Zentrum für Rhinologie • Allergologie

Universität Zürich[UZH]

USZ Universitäts Spital Zürich

HFV Research Project - Project Plan

COMPARISON OF INNATE IMMUNE RESPONSES INDUCED BY ALLERGY IMMUNOTHERAPY (AIT) WITH DIFFERENT ADJUVANTS

USZ-AZW_MCT001

Sponsor and Project Leader	University Zurich Department of Dermatology Prof. Pål Johansen University Hospital Zurich Gloriastrasse 31 8091 ZURICH Switzerland
HFV Category	HFV A: Non–clinical research project with sampling and further use of samples and health related data
Version Number and Date	Version 2, 8.8.2019

Project Synopsis

Sponsor / Project Leader:	Pål Johansen Department of Dermatology, University Hospital Zurich & University of Zurich
HFV category:	A – research project – sampling and subsequent use of samples
Title:	Comparison of innate immune responses induced by allergy immunotherapy with different adjuvants
Short Title:	USZ-AZW_MCT001
Project Population:	Adults, males and females
Coding:	Coded samples and data
Objectives:	Analysis of inflammatory responses in blood of patients receiving allergen immunotherapy (AIT) with different adjuvants
Outcomes:	Inflammatory cytokines, acute-phase proteins, and antibodies are measured in serum from patients receiving AIT
Number of Subjects:	24
Inclusion Criteria:	• Indication: Scheduled AIT • Male and Female subjects 18 years to 50 years of age • Written informed consent to the research project by the participant after information about the project
Exclusion Criteria:	• Objection of subsequent use of biological samples and personal health data • Systemic or local immunosuppressive treatment last 30 or next 7 days • Previous AIT • Chronic inflammatory diseases • Infections • Blood donations last 30 or next 7 days • Drug or alcohol abuse • Pregnancy or breast feeding
Project Duration:	Planned start: 09/2019 Planned project close: 09/2020
Statistical Methodology:	The study is a pilot study and it aims to obtain descriptive information on the type of inflammatory immune responses measured in serum after AIT and will be analysed using descriptive statistics.
Statement:	This project will be conducted in compliance with the protocol, the current version of the Declaration of Helsinki as well as all Swiss and German legal and regulatory requirements, in particular data protection regulations.

Table of Content

Page

Abbreviations

AIT	Allergen Immunotherapy
ALAT	Alanine aminotransferase (or Ala transaminase)
Alum	Aluminium hydroxide or phosphate (adjuvant)
ASAT	Aspartate-aminotransferase (or Asp transaminase)
AZW	Centre for rhinology and allergology (*Allergiezentrum Wiesbaden*)
CRF	Case report form
CRP	C-reactive protein
EDTA	Ethylene diamine tetra acetic acid (blood anticoagulant)
Gamma-GT	Gamma-glutamate-transferase
HFV	Human research ordinance (German: Humanforschungs-Verordnung)
HRO	Human Research Ordinance (HFV – Humanforschungs-Verordnung)
ICH	International Conference on Harmonization
IEC	Independent Ethics Committee
IgG, IgE, IgM	Immunoglobulin G, Immunoglobulin E, Immunoglobulin M
IRB	Independent review board
MCT	Microcrystalline tyrosine (adjuvant)
MPLA	Monophosphoryl lipid A (adjuvant)
USZ	University Hospital Zurich (German: *UniversitätsSpital Zürich*)

Project Personnel and Administration

Sponsor and Project Leader:	Prof. Dr. Pål Johansen University Hospital Zurich Department of Dermatology Gloriastrasse 31, 8091 Zurich Switzerland EMail: pal.johansen@usz.ch Phone: +41 044 255 86 16
Department of Dermatology University Hospital Zurich Gloriastrasse 31, 8091 Zurich Switzerland	Dr. med. Deborah Leuthard Dermatology, USZ Email: deborah.leuthard@usz.ch Phone: 043 253 05 68 Dr. med. Sara Micaletto Allergy Unit, Dermatology, USZ Email: sara.micaletto@usz.ch Phone: 043 253 76 30 Dr. med. Eugen Bersuch Allergy Unit, Dermatology, USZ Email: eugen.bersuch@usz.ch Phone: 043 253 88 50 Elvira Schmid Schwotzer Allergy Unit, Dermatology, USZ Email: elvira.schmid@usz.ch Phone: 044 255 24 41

	Master med. Alina Müller Dermatology, USZ Email: alina.mueller@usz.ch Phone: 076 391 18 35
Allergiezentrum Wiesbaden An den Quellen 10, 65183 Wiesbaden Germany	Prof. Dr. med. Ludger Klimek Allergiezentrum Wiesbaden Email: ludger.klimek@allergiezentrum.org Phone: +49 611 308 608 0 Dr. med. Annette Sperl Allergiezentrum Wiesbaden Email: annette.sperl@allergiezentrum.org Phone: +49 611 308 608-0 Master med. Alina Müller Allergiezentrum Wiesbaden Email: alinamueller@gmx.net Phone: +41 76 391 18 35 Dr. Ingrid Casper Allergiezentrum Wiesbaden Email: Ingrid.Casper@allergiezentrum.org Phone: +49 611 308 608-0
Biostatistics	Dr. Nicole Graf Amelenweg 5, 8400 Winterthur Email: graf@biostatistics.ch Phone: +41 79 270 6772
Data codes	Séverine Buffoni Dermatology, USZ severine.buffoni@usz.ch

1. Introduction

This research plan describes a scientific research project (Swiss HFV category A) and includes the re-usage of human blood serum samples collected from patients receiving allergen immunotherapy (AIT) at University Hospital Zurich and at the Centre for Allergy and Rhinology in Wiesbaden (*Allergiezentrum*, AZW). The USZ research project is led by Prof. Pål Johansen, USZ, while Prof. Ludger Klimek holds the lead at AZW; Prof. Klimek is also professor at the University Mannheim/Heidelberg.

