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List of Abbreviations

ABC	Ammonium Bicarbonate
ABPA	Allergic Bronchopulmonary Aspergillosis
ACN	Acetonitrile
ADP	Adenosine Diphosphate
AP	Alkaline Phosphatase
APS	Ammonium Persulfate
Aqua dest.	Aqua Destillata
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BCR	B-Cell Receptor
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony Forming Unit
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate
cholanoic acid	3α,12α- Dihydroxy-5β-Cholan-24-Oic Acid
CID	Collision-Induced Dissociation
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol

ECL	Enhanced Chemiluminescence
e.g.	Exempli Gratia
ESI	Electrospray Ionisation
et al.	Et Alia
FLTR	From Left to Right
FTMS	Fourier Transformation Mass Spectrometer
GnHCl	Guanidine Hydrochloride
GpFI	Putative Prophage Major Tail Sheath Protein
HDP	Host-Defence Peptide
HPLC-H_2O	High-Performance Liquid Chromatography-Grade Water
HRP	Horseradish Peroxidase
IAA	Iodoacetamide
IEF	Isoelectric Focusing
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG4	Immunoglobulin G4
IgM	Immunoglobulin M
IL	Interleukine
IMPa	Immunomodulating Metalloproteinase
INF-γ	Interferon–γ
IP	Isoelectric Point
LC	Liquid Chromatography

LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
m/z	Mass-To-Charge Ratio
MHC	Major Histocompatibility Complex
MS	Mass Spectrometry
MS/MS	Tandem Massspectrometry
MW	Molecular Weight
NAD^+	Nicotinamide Adenine Dinucleotide
NBT	Nitroblue Tetrazolium
$NADP$	Nicotinamide Adenine Dinucleotide Phosphate
$NADPH$	Reduced Nicotinamide Adenine Dinucleotide Phosphate
OD_{595}	Optical Density at 595 nm Light Wavelength
$\log OD_{595}$	Base 10 Logarimic Optical Density at 595 nm Light Wavelength
PAMP	Pathogen-Associated Molecular Pattern
PLL	Periciliar Liquid Layer
PRR	Pattern Recognition Receptor
P. aeruginosa	*Pseudomonas aeruginosa*
PSGL-1	P-Selectin Glycoprotein Ligand-1
PVDF	Polyvinylidene Fluoride
RHB	Rehydration Buffer
rpm	Revolutions per Minute
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium-Dodecyl-Sulfate Polyacrylamide-Gel Electrophoresis
S. aureus	*Staphylococcus aureus*

T_FH	Follicular T Helper Cell
TBS	TRIS-Buffered Saline
TBS-T	TRIS-Buffered Saline with 0.05% Tween20
TCA	Trichloroacetic Acid
TCR	T-Cell Receptor
TEMED	N,N,N',N'-Tetramethyl-Ethylenediamine
TGF-β	Transforming Growth Factor–β
Th cell	$CD4^+$ T Helper Cell
Th_1 cell	$CD4^+$ Type 1 T Helper Cell
Th_{17} cell	$CD4^+$ T 17 Helper Cell
Th_2 cell	$CD4^+$ Type 2 T Helper Cell
TNF-α	Tumor Necrosis Factor–α
T_{reg}	Regulatory T-Cell
TSB	Tryptic Soy Broth

1. Introduction

The human immune system is characterised as a complex network of immune cells, soluble mediators and its interactions with external influences. It is not only controlled by genetically predetermined pathways but significantly by its interaction with the environment. Robust enough resist the daily contact with pathogens, but at the same time flexible enough to adapt quickly to new challenges, it is one of the most intricate organs of the human body. As such, immunology, as the study of the immune system, operates in an interesting area between clinical application and scientific research. New findings here allow for a better understanding of infections and interactions with the environment, which leads to the design of new diagnostic and therapeutic approaches.

However, the immune system is itself not without failings. Studying bacterial immune escape mechanisms, or pathological immune processes such as allergies or autoimmunity, paves the way for developing preventative measures. This thesis is focused on the capabilities of the bacterial species *Pseudomonas aeruginosa* (*P. aeruginosa*) to skew the immune response to their advantage. For this purpose, the immunogenicity of antigens in the secretome of *P. aeruginosa* was investigated in the specific context of cystic fibrosis (CF).

1.1. General Concepts in Immunology

1.1.1. Innate Versus Adaptive Immunity

Innate Immunity

The term "innate immunity" collectively describes all anatomical, cellular and molecular structures and the processes that provide immunity from birth, and do not require specific antigen recognition. The first defence mechanism is provided by the external barriers of the body, like the skin and mucous membranes. The slightly acidic pH of the skin and the

resident skin flora act as barriers to the invasion of pathogenic microbes. On the mucous membranes, additional defence molecules such as defensins and cathelicidins are present, which are collectively known as host-defence peptides (HDPs)[1]. If these physicochemical barriers are breached, innate cellular and acellular mechanisms of immune defence are activated. One example is the complement system, which attacks the cell walls of bacteria, and does not rely on a cellular response. Conversely, a cell-dependent mechanism is triggered by tissue-resident cells such as dendritic cells, macrophages, and mast cells, which detect the presence of pathogens by recognising evolutionarily highly conserved structures (so-called pathogen-associated molecular patterns (PAMPs)) by means of pattern recognition receptors (PRRs). These cells then release cytokines that start an inflammatory response, which attracts other cells of the innate immune system, exempli gratia (e.g.) neutrophils to the site of infection. When these measures fail, the most effective weapon of the immune system is activated: adaptive immunity[2].

Adaptive Immunity

Unlike innate immunity, the adaptive arm of the immune defence takes more time to mount a response. However, once active, it counters pathogens much more specifically with antibodies (also called immunoglobulins (Igs)), or destroys infected cells with cytotoxic T lymphocytes (CTLs). For this, so called "antigens" serve as the target structure. In most cases, antigens are peptides, but other complex molecules like lipids or oligosaccharides may also be recognised. The adaptive immunity also consists of acellular mechanisms (Igs), as well as cellular (among others: $CD4^+$ T helper cells (Th cells), CTLs and B-cells) actors.

In brief, the adaptive response is based on the activation of antigen-presenting cells, such as dendritic cells (DCs). These themselves are part of the innate immune system, and are located in the tissue and take up antigens from their environment by means of phagocytosis or macropinocytosis. If an antigen is recognised as "foreign" and presented simultaneously with stimulating mediators, the DC is activated, and migrates to the nearest lymph node. Here, the absorbed and processed antigens are presented to passing naïve T cells via either the major histocompatibility complex (MHC) I or II. A naïve T cell is activated when it recognises its specific antigen and is stimulated by mediators signalling infection. It then differentiates into one of several types of effector T-cells. Details on this are described in the

next Section. B-Cells, which produce Igs, are activated similarly. However, they generally require T-cell help for a sustained response.

The immune system is capable of producing antibodies against almost any antigen (conservative estimates assume 10^{11} different specificities[3]). This requires only a few genes, which is made possible by complex processes of genetic, so-called "somatic", recombinations. These result in a variety of Ig specifities and B-cell receptors (BCRs), as well as corresponding T-cell receptors (TCRs). However, describing this process in detail here would exceed the scope of this thesis.

