnostic test in peanut allergic, n=73 (severe and not severe) and peanut sensitized, n=60 subjects with LAD2 mast cells evaluated

by $CD63^+$ expression in flow cytometry [32]. The test could discriminate between peanut allergics and peanut sensitized, but with a cut off at 17.2% $CD63^+$ LAD2 mast cells. Patients with severe peanut allergy had higher proportions of $CD63^+$ (>24.8%) than the non-severe group ([32]) but they could not be differentiated from the non-severe group, with a low specificity and positive predictive value. This supports that the reactivity of mast cells, based on humoral components, cannot directly distinguish differences by grouping in the clinical response. But it does reflect that severity, at least in food allergy, elicit a higher mast cell reactivity. The mast cell reactivity in this present study was generally high for all patients with low variation, median(IQR) of 66.60(57.65-73.10)% $CD63^+$. This is also the case for the allergen reactivity/anti-FcϵRI antibody reactivity ratio, which were all close to 1, meaning all patients elicited a response close to the maximum reactivity potential of the mast cells. The mast cell reactivity in this study may not reflect the AIT-response groups, but it may reflect that the patient group selected had more severe symptoms. However, in a study by Nopp et al. 2006, they did not find that basophil reactivity correlated to provocational clinical manifestations based on skin sensitivity to grass allergen in a SPT in patients with grass dependent asthma or AR (n=27)[62]. However, basophil sensitivity did correlate with sensitivity to skin allergen threshold and were able to reflect the groups receiving anti-IgE treatment from non-treated.

Baseline sensitivity in this present study could neither explain the global evaluation response in the groups. There was no relevant pattern in the medians of the groups, as it was hypothesized that the high group would be more sensitive (needing less allergen to elicit a response) than the median and NR groups. The relationship with sensitivity and severity in symptoms has further been investigated by Konradson et al. 2012, in regard to basophils and asthma [63]. Children with cat-dependent problematic severe asthma with (n=11) had a higher basophil sensitivity (CD-sens) to cat allergen compared to children with controlled cat-dependent asthma (n=11), why sensitivity may be associated with severity.

The "late" responders, which consisted of an extra patient group with delayed treatment effect, could have been interesting to place in the MAT response. However, its role was dependent on clearly defined effect groups when compared to high, medium and NR, to explore the context of a late treatment responder.

The extended analyses in section 3.5.5 with weighted data analysis revealed a significant difference in sensitivity between medium and NR group. This result must be regarded as not representative of the data, as it could not be confirmed in the other data groups or by linear model analysis from ALK-Abelló.

A small part of the samples (n=13) of remaining sera were used to test if mast cells from an atopic patient could discriminate between the response groups, but no statistical differences were observed in reactivity and sensitivity between the groups. There were some limitations to this; 1) a very small sample size was investigated, 2) there was an experimental inconsistency, in which plastic ware for activations and the flowcytometer instrument was changed in 1/3 of the samples due to technical issues. This has been observed to cause some differences in the $CD63^+$ measurements, but it did not seem to impact the statistical measurements. As previously confirmed in section 4.3.1, mast cells performed similarly regardless of donor origin.

The clinical readouts were not correlated to mast cell responses, but sensitivity and reactivity correlated to baseline levels of sIgE. Christensen et al. 2008 has shown that mast cell degranulation was dependent on the fraction of sIgE of total IgE [6]. Though the fraction of sIgE of total IgE was not investigated here, the sIgE values might still reflect a relationship. Bahri et al. 2018 did a MAT from human mast cells, passively sensitized with serum from peanut allergic subjects. Serum-specific peanut IgE values were strongly correlated to the degranulation (% $CD63^+$ expression by flow cytometry), when activated with peanut extract, R^2=0.89 ($p<0.0001$) [4]. The sensitivity measured by CD-sensitivity also correlated with serum-specific peanut IgE levels, R^2=0.61 (p=0.022). However, they also found that it was not the amount of sIgE alone, which affects MAT response. It is also affected by affinity and the combination of IgE specifities, which is confirmed in the work by Hjort and Christensen [5, 6]. No correlation was observed in baseline sIgG4 to reactivity and sensitivity, nor to baseline sIgE. This can be explained by the sIgG4 being induced after initiation of AIT [31, 61].