All serum samples from USZ and AZW will be all assessed at the USZ.

Ethical approval for the blood sampling at AZW will be obtained by Prof. Ludger Klimek from the Independent Ethics Committee (IEC) in Mannheim (*Ethik-Kommission der Medizinischen Fakultät Mannheim/Medizinische Ethikkommission der Universität Heidelberg*).

1.1 Background and purpose

IgE-mediated allergy represents a serious health problem in industrialized countries with about 30% of the population suffering from allergic symptoms such as rhinitis, conjunctivitis, bronchial asthma, or atopic dermatitis [1]. Allergy also represent a frequent cause of morbidity and disability when it manifests with bronchial asthma and anaphylaxis. These atopic diseases are characterized by production of IgE from B cells and a T-cell population that preferentially produces T-helper like 2 (Th2)

cytokines such as interleukin (IL)-4, IL-5, and IL-13. In contrast, protective biomarkers in allergy is thought to be Th1-like cytokines, regulatory T cells (Tregs), and allergen-neutralising IgG antibodies [2].

Since more than 100 years, the only causative treatment of IgE-mediated allergy was allergen immunotherapy (AIT) with allergen extracts, or more lately in clinical trials, purified, synthetic, or recombinant allergen proteins [3,4]. In Europe, but not in the USA, the AIT products usually contain adjuvants [5]. The adjuvant should provide a depot of the therapeutic allergen, facilitate protective immune responses, and in part to improve the AIT safety by adsorption of allergen. For a long time, hydroxide or phosphate salts of aluminium (Alum) were the only registered adjuvants for use in human [6]. However, new adjuvants that more specifically stimulate the inflammatory responses required for protection in allergy is ascribed an important role in the development of better and safer AIT regimens.

The adjuvant effect of Alum has been ascribed to its particle nature and its depot formation, but more lately, the mechanism of action has been shown to include the activation of inflammasomes. Inflammasome activation results in the immediate release of inflammatory cytokines such as IL-1β and IL-18 [6]. It is also well recognised that Alum is a Th2-polarising adjuvant and facilitates IgE production [7]. Hence, it is an immunological paradox that the Th2-biasing Alum is used to cure a Th2-mediated allergy, because improved AIT regimens are thought to be associated with Th1-polarising immune responses. To what extent Th1 or Th2 immune responses are triggered, very much depends on the initial and innate inflammatory reaction which can immune responses. Hence, the development of more beneficial adjuvants is a focused research area in allergy.

More lately, microcrystalline tyrosine (MCT) and combinations of MCT and Monophosphoryl Lipid A (MPLA) have been introduced as alternative AIT adjuvants [5]. MPLA is known to mediate the release of inflammatory immune responses through stimulation of Toll-like receptor 4 (TLR4) on antigen presenting cells (APCs) [8]. Mechanistic knowledge on MCT is by comparison incomplete, although we recently demonstrated that mice immunised with antigen and Alum or MCT produced comparable IgG antibody responses, but with less IgE antibodies and more Th1 cytokines in MCT-treated mice [9]. In humans, a side-by-side comparison of the inflammatory responses induced by AIT with the different adjuvants is totally missing.

The primary purpose of the current project is therefore to collect blood from patients that receive grass pollen AIT with Alum (Allergovit®), MCT (Polvac™), or MCT-MPLA (Pollinex Quattro™) as part of their standard AIT. How and to what extent the adjuvant system contributes to the stimulation of innate immune responses will subsequently be measured by means of antibody arrays and other immunological methods on patient serum samples obtained prior to AIT as well as 1, 7, and 49 (±3) days after AIT. This study will be the first in its kind, and the results of this analysis may provide clues to the mechanism of action and safety of the various adjuvants used in AIT, which again should allow us to optimize such adjuvants. The study should also allow us to correlate inflammatory responses with historical data on efficacy, which again may help the rational development of new and better AIT and AIT adjuvants. Bearing in mind that 90% of the allergy patients are currently reluctant to receive treatment by the only causative and disease-modifying method, safer and more efficient AIT is probably the most important goal as to make AIT more attractive to the vast number untreated allergy patients.

1.2 Project site Centre for Rhinology and Allergology, Wiesbaden (AZW)

Blood samples and data from patients that receive AIT with Pollinex Quattro™ (8 patients) is obtained from the AZW. The AZW is one of the leading German institutions in the area of research and treatment of diseases of the nose and the upper respiratory tracts and the only institute specializing exclusively in this area. Scientifically, AZW is part of the University Mannheim/Heidelberg. It has a special focus on the diagnosis and treatment of chronic inflammatory diseases of the nose and the paranasal sinuses – in particular also the identification of allergic causes.

http://www.allergiezentrum.org/de/

The Pollinex Quattro™ patients are not available in Switzerland, since Pollinex Quattro™ is not generally approved for use in Switzerland.