1.1.2. Physiological Immune Reactions

The following Section gives an overview of the various directions of differentiation for naïve T cells and the functions of both Th cells and CTLs will be given. The type 2 immune response will be a focal point. However, for a detailed description of how naïve B-cells are affected, the reader is invited to refer to more detailed immunology textbooks.

Type 1 Immune Responses – Defence Against Intracellular Pathogens

The type 1 immune response describes an immune reaction that is primarily directed against intracellular, cytosolic and intravesicular pathogens, including bacteria such as *Mycobacterium tuberculosis*, protozoa like *Leishmania major*, or viruses such as *Herpes simplex*. A $CD4^+$ type 1 T helper cell (Th_1 cell) dominated response is crucial for the defence against these pathogens. The differentiation of Th_1 subpopulation is driven by interleukine (IL)-12, which is secreted by activated DCs as they present their antigen to naïve T cells via MHC-II. Other cellular players are CTLs (which have their antigen presented via MHC-I), and natural killer cells[4]. Th_1 cells themselves secrete interferon–γ (INF-γ) and IL-2, promoting the differentiation of additional Th_1 cells. Furthermore, macrophages are stimulated to more easily degrade intravesicular pathogens[5]. The humoral arm of the type 1 immune response is dominated by immunoglobulin G (IgG) isotypes 1 and 2.

Type 2 Immune Responses – Defence Against Parasites

The type 2 immune response is directed against multicellular macropathogens such as helminths, that cannot be controlled by phagocytosis due to their size. It is orchestrated by

$CD4^+$ type 2 T helper cells (Th_2 cells). These differentiate from naïve T cells after recognition of their antigen via MHC-II under the influence of IL-4.

The aim of a type 2 immune response is to expel parasites and strengthen the epithelial barrier function. This occurs, for example, through increased epithelial turnover and IL-13 stimulated smooth muscle cell contraction. In addition, there is increased differentiation of alternatively activated M2 macrophages, which maintain the local immune reaction and promote tissue remodelling and reconstruction. Furthermore, eosinophilic granulocytes are activated by IL-5. These are effector cells of the innate immune system, which mount a response against said macropathogens[6].

Particularly relevant with regard to pathological reactions is the follicular T helper cell (T_FH)-induced class change of activated B-cells to immunoglobulin E (IgE). IgE is an immunoglobulin subtype that is bound by mast cells and primes them. Mast cells are typically tissue-resident cells that induce and maintain local inflammation. In doing so, they release mediators such as tumor necrosis factor–α (TNF-α) and histamine, but also interleukins such as IL-4, which can initiate and promote local adaptive immune responses[7]. Mast cells are activated by the cross-linking of the surface-bound IgEs, whereupon they release their mediators[8]. Such a response might be triggered by an innocuous antigen, a phenomenon which is called an allergy. This is further elaborated on in Section 1.2.1.

It must be emphasised that not every type 2 immune response leads to the formation of specific IgE. Alternatively, the formation of immunoglobulin G4 (IgG4) can occur, a subtype associated primarily with a long-lasting tolerogenic response. The relationship between IgE and IgG4 is discussed in detail in the Section 1.2.3.

Type 3 Immune Responses – Defence Against Fungi and Extracellular Bacteria

The type 3 immune response is primarily directed against single-cell, extracellular pathogens, as is the case for most bacteria and fungi. Like the other immune responses, the triggering antigen is recognised by a naive T cell via MHC-II. The Transforming growth factor–β (TGF-β) then drives the differentiation into $CD4^+$ T 17 helper cells (Th_{17} cells), as well as IL-1, IL–6, IL–13. The Th_{17} cells themselves secrete IL-17 and IL-22, which strengthen

the neutrophil response and promote their recruitment to the site of infection. In addition, the production of HDPs is increased. The type 3 immune response is mediated by the IgG subtypes 1 and 3, which are potent opsonising agents[4].

1.2. Pathological Immune Reactions

1.2.1. Allergy and Atopy

In contrast to the previously described reactions, not every immune response is accurate or adequate. Several forms of aberrant responses can be distinguished. Of particular interest for this thesis are allergies. These are defined as the exaggerated or misguided immune response to foreign and innocuous antigens[8].

Current research indicates a multifactorial pathogenesis involving genetic and environmental components that define the susceptibility of an individual for allergies, which are most commonly type I allergies. These rely on the production of IgE specific to an innocuous, environmental antigen. The IgE then primes mast cells. This process is called "sensitisation". Physiologically, IgE is produced during a type 2 immune response against multicellular parasites (see Section 1.1.2). IgE-sensitised mast cells then degranulate on subsequent exposure to the triggering antigen. This releases mediators like histamine and proteases, which cause the typical symptoms of local swelling, hyperaemia and itching. Systemic degranulation of mast cells can lead to life-threatening anaphylaxis[9]. The reason for the generation of such a misguided response is believed to lie in the route and context of antigen exposure. Generally, a prolonged, low dose and transmucosal exposure is thought to favour the generation of an IgE dominated allergic response[10]. To protect itself from such a misguided response, a healthy immune system develops regulatory T-cells (T_{reg}s). These are $CD4^+/CD25^+$ cells, that suppress an immune response upon antigen recognition[8]. Their generation is forced by DCs in the absence of danger signals. However, if this process is disrupted, no T_{reg}s are formed, and an allergy can develop. The general disposition of an individual to generate exaggerated IgE dominated immune responses is known as "atopy". As long as there are no symptoms, atopy is not a disease. However, atopy can develop into manifest disease, which encompasses the classical allergy as well as multiple other illnesses, like allergic rhinitis, atopic dermatitis, and allergic asthma[11].

1.2.2. The Question of Bacterial Allergens and Immune Escape

A new field of open questions emerged with the concept of bacterial allergens. These are proteins produced by bacteria that tend to elicit a type 2 immune response from the host's immune system. While not matching the classical definition of an "allergen" (meaning: an innocuous antigen), their potential to trigger said reaction lead to the adoption of the term "bacterial allergen". Some research suggests, that these proteins might be constructed to specifically skew the immune response. Bacteria that produce these proteins might benefit from doing so by redirecting the immune system into an ineffective response. This is due to the effector molecules of a given response inhibiting the formation of other types of immune reactions, thus masking the producing bacteria from further attack. This phenomenon is one of several "immune escape" mechanisms. Most often, a misdirected type 2 response has been observed: Experimental evidence exists which links atopy to the presence of bacterial allergen-specific IgE[12, 13], as well as research that associates the exacerbation of an atopic disease to a recent exposure to bacterial allergens[14]. However, demonstrating a causal link between the described phenomena has been difficult. Further research aiming to characterise properties of antigens that preferentially elicit a type 2 immune response is needed. While no definitive molecular structure has been identified so far, some general properties of allergens have been determined, which are listed in Table 1.1. Furthermore, many of the suspected triggering proteins are enzymes and especially proteases. For example, it was demonstrated that secreted proteases from *Staphylococcus aureus* (*S. aureus*) posess allergenic properties [15, 16]. Their enzymatic activity was associated with severe allergic inflammation in pulmonary tissue in mouse models. Based on this, an active search for proteins that posess these properties in conjunction with consistent Th_2 cell stimulation has begun.