The ratio of baseline sIgE/sIgG4 in this study did correlate with mast cell reactivity or sensitivity, but it seems that it was driven by the sIgE-levels. Santoz et al. 2015 did a study on sIgE and sIgG4 levels of the inhibitory effect on basophil and mast cell activation tests with passive sensitization in peanut sensitized and peanut allergic subjects [61]. The peanut allergic subjects were only able to induce inhibitory effect in BAT and MAT in the same level as peanut sensitized subjects after AIT. The ratio of baseline sIgE/sIgG4 was greater in peanut sensitized than the peanut allergic subject.

All together, these findings in this present study suggest that it is not the baseline levels of mast cell reactivity or sensitivity that determine the outcomes of allergen immunotherapy. It may also be that the patient population in the GT-08 study could be too homogeneous, as patients included in clinical studies is often more homogeneous, than those seen in the clinics. It should be noted that, the groups designed for this project, do not reflect the true treatment outcome of the GT-08 trial. The treatment outcome was based on reduced nasal and eye symptoms and reduced medication intake in the active group compared to a placebo group [43]. The treatment effect therefore not directly translated into individual subjects.

So far the closest predictive biomarker for treatment effect near baseline levels has been identified for SCIT, with a change in basophil sensitivity pre-treatment and after three weeks treatment [45]. Other biomarkers used in the evaluation of clinical efficacy of AIT may also be a desired target for predictive efficacy, serum inhibitory activity (FAB), chemokines and cytokines as IL10, cellular markers (Treg, Breg cells, DCreg) or provocation tests [41, 64].

4.5 Basophil Activation Test with Itulazax

BAT was performed on 12 patients with birch pollen-induced allergy at baseline levels. Basophil degranulation in patient 4 and 11 presented very reactive and highly sensitive basophils, with no decrease in CD63 expression at the lowest allergen concentration. By increasing the number of samples analyzed from singlets to duplicates or triplicates, the data would be more robust. Two of the BATs were performed in duplicates to evaluate the reproducibility of the individual measurements, in which the reactivity was within an acceptable mean variation($\pm$SD=2.47 and 2.10 %CD63$^+$). This was performed in patient 10 and 11, which had very different dose-response curves. But it should be noted that the statistical software used to calculate sensitivity, Prism, was unable to calculate a complete confidence interval using variable slope (4PL) for some of the dose-response curves. Sensitivity in the BAT could not be calculated for patient 4 and 11 and was estimated. Patient 2, 3, 4 and 5 had an ambiguous slope and sensitivity was approximate. 95% confidence interval could neither be calculated for patient 6, 9, 10 and 11. Sensitivity data should be interpreted carefully. A way to increase statistical strength for the sensitivity calculations, could be to narrow the values between the allergen dilution. Allergen was serially diluted 1:10, but a 1:4 dilution scale might have worked better and could better identify outliers. Basophil sensitivity and reactivity did not correlate, and though sensitivity values are uncertain in this study, Nopp et al. 2006 (n=27 grass dependent allergic subjects) also found no relationship between those two parameters from a BAT [62].

4.5.1 BAT and MAT

The valid BATs of those patients, who had birch pollen-induced allergy, was compared to a MAT (n=10). The buffy coat derived mast cells showed dose-dependent degranulation with patient plasma or serum, but the BAT did not. The BAT and MAT had low comparability in reactivity and sensitivity. Variations were $\pm$12.56% CD63$^+$ and $\pm$29.70 μDDE/ml, respectively. The basophils were more reactive than the mast cells with a mean difference of 20.15% CD63$^+$ and more sensitive with a mean difference of -17 μDDE/ml.