Although the current study is non-interventional, the study participants will receive AIT between the first (V1) and the second (V2) visits. The AIT is performed by well-trained and experienced allergologists, and AZW has all required infrastructure for treating patients that present with serious adverse event (SAEs) within the timeframe of the duration of the study. In case of SEAs because of the venous blood collection, the AZW has all infrastructure and medical personnel required to care for these study subjects.

Laboratories

- Labor Fleischauer: since 2006, accredited by DAKKS (*Deutsche Akkreditierungsstelle*) according to DIN EN ISO 15189

1.3 Project site University Hospital Zurich (USZ)

Blood samples and data from patients that receive AIT with Allergovit® or with POLVAC™ is obtained from patients treated in USZ (16 patients) All the blood samples are analysed at the USZ. The USZ has accredited and otherwise certified laboratories that can perform the serological analysis of acute-phase proteins and antibodies with high quality. Moreover, the Department of Dermatology is one of the most active dermatological research centres in Europe with a strong focus on skin cancer, inflammatory skin diseases, allergology, and immunotherapy. Here, the Allergy Unit is part of a network of 19 EU allergy centres joined in the so-called Global Allergy Asthma European Network (GA2LEN), which strive and commit to high quality standards in patient care, training, and quality management, and are regularly reviewed through external audits.

Laboratories

- USZ Haematology: accreditation no. STS 0445
- USZ Dermatology: accreditation no. STS 0610
- USZ Immunology: accreditation no. STS 0227
- USZ Clinical chemistry: accreditation no. STS 0206

2 Project Objectives

2.1 Project Objectives

The primary objective is to compare inflammatory responses in blood sera from patients receiving first AIT with Alum, MCT, or MCT-MPLA as adjuvants. The AIT products are containing allergen extracts of non-specified origin (pollen, dander, dust mite, venom) and provided by Allergopharma (with Alum) or Bencard Allergie (with MCT or MCT-MPLA).

Since no MPLA-containing allergy vaccines or AIT preparations are approved for use in Switzerland, the study subjects enrolled into this study will be recruited among AIT patients at the AZW.

2.2 Project Outcomes

The following secondary objectives will be investigated on all study subjects, but not on all time points:

- the composition of blood leukocytes is measured on days 0, 1, and 7
- allergen-specific antibodies are measured on days 0, 7, and 49
- acute-phase proteins including liver enzymes are measured on day 0, 1, and 7.

3 Project Design

This study is an observational study with subsequent use of coded biological material. The blood sera are collected and prepared at the Allergy Units at USZ (Zürich) or AZW (Wiesbaden). The AZW serum samples will be transferred to Zurich for serological analysis at USZ. The flow chart in **Figure 1** illustrates the overall design, content, and schedule of the study.

The AIT patients will be allocated to three arms based on their scheduled AIT and as indicated in **Figure 1**. Two arms will be allocated to patients at the USZ Allergy Unit, while the last arm is allocated to patients at AZW Wiesbaden.

Sixteen study subjects will be recruited from allergy patients that visit the Allergy Unit at USZ to receive AIT as part of standard of care treatment for their allergy. If the patients receive AIT, they can be included in the study. The decision on performing AIT is done by the USZ allergologist, and the therapy itself is not part of the current research project. The 16 USZ patients are split in two study arms. One arm of study subject is scheduled for AIT with Allergovit® and the second arm is scheduled to receive AIT with POLVAC™.

Eight study subjects will be recruited from allergy patients that visit the AZW to receive AIT as part of standard of care treatment for their allergy. If the patients receive AIT, they can be included in the study. The decision on performing AIT is done by the AZW allergologist, and the therapy itself is not part of the current research project. The 8 patients comprise the third arm of the overall study, and they receive AIT with Pollinex Quattro™. The Pollinex Quattro™ patients are not available in Switzerland.