1.2.3. The Relationship Between IgE and IgG4

Immunoglobulin G4 is a subclass of the immunoglobulin G protein family. IgG4 and its regulation have not been a target of extensive research thus far. However, this subclass of antibody possesses interesting qualities not found in other Igs.

Like other IgG-type antibodies, it is capable of neutralising antigens, and easily diffuses into extravasal sites. However, it provides little stimuli for monocytes, does not trigger the complement system[17] and is almost unable to cross the placental barrier[18]. Most in-

Table 1.1.: Properties of Antigens Favouring a Type 2 Immune Response.
Modified for Secreted Bacterial Proteins in a Pulmonary Environment after Murphy et alia (et al.)[8].

Feature	Mechanism
Protein (often glycosylated)	Especially glycosylated proteins are readily recognised by Th cells. Glycosylation is often found in extracellular proteins.
Low dose	Low dosage of antigens seems to physiologically favour a Th_2 cell driven response.
Low molecular weight	Facilitates antigens diffusing from the broncheolar/alveolar compartment into the mucosal compartment.
High solubility	Favours elution from carrying particles in the broncheolar compartment.
MHC-II binding peptide sequences	Required for T-cell recognition

terestingly, IgG4 is able to form bi-specific antibodies, meaning antibodies which can bind two different antigens. This is due to the fact that the disulphide-bonds linking both heavy chains in immunoglobulins are prone to undergo chemical reduction in IgG4. This leads to the molecule splitting into "half-antibodies", which can spontaneously reassemble with other halves. IgG4 has been linked to reduced severity of allergic symptoms in patients, and has been hypothesised to be a marker of a tolerogenic Th_2 cell immunoresponse[19]. However, the mechanism by which this protection is achieved is still a topic of debate.

IgG4 production is – like IgE production – governed by a Th_2 cell response, and thus, by type 2 cytokines like interleukins 4, 5, 7, 9 and 13, in addition to IL-10[17]. It should be stated, that the IgE class switch can be achieved in two ways: with either a direct switch from immunoglobulin M (IgM)-positive B-cells, or via IgG4 producing plasma cells. This is due to the line-up of the genetic elements encoding the constant domains of the Ig heavy chains in the Ig gene locus. Previous work by Aalberse et al. showed, that not every exposure to typical antigens of allergy leads to an IgE dominated Th_2 cell response. Instead, exposure in sufficient dose or over a prolonged time period results in high levels of IgG4, and no allergic symptoms[20], with no or low levels of IgE. This research suggests, that in a healthy individual, exposure to a potent Th_2 cell inducer leads to a tolerogenic IgG4 re-

sponse with sustained IgG4 production and strong presence of memory cells, and only little IgE during the primary response via secondary plasma cell class switch as described above. This response requires a strong germinal centre reaction only achieved by sufficient antigen exposure. In this scenario, primary IgE-producing B-cells do not survive in the germinal centre, as they are unable to receive the necessary stimulation form T_FHs[21]. On the inverse, the development of classical allergy requires long-lasting, IgE-producing plasma cells that have escaped the negative selection in insufficiently matured germinal centres. This may be due to a short, low-level exposure, which would only lead to an insufficient germinal centre response without memory cell formation.

While IgG4 is not a marker of allergy and is not always associated with the presence of IgE, it has a strong advantage as a target in research: its prevalence. IgG4 is much more abundant in healthy individuals (0.5 mg/ml), in contrast to the low serum levels of IgE (3×10^{-5} mg/ml)[18]. As such, IgG4 is easier to detect in screening assays and is a reliable marker for a Th_2 cell dominated immune response. Thus, it was chosen as a target to identify Th_2 cell stimulating proteins secreted by *P. aeruginosa*.

1.3. Cystic Fibrosis

1.3.1. Aetiology and Pathogenesis

CF is one form of expression of a monogenic, autosomal recessive gene defect caused by a single major or multiple minor mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene encodes a chloride channel, which is significantly involved in the production of serous secretions through the transport of chloride ions into extracellular spaces[22]. The responsible mutations are divided into six categories[23]. Most critical are class I mutations, in which no protein is expressed, followed by class II – IV, in which there is misfolding or loss-of-function. Lastly, there are class V and VI mutations, which are characterised by reduced expression or increased turn-over. So far, over 1900 single mutations have been identified[24].

1.3.2. Symptoms of Cystic Fibrosis

As described above, mutations in the CFTR gene fall into different categories. As such, CFTR-related defects comprise a spectrum of illnesses[25], of which CF is the most severe. It is often caused by the ΔF508 deletion, which is present in around 70% of all CF cases[26].

In public perception, CF is often just recognised as a deterioration of a patient's lung function which leads to a shortened life span. However, CF is more than a pulmonary disease. For example, exocrine – but also endocrine – pancreatic dysfunction is typical. This results in maldigestion and malabsorption, but also in CF-associated type III Diabetes mellitus[27]. In addition, patients often suffer from osteoporosis and have an increased risk of developing liver cirrhosis[28]. Furthermore, due to aplasia of the vasa derferentia associated with CF, almost all male patients are sterile.

However, in the context of the phenomena discussed here, the consequences for lung function and structure are the most relevant. In homeostasis, the smallest air-conducting airways (bronchioles) are covered by a so-called periciliar liquid layer (PLL) (sometimes called "bronchioepithelial lining fluid"). It acts as a protective layer between the epithelium and the overlying mucus, allowing for the proper operation of the ciliary epithelium, whose function it is to expel the mucus together with bound microbes, dust and dirt. Due to the dysfunction of the CFTR-encoded channel, the PLL contains less water and is more viscous, which hinders the ciliary beat and impedes mucociliary clearance[29]. These conditions not only limit lung function, but also lead to structural changes in the bronchioli and bronchi, for example through the development of bronchieectasias, which in turn provide safe niches for microorganisms. This self-reinforcing cycle is deemed to be one key reason for the progressive deterioration of lung function in CF patients[30].

1.3.3. Effects of Cystic Fibrosis on the Lung Micro-Environment and the Host's Immune Function

In homeostasis, a layer of mucus lies on top of the PLL, protecting the delicate airways. To prevent the accumulation of microorganisms, this layer is expelled together with the PLL through mucociliary clearance. If the PLL is too viscous, the mucus can not be expelled. In

this layer, numerous bacteria find ideal growth conditions despite the antimicrobial properties of the mucus[1]. Typical bacteria found in the sputum of CF patients are *Stenotrophomonas spp.*, *Burkholderia spp.*, and *P. aeruginosa*[31], all of which are facultative pathogens. Thus, the antigen-rich mucus is in constant contact with the epithelial lining of the airways, which contains epithelial cells, as well as, numerous immune cells such as DCs, but also lymphoid tissue[32]. Accordingly, the immune system of CF patients is in close and prolonged contact with their pulmonary microbiome. This route of exposure may play a role in the elicited immune response.