As mentioned in section 4.4.3, basophil sensitivity data should be interpreted carefully. Mast cell sensitivity in patient 2, 9 and 12 were also approximated with an ambiguous slope. However, MAT sensitivity values did appear reproducible in the sensitivity calculations when tested in two buffy coat derived mast cells cultures. Santoz et al. 2018 found a correlation in the CD63$^+$ mast

cells of ρ=0.649 for patients suffering of peanut allergy. Sensitivity was not compared. In this study no significant correlation in reactivity could be confirmed, but the smaller sample size should be kept in mind. In the study by Bahri et al. 2018, where they used a MAT sensitized with sera from peanut-sensitized subjects, they also compared the MAT performance to a BAT and reliability of between the two tests. But some differences in the assays were identified [4]. The MAT consisted of pooled mast cells from at least three different donors. However, they found that MAT between to separate measurements were more reproducible based on intraclass correlation (ICC)=0.96 values, whereas BAT was only ICC=0.43. It is unknown whether these comparisons were based on AUC or %CD63$^+$. They found a 2-log difference in the expression of CD63 in MAT compared to BAT, showing that the MAT needed a lower concentration of peanut allergen. It should be noted that basophils express a higher density of FcϵRI on the surface than mast cells [34]. This present study found that the basophils were slightly less sensitive than the mast cells, mean difference($\pm$SD) in sensitivity of -17.00($\pm$29.70) μDDE/ml. But this needs to be further explored, as this could be explained by the various quality and variation of the basophil sensitivity data.

4.5.2 Predictive Value of Activation Assays and Treatment Effect of Itulazax

Of the 12 patients recruited, nine were qualified to complete the RQLQ at the follow-up time in April 2020. Only seven patients completed the the RQLQ in a timely manner, which resulted in a very low participation. This study found a mean reduction in symptoms (RQLQ-score) of 49.15%. This was higher than the relative differences of 31% in the RQLQ-scores found in an efficacy and safety study of Itulazax with n=280 birch and tree-dependent AR patients in the active group by Biedermann et al. 2019 [44]. But this can be due to the heavy difference in sample size. It should be noted that the primary end-point for efficacy in the study by Biedermann used total combined scores based on daily medication scores and daily symptom scores in the birch-pollen season against a placebo group, where the efficacy was 40% [36].

In this study, no significant correlation was found between reduction in RQLQ-scores and baseline cell activation tests with basophils or mast cells. Interestingly, patient 3 and 12 who had the lowest effect did already receive AIT therapy at baseline for unrelated allergens. Most literature on efficacy of AIT is on single-allergen products and the efficacy on multiple allergen products still needs further data for validation [36]. It could not be established if the concurrent AIT treatment had an effect on the baseline activation assays as two patients had opposite values in both reactivity and sensitivity (Figure 19D and E), but it may have other immuno-modulatory effects. Patient 2 began SLIT treatment for grass-allergy after recruitment, but this did not seem to have an effect on the treatment effect. This study needs further investigation in order to explore any relationship with baseline activation tests as a predictive tool for AIT. This patient population does reflect the diversity of the patients, who are offered AIT. However, further investigations should be conducted in patients without multiple AIT treatments first. Another end-point for clinical efficacy would be to include daily medication scores, provocation tests and focused nose and eye symptoms as these are used in other AIT-studies with AR patients [36, 41, 43, 44]. Preferably combined scores of medication and symptoms as primary end-points with secondary end-points of life quality and provocation tests could be used in future studies [41]. Power calculations of sample sizes revealed high numbers of subjects to have a power of 95%. However, BAT sensitivity may to be the best option to investigate the role of predictive biomarker for AIT.

5 Conclusion

The following conclusion is divided into two parts, one for each main study:

- When sensitized with different IgE affinities, human derived mast cells elicited a stronger degranulation response with higher affinities, whilst sensitivity increased with decreasing affinities. This confirms the relationship of IgE affinity on effector cell degranulation. This study did

not find a statistical difference in mast cell reactivity nor sensitivity between severe asthmatics and healthy controls suggesting cultured mast cells to be independent of donor status.

- MAT with serum or plasma passive sensitization, on buffy coat derived mast cells, is reproducible. Reactivity showed a small variation, whereas sensitivity (EC50) is very reproducible. Buffy coat derived mast cells with baseline patient serum did not show predictive properties in grass allergen SLIT treatment outcome. Patient stratification based on effector cell reactivity and sensitivity did not show patterns congruent with the treatment outcomes from the GT-08 study, which suggests that the activation tests cannot discriminate between the poor and good responders. The humoral markers of sIgE and sIgG4 was neither effective in explaining treatment outcome, but IgE-baseline levels correlated with mast cell reactivity and sensitivity. The role of pre-AIT-BAT as a predictive biomarker for efficacy needs further investigation.

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