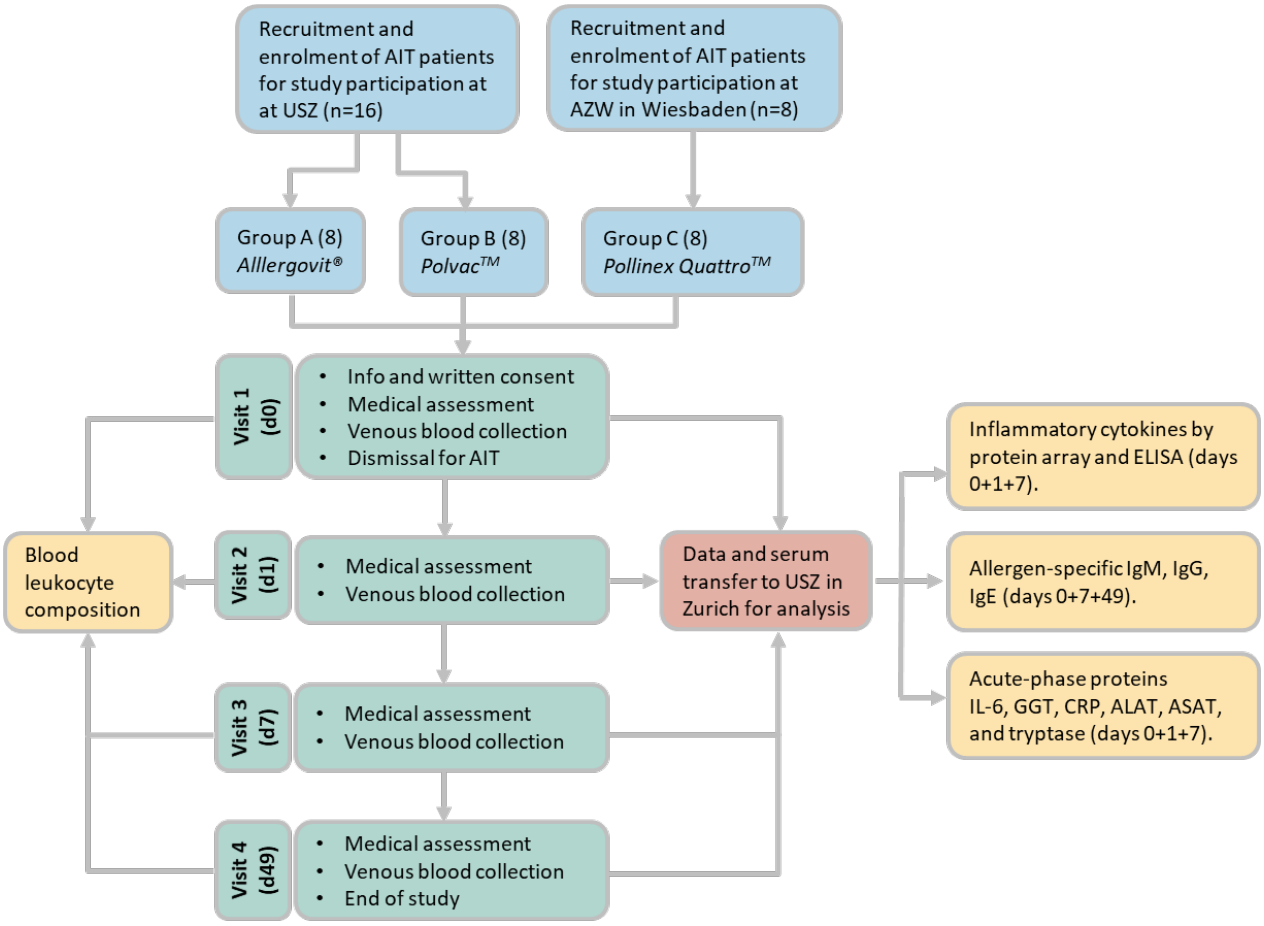


Figure 1. *Flow chart showing the study design, the allocation of the groups of patients, and the study progression included into the trial at the ASW study site in Wiesbaden. Patient sera are transferred to Zurich for assessment of inflammatory proteins and antibodies at USZ.*

The trial requires four visits (**Fig. 1**). Screening visit (V1) takes place on the day of the scheduled AIT. Prior to AIT (not part of the study), patient blood is drawn. Three subsequent visits are planned one (V2), seven (V3), and 49 (V4) days after the first visit. Patient blood is drawn on all visits.

3.1 Project Schedule

The individual duration of study participation for patients completing the study is 49 days. The total number of patients required for the study is expected to be recruited an enrolled within two months. The estimated time to complete the subsequent lab analysis of blood from patients included in the trial is ca. 6 months.

- Duration of the recruitment phase: Sep 2019 to Jan 2020
- Planned project start (first sampling of blood): Sep 2019
- Planned project end (last sampling of blood): Jan 2020
- Definition of the project end (finalised lab analysis): Sep 2020
- Duration of the project in total: Aug 2019 to Sep 2020

4 Population of Biological Samples

Blood samples will be obtained from a total of 24 patients with IgE-mediated allergy as diagnosed or confirmed by an allergologists at the Allergy Units of USZ (16 samples) and AZW (8 samples).

For blood obtained from patients at the USZ, all samples will be processed and analysed at USZ. For blood obtained from patients at AZW, whole blood will be directly analysed at *Labor Fleischauer* in Wiesbaden for leukocyte composition, while part of the blood will be centrifuged for preparation of serum that is frozen (-20°C) and transferred to USZ for further analysis.

Patients that will be considered for inclusion into the study should have been scheduled for treatment with AIT by means of subcutaneous injections of Allergovit® (USZ), Polvac™ (USZ), or Pollinex Quattro™, (AZW), the goal being to include eight patients from each of the three treatment regimens.

Important, the study subjects are only recruited among patients that are to receive their very first AIT. The AIT is not part of the study, but takes place due to prior decisions by the allergologists Dr. med. Sara Micaletto and Dr. med. Eugen Bersuch at the Allergy Unit USZ or by Dr. med. Annette Sperl at AZW.

Patients that will be considered for inclusion into the study are scheduled for treatment with AIT. The study subjects are primarily recruited by medical doctors at USZ (16 subjects) and AZW (8 subjects) during allergological consultation. The method of recruitment both at USZ and AZW is by interviews with the allergologist during pre-AIT consultancy. Alternatively, telephone interviews of patients scheduled for an AIT appointment may be applied. An USZ study doctor (Alina Müller) will join the team at AZW and help recruiting and supervising the study subjects.

4.1 Inclusion Criteria

Subjects, who will fulfil all the following inclusion criteria, may be included into this project:

- Males and females of age 18 to 50 years
- History of allergy due to IgE sensitisation to any allergen that is treatable by means of AIT, e.g. grass pollen allergens, three pollen allergens, animal dander allergens, dust mite allergens, or insect venom allergens.
- Scheduled to receive first AIT with Allergovit® oder Polvac™ or to receive Pollinex Quattro™ at AZW.
- Signed written informed consent for subsequent use of coded blood samples including blood leukocytes data and serological data.