Furthermore, the elicited immune response itself has an impact on the disease progression. Persistent inflammation of the lower airways has been linked to worse lung function[33], possibly caused by pulmonary remodelling[34]. This is of particular interest in the context of a frequent CF complication: allergic bronchopulmonary aspergillosis (ABPA)[35]. ABPA is characterised as the bronchiolar hyperreactivity to antigens from *Aspergillus fumigatus*, a common mould[36]. Previous research demonstrated a possible link between an atopic, Th_2 cell dominated phenotype, and both CF and ABPA[37].

1.4. *Pseudomonas aeruginosa*: a Critical Pathogen in the 21st Century

Pseudomonas aeruginosa is a rod-shaped, gram negative and lophotrichously flagellated bacterium. As a species of the genus *Pseudomonas*, it can be found in almost all moist environments. Both natural surfaces such as stone or soil but also artificial substrates such as plastic (especially ventilation tubes) and ceramics can be colonised by pseudomonads. Furthermore, they are extremely resistant to both biotic and abiotic environmental stressors. *P. aeruginosa* colonises not only the environment but can also be detected on the mucosa of the human respiratory tract. Here, *P. aeruginosa* shows some adaptations to this inhospitable environment. For example, bacterial colonies protect themselves from the immune system by forming biofilms[38]. Furthermore, the lack of available iron, essential for the biochemistry of most bacteria, is countered by pseudomonads producing siderophores. These are iron-collecting proteins that are released into the environment[39]. The infections caused by *P. aeruginosa* cover a broad spectrum, from wound infections, to severe – especially

ventilator-associated – pneumonias. In addition, *P. aeruginosa* is intrinsically resistant to many commonly used antibiotics. Resistance to most cephalosporins, a widely used and broadly effective class of antimicrobials, is particularly critical. In recent years, the situation has worsened and the antibiotic resistance crisis is a major health problem of the 21st century. For example, strains acquired in hospitals are particularly often resistant to the antimicrobial drug class of carbapenems, which are already considered reserve antibiotics[40, 41]. These developments led to the inclusion of *P. aeruginosa* on the priority list for the development of new antimicrobial agents of the World Health Organisation[42] as early as 2017.

1.5. Cystic Fibrosis and *Pseudomonas aeruginosa* as a Model

The pulmonary micro-environment of CF patients is immunologically interesting, as any immune response generated is highly dependent on the route and duration of antigen exposure. In this case, the proteins secreted by bacteria are in direct and persistent contact with the epithelium of the airways, which contains immune cells. This continuous stimulation is suspected to provoke a Th_2 cell dominated immune response[43]. Furthermore, previous research by Clerc et al. demonstrated that CF patients show significantly increased IgG4 titres[44]. This in turn indicates a strong Th_2 cell-dominated response, preventing an effective antibacterial defence. Additionally, *P. aeruginosa* is particularly prevalent in patients with CF. By age 18, around 60% carry *P. aeruginosa* within their respiratory tract, and the prevalence increases with age[45]. These factors combined qualify an examination of the immune reaction of CF patients as particularly interesting for a preliminary study of putative Th_2 cell stimulating bacterial proteins.

2. Aims

This thesis aimed to identify secreted proteins from *P. aeruginosa*, that are bound by specific IgG4. This implies that they are likely recognised by Th_2 cells in the context of a type 2 immune response. Type 2 responses are physiologically targeting multicellular parasites, but are ineffective against bacteria like *P. aeruginosa*. Bacteria are known to manipulate the host's immune system by secreted proteins. Proteins that are prone to elicit a type 2 immune response, including IgE and Th_2 cell cells, are known as allergens. Since IgE and IgG4 are generated under similar immunological conditions, IgG4 was used as a proxy for IgE. This increases the sensitivity of the search, as IgG4 is present in plasma at much higher concentrations than IgE.

To further optimize the sensitivity of the search for proteins of *P. aeruginosa*, CF patients who are extensively exposed to *P. aeruginosa* in their lungs over a long time were studied. The overarching goal was to identify secreted, IgG4 reactive proteins of *P. aeruginosa*, and contextualising their specific qualities with other secreted proteins of *P. aeruginosa*.

Examining host-pathogen interactions is crucial for understanding the way the human immune system interfaces with its environment. Furthermore, discovering new putative Th_2 cell stimulating proteins could hint at causative structural and functional properties of allergens, which is of general interest considering the high prevalence of allergic diseases. This would have further implications in treating misdirected immune reactions like allergies and auto-immune diseases, but also in the design of sub-component vaccines.

3. Materials and Methods

3.1. Protein Extraction and Purification

The basis for the conducted experiments were formed by 23 *P. aeruginosa* isolates from 7 CF patients, in conjunction with 33 serum samples obtained from the same patients. These were acquired during hospitalisations for exacerbations of CF (e.g. worsening lung function, acute bronchitis, pneumonia). All samples were provided by the Department of Clinical Microbiology of the University Hospital of Münster. Furthermore, the *P. aeruginosa* reference strain PAO-1 was examined. Table A.1 gives an overview of the association of strains and sera.

3.1.1. *P. aeruginosa* Growth Curves

The aim of this thesis was to investigate the secreted proteins of *P. aeruginosa*. Therefore, it was decided to precipitate the targeted proteins from the supernatant of liquid cultures. For this purpose, it was necessary to characterise the growth behaviour of the investigated strains. The precipitation method had to yield sufficient amounts of protein, but also minimise contamination with intracellular proteins caused by cell lysis. Preliminary experiments determined the optimum point in time for precipitation to be around 3 hours after reaching the stationary growth phase.

Hence, the time for transitioning into stationary growth had to be experimentally determined for each strain. For this purpose, the strains were first plated out of glycerol stocks on Columbia blood agar and incubated overnight at 37 °C. From these, one colony forming unit (CFU) was added to each of a 10 ml tryptic soy broth (TSB) pre-culture in a 50 ml Falcon tube and incubated overnight in a rotary incubator at 200 revolutions per minute (rpm) and 37 °C. The following day, the optical density at 595 nm light wavelength (OD_{595}) of these

$$V_{in} = \frac{V_{culture} \times OD_{595}(culture)}{OD_{595}(PC)} \tag{3.1}$$

Equation 3.1: Calculation of Inoculation Volumina.
V_{in}: Volume for Inoculation. $V_{culture}$: Target Culture Volume. $OD_{595}(culture)$: Targeted OD_{595}. $OD_{595}(PC)$: OD_{595} of the Pre-Culture

cultures was measured. A plain TSB sample served as a reference. From the OD_{595} of the pre-culture, the required inoculation volume for the main cultures could then be calculated according to formula 3.1. An OD_{595} of 0.05 was set as the target for inoculation.
Cultures were inoculated from the pre-cultures with the calculated volume and incubated in a linear shaker water bath at 100 rpm and 37 °C. A sample was taken every 60 minutes and the OD_{595} was measured. To ensure accurate results, samples with a OD_{595} greater than 1.0 were diluted 1:10 with TSB and measured again. This accounted for possible technical errors caused by the photometer. The measured density was then plotted both linearly and logarithmically. Further values were recorded until the logarithmic curve showed the transition from exponential to stationary phase. After this, three more data points were obtained to ensure transition into stationary growth.

3.1.2. Precipitation of Secreted Proteins

Analogous to the growth curves, the strains for production cultures were first plated from glycerol stocks on Columbia blood agar and incubated at 37 °C overnight. After this, one CFU was transferred into a 10 ml TSB pre-culture and incubated again overnight at 37 °C and 200 rpm in a circular shaker. Subsequently, the 600 ml production cultures were inoculated from the pre-cultures, according to the target OD_{595} of 0.05, and then incubated at 100 rpm in a linear shaker water bath at 37 °C until their specific precipitation time was reached.

For protein precipitation, the cultures were transferred to centrifuge containers and centrifuged for 10 minutes at 8500 rpm and 4 °C. The supernatants were decanted and sterilised using a 0.22 nm water jet filter. A mixture of 99.6% trichloroacetic acid (TCA) and 0.4% cholanoic acid[1] was then added to the supernatants at a ratio of 1:10. The precipitation process was carried out at 4 °C for 36 hours. Subsequently, the supernatants were centrifuged

[1] 3α,12α- dihydroxy-5β-cholan-24-oic acid (cholanoic acid)

again at 13000 rpm and 4 °C for 10 minutes. The supernatants were decanted again and the pelleted proteins resuspended in 70% ethanol cooled to -20 °C.

3.1.3. Cleaning of Protein Samples

The ethanolic protein suspensions were divided into 1 ml aliquots and kept on ice permanently. For purification, the aliquots were centrifuged at 13000 rpm for 7 minutes, decanted and taken up again in 70% chilled ethanol, and then placed in a shaker at 1400 rpm until complete resuspension. This process was repeated 3 times and a final time with pure ethanol ($\sim$ 96.9% purity). Samples intended for immunoblotting and liquid chromatography tandem mass spectrometry (LC-MS/MS) were additionally treated with a commercial kit ("2-D Clean-Up Kit") according to the manufacturer's instructions.

For storage, the samples were decanted and dried with a vacuum rotary evaporator (setting V-AL) for 10 minutes. The resulting pellet was transferred to 2D immunoproteomics rehydration buffer (RHB) or (for LC-MS/MS) CHAPS[2]-free RHB. Composition of the buffers can be found in Appendix C.

3.2. Protein Quantification by Bradford Assay

Bradford protein quantification is an established and well verified method for rapid concentration determination of a protein mixture[46]. The process is based on non-covalent interactions of Coomassie G-250 with functional groups in proteins. This results in an absorption shift in UV-Vis photometry from 465 nm to 595 nm, which can be easily detected. The protein concentration is calculated by means of a linear regression from a standard curve, which is generated utilising bovine serum albumin (BSA) as a reference protein.

Table 3.1.: Set-Up for Bradford Standard Curve.

µl BSA (0.1 µg/µl)	0	10	20	40	60	80	100	120
µl water	800	790	780	760	740	720	700	680
µl Bradford stock solution	200	200	200	200	200	200	200	200

[2] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

$$OD_{595}(Stand) = ac_{stand} + b \tag{3.2a}$$

$$c_{Sample} = \left(\frac{OD_{595}(Sample) - b}{a}\right) \div k \tag{3.2b}$$

Equation 3.2: Protein Quantification Calculation.
3.2a: Standard Linear Regression Formula. $OD_{595}(Stand)$: Measured Extinction of a Given standard. c_{stand}: Concentration of a Given Standard. a,b: Derived Parameters of Linear Regression. **3.2b:** Calculation of Sample Concentration. c_{Sample}: Calculated Concentration. $OD_{595}(Sample)$: Measured Extinction of Given Sample. a,b: Derived from Equation 3.2a. k: Correction Coefficient to Adjust for Possible Dilution.

Table 3.2.: Bradford Concentration Determination: Sample Preparation.

	V_{Sample} (µl)	V_{Water} (µl)	$V_{Bradford}$ (µl)	coefficient k
Pres. low concentration	5	795	200	0,2
Pres. high concentration (Pre-dilution 1:10)	10	790	200	1
Pres. very high concentration (Pre-dilution 1:10)	1	799	200	10

The exact concentrations for the standard curve are listed in table 3.1. The samples were mixed with the Bradford reagent, vortexed and incubated in darkness for 5 minutes. Afterwards, the OD_{595} was measured. A linear regression curve was formed from the data obtained (see equation 3.2a). The regression curve was accepted as valid if the coefficient of determination $R^2 > 0.95$. To calculate the concentration of unknown solutions, samples were prepared and measured according to Table 3.2 and treated as detailed above. Protein samples likely to have a particularly high concentration of proteins were pre-diluted 1:10 with TSB. The recorded OD_{595} was then entered into the regression formula together with the corresponding correction coefficient k (see equation 3.2b). To obtain reliable results, each sample was measured with a technical replicate.

3.3. SDS-PAGE

After sample purification, integrity was verified by one-dimensional sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE). For this purpose, self-cast polyacrylamide-based separation and stacking gels were used (See Appendix C for the exact composition). Each well was loaded with 12 µl of protein mixture. This mixture was composed of 3 µl

3× loading buffer, a volume corresponding to 15 µg protein sample and high-performance liquid chromatography-grade water (HPLC-H_2O) up to the final volume to 12 µl. These samples were then denatured at 95 °C for 5 minutes. The prepared gels were fixed into the electrophoresis chamber, covered with sodium dodecyl sulfate (SDS) running buffer and loaded with the protein samples and a mixture of markers (range 10 – 245 kDa).

Electrophoresis was then started at 80 V until the samples had migrated from the stacking gel to the separation gel. After this, the voltage was increased to 120 V. The electrophoresis ran until the running front reached the bottom of the gels. To visualise the protein bands, the gels were stained overnight in a colloidal Coomassie G-250 staining solution and rinsed the following day followed by photo-documentation.

3.4. Semi-Automated One-Dimensional Immunoblotting Using PEGGY-Sue

In order to focus further experimentation to the most promising combinations of secretome and reactive serum, a screening assay was needed. Semi-automated, one-dimensional immunoblots were the most suitable choice. The PEGGY-Sue system from Protein Simple was available as a commercial solution. It consists of a capillary electrophoresis system coupled with an enhanced chemiluminescence (ECL) detector array. Up to 96 samples per run (consisting of 8 cycles, 12 samples per cycle) can be separated according to their molecular weight and tested in adjustable combinations against any primary antibody. As a detection system, the use of horseradish peroxidase (HRP)-conjugated secondary antibodies for ECL is available. For this study, murine anti-human-immunoglobulin G1 (IgG1)- and anti-human-IgG4-HRP conjugates were used as secondary antibodies.

The assays were prepared with a kit supplied by the manufacturer. Molecular weight (MW) target range for protein size was set at 12 – 230 kDa, which determined the separation matrix used. Sample concentrations were first normalised to 1 µg/µl using 0.1× sample buffer and 5× master mix, with a target volume of 5 µl per well. In addition, a mixture of biotinylated ladder proteins was prepared following the manufacturer's instructions. This allows the detector array to determine the mass of a protein located at any given measurement point. All

samples were then denatured at 95 °C for 5 minutes. The primary and secondary antibody dilutions were prepared 1:50 with the diluent provided. This diluent also acts as a blocking buffer. Finally, samples, antibody dilutions, a provided streptavidin-HRP-conjugate, detection solution, block buffer, liquid separation matrix and cooling water were pipetted into a 384-well assay plate. The plate was then loaded into the PEGGY-Sue together with the run and wash buffers and the required sample capillaries.