4.2 Exclusion Criteria

If a subject fulfils any of the following exclusion criteria, will not be included in the project:

- Previous AIT
- Chronic inflammatory diseases (rheumatic diseases, pyelonephritis, osteomyelitis or others)
- Acute infections
- Drug or alcohol abuse within the last 5 years
- Relevant anaemia (as judged by investigator)
- Blood donation within the last 30 days or during the next 7 days
- Pregnancy or breast feeding
- Systemic glucocorticoid or antihistamine therapy within the last 30 days or during the next 7 days.
- Systemic or local immune drug therapy within the last 30 days during the next 7 days.
- For linguistic and/or cognitive reasons unable to understand the study procedures

4.3 Premature withdrawal criteria

Samples will be withdrawn from the trial if the patients, from whom the blood samples were drawn, meet any of the following criteria:

- marked deterioration of their allergic status as judged by the investigator
- patient or investigator decision
- protocol violation

The samples will then be replaced by samples from another patients who fulfil the inclusion criteria but none of the exclusion criteria.

5 Subject Information and Informed Consent

The patients are informed in writing and verbally on:

- The purpose and duration of, and procedure for, the research project.
- Their right to withhold or to revoke their consent at any time without giving reasons.
- The consequences of revocation of consent for the biological material and personal data used up to this point.
- Their right to receive information at any time in response to further questions relating to the research project.
- Their right to be informed of results concerning their health, as well as their right to disclaim that information; or to designate a person who is to take this decision for them.
- Measures to protect the biological material and the personal data.
- The main sources of financing for the research project.

6 Project Data and Samples

Upon enrolment in the trial, a patient will undergo a short physical examination and demographic details will be obtained and recorded in the CRF. Blood is then sampled for baseline determination

of test parameters (blood and serum analysis). On the next experimental study days (1 and 7 days later), a short examination with questionnaire is performed and blood is again drawn for analysis.

6.1 Sample generation and processing

Visit 1 (V1; screening and baseline laboratory)

A signed “Informed Consent” will be obtained from each patient prior to initiating any trial procedure. The patient eligibility will be determined in accordance with the inclusion/exclusion criteria. Further, a medical history and medication use will be obtained and recorded, and a short physical examination will be performed. Venous blood will be taken for the determination of lab parameters: two red-cap vials (serum) of each approx. 6 ml for serum analysis, one green-cap vial (heparin) of approx. 3 ml for acute-phase protein analysis and one violet-cap vial (EDTA) of ca. 2 ml for whole blood analysis. The study subjects will then be dismissed to receive AIT with their allergologists and asked to return next day at the same time point. The study subjects will also be asked to consume foods and drinks similar to that of the visit 1 day.

Visit 2 (V2; one day post V1)

Visit 2 will be performed 24 hours after visit 1. It is important that the patient present at the same time of the day as for visit 1. Venous blood will be sampled for the determination of lab parameters as for the previous day: two red-cap vials (serum) of each approx. 6 ml for serum analysis, one green-cap vial (heparin) of approx. 3 ml for acute-phase protein analysis and one violet-cap vial (EDTA) of ca. 2 ml for whole blood analysis. The patients will be asked to return six days later at the same time point and to consume foods and drinks on the morning of the visit similar to that of the days of visits 1 and 2.

Visit 3 (V3, seven days post V1)

Visit 3 will be performed seven days after visit 1 and six days after visit 2. It is important that the study subjects present at the same time of the day as for visits 1 and 2. Venous blood will be sampled for the determination of lab parameters: two red-cap vials (serum) of each approx. 6 ml for serum analysis, one green-cap vial (heparin) of approx. 3 ml for acute-phase protein analysis and one violet-cap vial (EDTA) of ca. 2 ml for whole blood analysis.

Visit 4 (V4, 49 day post V1, final visit)

Visit 4 will be performed ca. 49 (±3 days) after visit 1. It is important that the study subjects present at the same time of the day as for the previous visits. Venous blood will be sampled for the determination of lab parameters: one red-cap vials (serum) of 10 ml for serum analysis.

Physical examination and questionnaire

Mild adverse events may follow AIT, and although not part of the study and not mandatory for observational studies, the study doctor will assess by examination of the site of AIT injection and by questionnaire, to what extent the study subjects reacted to the AIT and whether the reactions varied across the three AIT regimens applied. The parameters assessed are local reactions such as pain, itch, erythema, and swelling, as well as systemic reactions such as fever, irritability, drowsiness, loss of appetite, vomiting. In addition, a photograph of the site of AIT injection is made. Questionnaires and the photographs are stored in the CRF.

6.2 Analysis of samples and data

Whole blood and serum will be sampled and analysed. The volume of blood sampled from patients completing the whole study is approximately 85 ml within seven days (25 ml on each of the visits V1, V2, and V3 and 10 ml on V4). EDTA analysis is submitted for whole blood analysis to Haematology at USZ or *Labor Fleischauer* in Wiesbaden on the day of sampling. Serum blood is processed for production of serum at USZ or AZW on the day of sampling and stored at -20 °C for later protein and antibody analysis.

Blood leukocyte composition

The composition of blood leukocytes from whole blood is measured by standardized and certified methods. The blood collection is made in EDTA vacutainers and the analysis is made directly after sampling. Total leukocyte and total white leukocytes counts are determined as well as the frequency of leukocyte sub-populations such as T and B lymphocytes, neutrophils, monocytes, eosinophils, and basophils.