Instructions were set using the manufacturer's software before a run was started. Therein, the location of samples and reagents on the assay plate and experimentation cycles are defined. In a run, the equipment takes up a protein sample as well as the protein ladder into a capillary, and subjects it to electrophoresis through a liquid matrix. This separates the contained proteins by MW. The proteins are then probed with a set primary and secondary antibody, as well as the detecting agent. The equipment records chemiluminescence data for each capillary with different exposure times, and plots them against the detected molecular weight by comparing the signal position with the position of the ladder proteins. This allows a statement to be made about the reactivity of a secretome/serum pair. It also provides information about the MW of reactive proteins.

3.5. Isoelectric Focusing and Two-Dimensional SDS-PAGE

The combinations of secretome and serum sample determined using PEGGY-Sue were examined in more detail using two-dimensional SDS-PAGE. This method consists of two phases. First, proteins are separated according to their isoelectric point (IP), and then according to their MW, resulting in two dimensions. The IP is defined as the pH at which the positive and negative charges on a peptide balance out[47]. This can be determined experimentally by isoelectric focusing (IEF). Here, the proteins are subjected to a voltage and thereby forced to migrate through a matrix with a defined pH gradient. If the positive and negative charges on the protein equalise at a position, the protein begins to behave in an electrically neutral manner, whereby no further electrical drag is exerted on the protein.

To prepare the samples, 100 µg of protein in RHB was mixed with 94 mmol of dithiothreitol

(DTT). DTT is a stabiliser often used for electrophoreses, as it prevents the formation of dimers (e.g. via disulphide bridges). The native samples prepared in this way were then loaded into a IEF chamber and covered with a matrix strip with a fixed pH gradient. A pH-range of 4 – 7 was used for these experiments. To prevent drying out, the strips were covered with mineral oil. The protocol used for the IEF is detailed in Table 3.3. This is an adapted standard IEF protocol from Cornell University[48]. The duration of the individual steps is given in *Vh* (Volt hours). *Vh* are a measurement of the displacement of a charged particle in an electric field. This is expressed as a function of migration velocity of the particle and time[49]. Relying on *Vh* compensates for differences in gel size and conductivity (e.g. due to moisture, salinity, contact area) of different strips and thus, the results of different experiments are more comparable[50].

Table 3.3.: Set-Up of IEF Experiments.
Temperature was Maintained at 20 °C throughout the Procedure.

Step	Slope	Voltage	Duration
1	rapid	50	12:00 h (active rehydration)
2	rapid	200	00:01 h
3	rapid	3500	until 2600 Vh
4	rapid	3500	until 5200 Vh
5	rapid	500	on hold, at least until 8000 Vh

After IEF, the strips still had to be equilibrated. This denatures the immobilised proteins for separation by MW. For this purpose the strips were rinsed with SDS running buffer to remove mineral oil residues. The strips were then incubated in 130 mM DTT equilibration buffer for 15 minutes and rinsed again. This was followed by another 15 min incubation in 135 mM iodoacetamide (IAA) equilibration buffer, as well as with another rinse. After equilibration, the strips were either stored at -80 °C for later analysis or fixed on a SDS-PAGE separation gel together with a protein marker using agarose gel (see Appendix C for composition). The gel electrophoresis was then performed using the same parameters as detailed in Section 3.3. The resulting gels were either stained using colloidal Coomassie or used for immunoblotting.

3.6. Immunoblotting

Immunoblotting describes the process by which proteins are transferred and bound to a polyvinylidene fluoride (PVDF) membrane by means of western blotting, and then sampled using specific, so-called primary, antibodies. Antibodies that have bound their antigen on the membrane can then be detected using secondary anti-immunoglobulin antibodies. In this case, patient sera were used as the source of primary antibodies. The secondary antibodies were either anti-IgG1/anti-IgG4-HRP conjugates or anti-IgG-alkaline phosphatase (AP) conjugates (see Section 3.8).

First, the proteins separated by 2D-SDS-PAGE had to be transferred to the immunoblot membrane. For this, 6 Whatman filter papers per membrane were cut to size. The PVDF membrane was then activated with a 15 s incubation in 100% methanol and rinsed with Aqua destillata (Aqua dest.) for 2 minutes. The membrane, filter papers and gel were then saturated in transfer buffer for 5 minutes. After this, the transfer stack was assembled, consisting of 3 filter papers as a base, the membrane, the SDS gel and again 3 filter papers. This stack was then loaded into the transfer equipment and the transfer was started with 80 mA per stack for a duration of 100 minutes. The successful transfer was verified with a non-specific Ponceau-S stain. After photo-documentation, the stain was removed by several washes with TRIS-buffered saline (TBS).

To prevent non-specific binding of Igs, an initial blocking was performed by incubating in block buffer for one hour. This was followed by incubation with the primary antibodies. For this purpose, a 1:1000 antibody dilution was prepared in block buffer. The PVDF membranes were then placed in 50 ml conical tubes along with 5 ml of the antibody dilution, and incubated at room temperature in rotation overnight.

The following day, the membranes were rinsed three times in TRIS-buffered saline with 0.05% Tween20 (TBS-T) for 10 minutes each. This was followed by incubation with the secondary antibody, which were suspended 1:10,000 in block buffer. The antibodies used for ECL were murine anti-human-IgG1/IgG4 antibodies to which HRP was bound. After incubation, the membranes were again rinsed three times with TBS-T for 10 minutes.

For detection by ECL, the membranes were covered with 1 ml of a 1:1 mixture of luminol and buffered hydrogen peroxide and incubated for 5 minutes. Afterwards the membranes were scanned with an ECL imager (exposure 6.15 min, binning 1x1). Remaining substrate was rinsed with TBS-T and the membranes were stored wetted at 4 °C until further use.

3.7. Stripping and Reprobing of Immunoblots

A common way to limit variance between immunoblots and conserve samples is to remove the antibodies from already sampled PVDF membranes. After this, the membranes can be re-sampled. This process is called "stripping" and prevents further quantitative analyses, while still allowing for qualitative analyses. For this purpose, a protocol by Yeung et al. was adapted[51]. Membranes were first tested for bound IgG4, then stripped and subsequently checked for IgG1 reactive proteins.

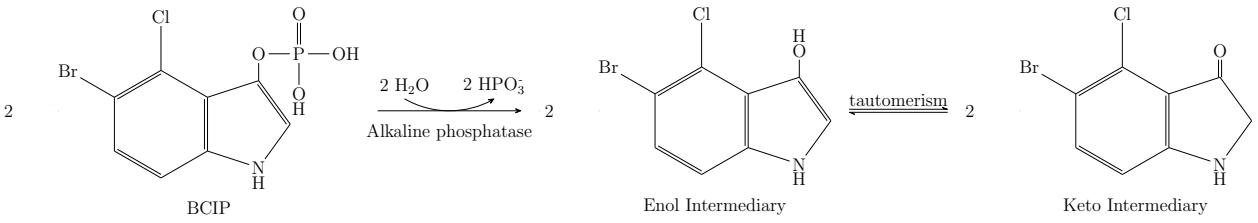
Membranes to be stripped were incubated twice in guanidine hydrochloride (GnHCl) stripping buffer for 5 minutes each (See Appendix C for composition). This was followed by four washes in wash buffer for 3 minutes each. Finally, the membranes were rinsed in TBS-T for 5 minutes.