Protein array

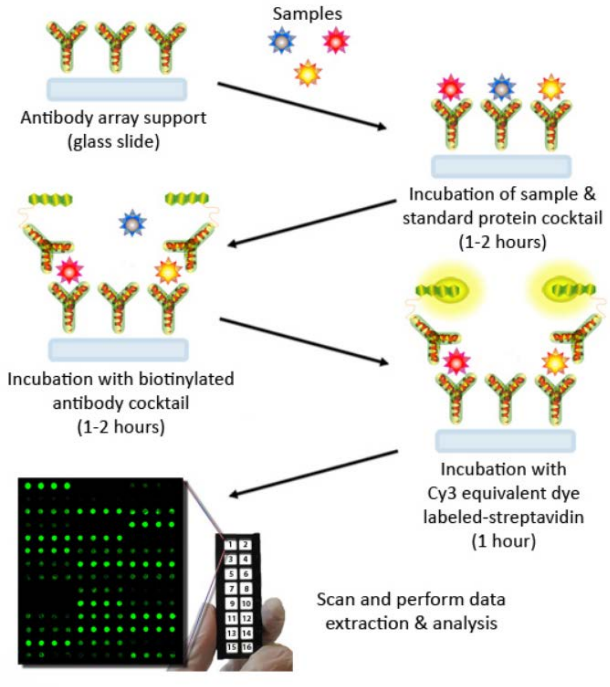
Briefly, inflammatory cytokines and other proteins will be measured in serum by means of a quantitative protein array according to a protocol provided by the producer (RayBiotech®). The array is a multiplexed sandwich ELISA-based quantitative array that combines the advantages of the high detection sensitivity & specificity of ELISA and the high throughput of arrays (**Fig. 2**). We will use glass slides that are pre-coated with antibodies against 120-200 inflammatory cytokines, chemokines, and growth factors.

Enzyme-linked ImmunoSorbent Assay (ELISA)

Proteins, which secretion into blood is notably reduced or increased as suggested from the results of the protein array, will be re-tested for confirmation by using specific quantitative ELISAs according to protocols provided by the producer (eBioscience®). These ELISA methods a well standardized and established in the laboratory at USZ.

Acute-phase protein analysis

Inflammation is accompanied by many changes, far from the site of inflammation and involving many organ systems [10]. We do not expect the secretion of acute-phase proteins to be highly elevated upon AIT. However, AIT and adjuvants are by nature inflammatory, hence, changes may be detectable, but such data is typically not collected for AIT in clinical testing or use. Therefore, we plan to analyse the secretion of acute-phase proteins such as, but not exclusively, aspartate-aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyl transferase (GGT), C-reactive protein (CRP), and IL-6 by accredited methods at the USZ Department of Clinical Chemistry.



AREG	CXCL10	IL12B	SPP1
AXL	CXCL11	IL13	TGFA
BDNF	CXCL12	IL15	TGFB1
BMP4	CXCL13	IL16	TGFB3
BMP5	CXCL16	IL17A	TIMP1
BMP7	CXCL2	IL17F	TIMP2
BTC	CXCL3	IL18BP	TNF
CCL1	CXCL5	IL1A	TNFRSF11B
CCL11	CXCL6	IL1B	TNFRSF1A
CCL13	CXCL8	IL1RN	TNFRSF1B
CCL14	CXCL9	IL2	TNFSF14
CCL15	EGF	IL31	TSLP
CCL16	EGFR	IL4	VEGFA
CCL17	FGF2	IL5	XCL1
CCL18	FGF4	IL6	
CCL19	FGF7	IL6R	
CCL2	FIGF	IL7	
CCL20	FLT4	IL9	
CCL21	GDF15	INS	
CCL22	GDNF	KDR	
CCL23	GH1	KIT	
CCL24	HBEGF	KITLG	
CCL25	HGF	LIF	
CCL26	ICAM1	LTA	
CCL27	IFNG	MIF	
CCL28	IFNL1	MST1	
CCL3	IFNL2	NGF	
CCL4	IGF1	NGFR	
CCL5	IGFBP1	NTF3	
CCL7	IGFBP2	NTF4	
CCL8	IGFBP3	PDGFA	
CSF1	IGFBP4	PDGFB	
CSF1R	IGFBP6	PF4	
CSF2	IL10	PGF	
CSF3	IL11	PPBP	
CXCL1	IL12A	PROK1	

***Figure 2**. Scheme showing the principle how the protein array works (left panel) and the list of anti-proteins antibodies contained in the array (right panel).*

Antibody ELISA

The sera will also be analysed to measure changes in allergen-specific antibodies. Total IgM and allergen-specific IgE are measured by accredited methods at the USZ Department of Clinical Chemistry. Allergen-specific IgM, IgG, IgG1, and IgG4 are measured by standardized and in part accredited methods at the USZ Department of Dermatology. Typically, strong induction of antigen-specific antibody responses may not expected within one week of AIT and by consequent, allergen-specific antibodies are normally monitored at later time points after AIT [11]. Nonetheless, since the AIT patients are already primed through natural sensitisation, the reactivity of the immune system is higher and antibody responses may be detectable earlier after AIT. However, while early detection of IgG or IgE is a result of boosting pre-existing immune responses, the induction of IgM is a primary immune response, precedes IgG and IgE iso-class switching, and typically peaks within 5-7 days of immunization [12]. Since the targeted clinical effect of adjuvants is the early activation of the innate immune system, the different adjuvants tested in the current study may be discriminated in their activation of IgM and other allergen-specific antibody responses.

6.3 Code Administration

When un-coded blood or serum samples are submitted to clinical chemistry (IKC), clinical immunology (AKI), haematology (HAD) or dermatology (DER) for analysis, this carried out internally within the USZ using the clinical information system (KISIM). The results of the analysis is subsequently available in KISIM. The access to KISIM is restricted and secured by password.

All data from analysis will be coded before entered into an Excel database specifically created for this project. Hence, the data analysis is only performed with coded data and patient identities are any more available for persons performing data analysis. The Excel database will be created according to the format and content of the CRF, the study protocol, and the blood and serum analysis performed.

When samples and data are transferred from AZW to USZ, only coded samples and data are transferred. The code for patients at AZW remains with the study-coordinator at AZW (Ingrid Casper). The code for patients at USZ is secured by the QM officer for clinical research at the USZ Department of Dermatology (Séverine Buffoni).

The coded data will be accessible to the project leader at USZ (Pål Johansen), two medical researchers at USZ (Deborah Leuthard and Alina Müller), and the statistician (Nicole Graf).

7 Statistical Plan

7.1 Sample Size Determination

The primary objective of this experimental study is to compare inflammatory responses in blood serum from patients receiving AIT with Alum, MCT, or MCT-MPLA as adjuvants. The study is purely descriptive with regards to an array of 120-200 inflammatory proteins that will be determined in blood serum. Moreover, no hypothesis is or can be made as to how the differential production of these inflammatory proteins can be expected. Therefore, this study does not satisfy any statistical requirement. Twenty-four allergy patients (3 groups of 8) is considered sufficient to acquire the necessary scientific screening.

7.2 Statistical Methods

The investigators and the appointed external biostatistics service group will be responsible for the statistical analysis of the collected data. The statistical software used will be Graph Pad Prism®, SPSS®, or open-source software "*The R Project*".

The results from the array analysis will used to generate heat maps, and for the hierarchical clustering of AIT-adjuvant and protein-secretion cluster phenotypes, we will apply *The R Project* with Euclidean metrics.

Data from all clinical assessments whether explicitly referred to in the statistics section or not, will be presented in summary tables. Data will be summarised primarily with respect to the type of AIT received. In addition, the data may be summarized with regards to demographic characteristics or with regards to clinical observations, e.g. reported side effects of AIT.

- Standard descriptive summary statistics will be calculated for continuous variables, e.g. serum concentration of proteins and antibodies (arithmetic mean, standard deviation, minimum value, lower quartile, median, upper quartile, maximum value, number of non-missing values).
- Heat map and cluster analysis will be applied for the original protein array data after normalisation of the data for appropriate heat-map analysis.
- Categorical data will be presented in frequency tables using counts and percentages.
- Individual patient data listings will be presented by parameter and will be sorted by treatment group, patient number, and visit number. Summary tables will be displayed by treatment group and for the total of the sample.
- All p-values and confidence levels of inferential statistical methods will be interpreted in the exploratory sense.

***Analysis sets**:* A criterion to enter the study analysis is that the study subject completed the study with four blood samples drawn according to protocol (no protocol violation), and that all samples could be analysed according to plan. Hence, only one data set will be analysed. This full analysis set will consist of all patients for whom values for inflammatory protein assessment and specific-antibody assessment have been obtained. The data set will be divided in three subgroups, namely patients that originally reived AIT with (i) Allergovit®, (ii) Polvac™, and (iii) Pollinex Quattro™.

***Baseline and demographic characteristics**:* Assessments made at the screening (V1) and the experimental visits (V2+V3+V4) will be summarised. These assessments will include demographic characteristics and other relevant parameters, e.g. medical history and physical examination.

Summary tables will be provided for the full analysis set by means of descriptive statistics and frequency tables where appropriate.

8 Independent Ethic Committee

Before this project will be conducted, the protocol, the proposed participant information, consent form, and other project-specific documents will be submitted to a properly constituted Independent Ethics Committee (IEC), for formal approval, the IEC being the *Kantonale Ethikkommission Zürich*. The start of the project will not be commenced until after the decisive approval of the IEC is achieved in writing to the Project Leader. For blood samples obtained from patients visiting AZW, ethical approval is obtained from the IEC in Mannheim (*Ethik-Kommission der Medizinischen Fakultät Mannheim/ Medizinische Ethikkommission der Universität Heidelberg*).

Significant changes to an authorized research project must be approved by the IEC before being implemented. Exempt from this requirement are measures which have to be taken immediately in order to protect the participants.

The following are considered to be significant changes:

- changes affecting the participants' safety and health, or their rights and obligations;
- a change of research site or conducting the research project at an additional site; or
- a change of the project leader or sponsor.

The IEC will be informed within 90 days regarding the termination or completion of the project.

9 Confidentiality and Safe Handling of Samples and Data

Data generation, transmission, archiving, and analysis of health related personal data within this project strictly follow the current Swiss legal requirements for data protection and according to the Ordinance HFV Art. 5. Prerequisite is the voluntary approval of the participant given by signing the informed consent prior start of participation of the research project.

Health related personal data captured during this project from participants are strictly confidential, and the disclosure of such data to third parties is prohibited. Confidentiality will be ensured by using coded data. The code will be protected against unauthorized access and will be stored appropriately with the QM clinical research officer at USZ Dept. Dermatology (Séverine Buffoni), or with the study-coordinator at AZW (Ingrid Casper).