To verify removal of all primary IgG antibodies, stripped membranes were incubated with a 1:10,000 dilution of an ECL-compatible anti-IgG1 antibody in TBS-T block buffer for 60 minutes. This was followed by ECL detection as described in the previous Section. If all primary antibodies were completely removed, the membranes could be re-sampled with a primary antibody. Membranes that still showed signal were subjected to the same process again. If only the secondary antibodies had to be removed, the stripping was verified directly via ECL without exposure to secondary antibodies.

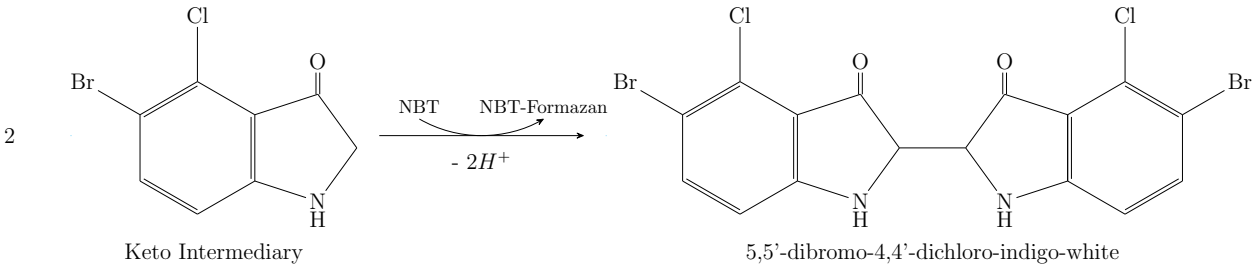
3.8. Alkaline Phosphatase-Based Total-IgG Assay

For further analysis of the secreted proteins, spots had to be cut from the immunoblot membranes. Since the chemiluminescence of ECL is not permanent and difficult to perceive with the unaided eye, a colorimetric method was used for the permanent visualisation of immunoreactive proteins. For this purpose, an AP-based technique was used. This involved using

a conventional immunoblot with patient sera as the source of primary antibodies. However, the secondary antibodies were AP-conjugated anti-human–IgG, which can detect all subclasses of human IgG. When 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) are added to the membrane with the bound AP conjugates, the AP enzymatically forms an insoluble, deep blackish-purple product which is deposited on the membrane. A reaction scheme is shown in Figure 3.1.



(a) Enzymatic Dephosphorylation of BCIP



(b) Dimerisation of Intermediates and Reduction of NBT

Figure 3.1.: Alkaline Phosphatase Colorimetric Assay Reaction Scheme.
BCIP is Hydrolytically De-phosphorylated by the AP, Forming an Intermediate. The Intermediate Dimerises into an Insoluble Indigo Form. This Releases Protons that Convert the NBT into an Insoluble, Black Formazan Compound. This Results in the Dark Purple Colour of the AP Assay.

For this process, immunoblots that were stripped after ECL detection were washed in TBS-T for 60 min. Afterwards, 5 ml of the patient sera, diluted 1:1000 in TBS-T block buffer, were applied as primary antibodies to the membranes and incubated overnight. The following day, the membranes were rinsed three times in TBS-T to remove unbound antibodies. This was followed by an incubation at room temperature for 60 min with the secondary antibody, a goat anti-human-IgG antibody as a 1:20,000 dilution in TBS-T block buffer. The membranes were rinsed four times with TBS-T for 5 minutes to remove excess antibodies. This was followed by 10 minutes in AP buffer (see Appendix C for composition). The used AP buffer was then discarded and 5 ml substrate (BCIP/NBT) was added to the membranes. The membranes were then incubated up to 15 minutes under constant agitation during which the precipitation reaction took place. After the desired intensity was reached, excess substrate

was removed with Aqua dest. for 10 minutes. Membranes were dried overnight in a drying cabinet at room temperature, photo-documented and stored at -20 °C.

3.9. Mass-Spectrometry

3.9.1. Sample Preparation and Trypsin Digestion

Trypsin digestion is a common method for the controlled digestion of proteins prior to further analysis, for example by mass spectrometry. Two different approaches were applied in the experiments carried out. One was to digest excidates of immunoblots that were reactive in ECL, the other was to treat whole culture supernatants with trypsin for comparison. This made it possible to contextualise the reactive proteins in comparison to the whole secretome.

Digestion of Immunoblots

First, a map was created to determine which areas of the immunoblots should be further investigated using trypsin digestion. For this, the ECL scans for IgG1 and IgG4 reactivity, as well as the AP assay of a membrane were digitally superimposed. Thus, interesting points could be marked and the AP staining could be used as a guide. In general, all definite IgG4 and all intensive IgG1 binding areas were further investigated, as well as some areas that bound both antibodies or neither of them but were positive in the AP assay. In addition, several negative samples were taken from each blot.

Excision was performed using a 2 mm biopsy punch, which was cleaned with ethanol between punches. For larger areas, several samples were taken per spot. Afterwards, the excidates were individually collected in low-binding micro-centrifuge tubes and labelled.

In order to cleave the proteins, the samples were mixed with 50 µl methanol and incubated for 30 minutes. They were then decanted and dried briefly at room temperature. 10 µl 30% acetonitrile (ACN) in a 20 mM ammonium bicarbonate (ABC) solution were added to the excidates. Following this, 20 µg trypsin was added to each sample. The samples were then vortexed and briefly centrifuged to ensure homogeneous coverage of the samples, which

was followed by incubation at 37 °C overnight.

The following day, the supernatant of each sample was collected separately. To improve protein extraction, 20 µl of an 80% ACN solution were added to the supernatants in HPLC-H_2O, vortexed and incubated at 37 °C for another 30 minutes. Thereafter, the samples were chilled at -80 °C until completely frozen through, and evaporated in a vacuum rotary evaporator (setting V-AL) until the liquid was completely evaporated, but not longer than one hour. Samples that were not completely dry by then were subjected to the process again. The completely dried samples were then eluted in 12 µl buffer A (see Appendix C) and stored at -20 °C until mass spectrometry.

Digestion of Culture Supernatants

A native protein digestion protocol was used to process the culture supernatants. For this purpose, 10 µg of protein in CHAPS-free RHB were adjusted to a volume of 10 µl. An aqueous solution of 20 mM ABC and 2.5 mM DTT was used as a diluent for this purpose. The samples were then incubated at 37 °C for 30 minutes. Alkylation was carried out by adding 10 µl of an aqueous solution of 20 mM ABC and 10 mM IAA and incubating the samples with the same conditions as described above. A trypsin solution (5 µg/µl) was added in a ratio of 1:25 for digestion. The prepared samples were incubated in darkness at 37 °C for 16 – 18 hours.