The anonymised serum samples will be stored at USZ for 5 years after termination of the project and then disposed.

9.1 Proof of secure handling of samples and data and the storage thereof

Health-related personal data captured during this project from participants are strictly confidential and disclosure to third parties is prohibited. Coding will safeguard participants' confidentiality.

The electronic case report forms (CRFs) will be entered into an excel sheet, which is secured using a password. The excel document is kept on stored on a USZ Server with restricted access. \\fs-group\der_forschungen\Der_FL_Immunotherapie

The project site will retain all essential documents according to national legal and regulatory requirements, including source data and CRF. Source data are all information in original records and certified copies of original records of clinical findings, observations, or other activities necessary for the reconstruction and evaluation of this project. All essential documents are stored until 10 years after the termination of the project, during which time the project leader guarantees access and availability of the data at any time.

Confidentiality will be ensured by entering only coded data into the analysis set. This coded data is entered into an Excel-based database. The coded data will be password-protected and accessible to the project leader at USZ (Pål Johansen) and two investigators two researchers at USZ (Deborah Leuthard and Alina Müller). The statistician will obtain copies of the Excel database after the data entry has been completed. Coded research data are not considered part of the essential documents and will be stored on a central data server at the USZ with restricted access: \\fs-group\der_forschungen\Der_FL_Immunotherapie

This project also concerns the re-usage of sera data generated at AZW in Wiesbaden. Patient names or details that identify the AZW patients will not follow the sera transfer to USZ in Zurich, i.e., only coded samples and data are transferred. The code remains in at AZW.

The code for data generated at USZ is not available for any researchers in this project, but is kept with the QM clinical research officer at the Department of Dermatology, USZ (Séverine Buffoni).

Processed or analysed coded data will be stored in the analysis software (Prism®, SPSS®, and The R project). This coded data will be accessible to the project leader at USZ (Pål Johansen), two medical researchers at USZ (Deborah Leuthard and Alina Müller), and the statistician, but the data will not be password-coded.

9.2 Coding of samples and data

At UZH, the code of the samples and the data is hold by the Clinical research Quality Manager at Department of Dermatology, USZ (Séverine Buffoni). At AZW, the code of the samples and the data is hold by the study-coordinator at AZW (Ingrid Casper).

The code holders will annotate numerical codes in a non-randomized way (at AZW: USZ-AZW 001-008; at USZ: USZ-AZW 009-0024) as patients enters the study blood and first blood is collected, i.e., first patient donating blood gets the code number “USZ-AZW 001” and last patient donating blood getting the number “USZ-AZW 024”.

9.3 Storage period after finalization of the research project

The anonymised serum samples will be stored at USZ for 10 years and then disposed.

The anonymised analysed data will be stored at USZ for 10 years and then erased.

The CRFs will be stored at USZ for 10 years and then disposed.

10 Funding and Support

All personnel and material are available with support by University Hospital Zurich (USZ) and the University of Zurich (UZH) and by *Allergiezentrum* Wiesbaden (AZW).

11 Publication

After the statistical analysis of this trial the project leader will make every endeavour to publish the data in a medical journal.

12 Signatures

The following people agree with the content of this project by signing this protocol. Changes concerning the responsibilities of every person signing this protocol need to be announced immediately.

Sponsor and Project Leader

I hereby confirm that this project plan was subjected to a critical review according the moral, ethical and scientific principles governing clinical research and approved/released by myself. I confirm that samples and data will be treated and used according to national legal requirements of data security.

Prof. Dr. Pål Johansen
University Hospital Zurich
Department of Dermatology

Zurich, 5.7.2019 

Place, Date | Signature

13 References

1. Gunawardana & Durham. New approaches to allergen immunotherapy. Ann Allergy Asthma Immunol 121, 293-305 (2018).
2. Shamji, et al. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper. Allergy 72, 1156-1173 (2017).
3. Calderon, et al. One hundred years of allergen immunotherapy European Academy of Allergy and Clinical Immunology celebration: review of unanswered questions. Allergy 67, 462-476 (2012).
4. Larsen, et al. Allergy immunotherapy: the future of allergy treatment. Drug Discov Today 21, 26-37 (2016).
5. Klimek, et al. Clinical use of adjuvants in allergen-immunotherapy. Expert Rev Clin Immunol 13, 599-610 (2017).
6. Ghimire. The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm. Springerplus 4, 181 (2015).
7. Jensen-Jarolim. Aluminium in Allergies and Allergen immunotherapy. World Allergy Organ J 8, 7 (2015).
8. Del Giudice, et al. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Semin Immunol 39, 14-21 (2018).
9. Leuthard, et al. Microcrystalline tyrosine and aluminum as adjuvants in allergen-specific immunotherapy protect from IgE-mediated reactivity in mouse models and act independently of inflammasome and TLR signaling. J Immunol 200, 3151-3159 (2018).
10. Gabay & Kushner. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 340, 448-454 (1999).
11. Senti, et al. Epicutaneous allergen-specific immunotherapy ameliorates grass pollen-induced rhinoconjunctivitis: A double-blind, placebo-controlled dose escalation study. J Allergy Clin Immunol 129, 128-135 (2012).
12. Colino & Outschoorn. Dynamics of the murine humoral immune response to Neisseria meningitis group B capsular polysaccharide. Infect Immun 66, 505-513 (1998).