After incubation, digestion was stopped by the addition of acetic acid to a concentration of 1%. For sample enrichment, a commercial solution was employed using the ZipTip® protocol. The pipette tips were first equilibrated according to the manufacturer's instructions. For the enrichment, the samples were then taken up into the prepared tips 10 times each, followed by 5 rinses with the diluent. For the elution, 5 µl 50% ACN in diluent were taken up and pipetted first, followed by 5 µl 80% ACN in diluent. Both fractions were combined in a micro-vial and concentrated to 2 µl by evaporation.

3.9.2. LC-MS/MS

The LC-MS/MS for both excised proteins and culture supernatants were performed using a nanoAcquity UPLC and a LTQ-Orbitrap Velos mass spectrometer with a pico-tip emitter

for electrospray ionisation (ESI). For liquid chromatography (LC) separation, the prepared samples were first enriched on a UPLC C-18 pre-column (2 cm length, 180 µm inner diameter and 5 µm particle size) and then aggregated on a UPLC C-18 column (10 cm length, 100 µm inner diameter and 1.7 µm particle size). Two different protocols were then used for separation.

Proteins excised from membranes were separated using a gradient of buffer A and buffer B (See Appendix C): 2 min in 1 – 5%, 23 min in 5 – 70%, 2 min in 70 – 99%, for a total of 27 mins.

A longer protocol was used for culture supernatants: 2 min in 1 – 5%, 63 min in 5 – 25%, 25 min in 25 – 60%, 1 min in 60 – 99%, for a total of 91 mins.

In both cases, elution was carried out after enrichment at a flow rate of 400 nl/min. For mass spectrometry (MS), the following conditions generally applied: ESI with 1.5 – 1.6 kV; no sheath or auxiliary gas flow, capillary temperature of 300 °C. Threshold for tandem massspectrometry (MS/MS) selection was 2000 counts, at an activation time of 10 ms and activation energy of 35% normalised. Only doubly and triply charged ions were subjected to MS/MS.

First scan was performed in the Fourier Transformation Mass Spectrometer (FTMS) mode (Orbitrap-MS) to determine the mass-to-charge ratio (m/z) of eluted and ionized peptides, operating in positive and profile mode. The second scan was a MS/MS (LTQ-MS/MS), acquiring data in centroid mode and performed in data-dependent mode (based on the fragmented peptides). The equipment automatically switched between Orbitrap-MS and LTQ-MS/MS. Survey scans of MS spectra (from a m/z of 325 – 1525) were acquired from the Orbitrap with a resolution of R=30000, for a target value of 1xE6. This allowed for sequential isolation of the twenty most signal intense ions, which were subjected to collision-induced dissociation (CID)-fragmentation (isolation-width of 2 Da, target value of 1xE4, with a maximum ion time of 100 ms). Target ions that were already selected for MS/MS were excluded for 60 s.

3.9.3. Spectrum Matching and Database Research

To match the spectra generated by MS to the corresponding peptide sequences, a search was performed using the MASCOT algorithm in "Protein Discoverer" (version 2.1). The detected peptide sequences were then checked for matches in a specially constructed database. This database contained the fully annotated proteome data from UniProt of the following *P. aeruginosa* strains: ATCC 15692 (PAO-1), UCBPP-PA14, PA7, LESB58, PAK, as well as proteins with the generic descriptor "Pseudomonas aeruginosa". The selected strains represent the most complete *P. aeruginosa* proteomes.

Furthermore, another database was constructed from the proteome data described above. This relational HSQLDB database (hereafter called "master database") contained further information from the UniProt database for all registered proteins such as: Acession ID, strain, description and subcellular location. Additionally, the molecular weight of the protein and its theoretical IP value were calculated from the peptide sequence using an ExPASy tool[3]. Moreover, information regarding the examined samples and spots, generated blots, registered binding capacity of immunoglobulins, was included in this database. All further data operations were based on this master database.

After spectrum matching, the proteins detected in the MS were compared with the master database, annotated accordingly and linked to the data already recorded. On the basis of this data, complex queries could now be made, as to how all the identified instances of a protein were distributed across all the immunoblots examined, or in what proportions which antibody was bound.

[3]https://web.expasy.org/compute_pi/; last checked 21/09/2021

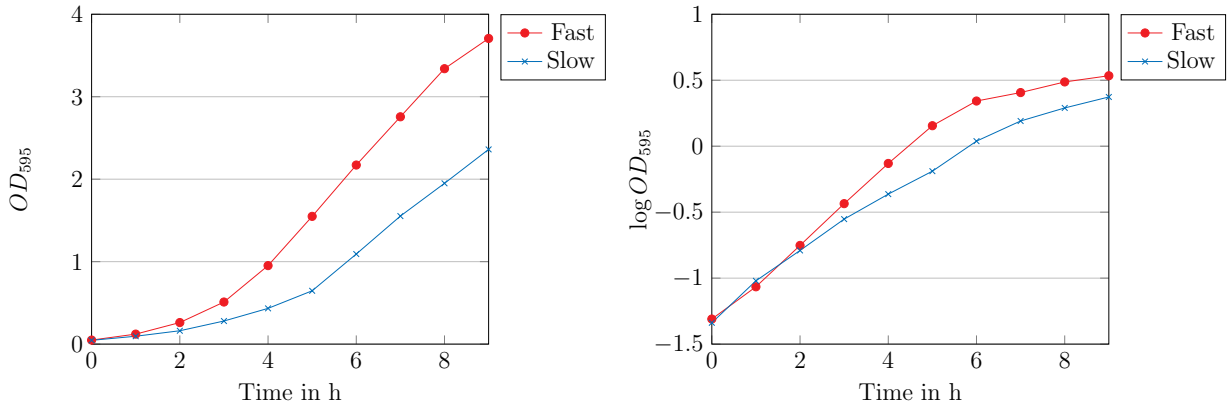
4. Results

4.1. Protocol Adaptation to *Pseudomonas aeruginosa*

4.1.1. Improved Precipitation and Purification of Secreted Proteins

Initial experiments showed, that the established protocols for *S. aureus* secreted protein precipitation were not applicable to *P. aeruginosa*, and therefore had to be optimised. Although *P. aeruginosa* is a rather undemanding bacterium, culturing it under laboratory conditions to produce a sufficient amount of secreted proteins proved to be difficult. Furthermore, preliminary growth curves revealed two groups of strains: fast-growing and slow-growing. To distinguish between the two, a cut-off (OD_{595} of ≥ 1; 5 hours post-inoculation) was defined. Figure 4.1 displays the differences in growth behaviour between both groups. Therefore strain-specific growth curves were generated. Based on these curves, the secreted proteins were precipitated 3 hours after entering the stationary phase for each strain. The time for this transition is shown in table A.2. Early experiments used culture volumes of 40 ml, which yielded insufficient amounts of protein. Thus, culture volumes were increased to 100 and 600 ml for growth curves and production cultures, respectively. Precipitation of the secreted proteins was originally carried out according to the previously established procedure with pure TCA 1:10 overnight. However, the protein yield was improved by adding 0.4% cholanoic acid and extending the incubation period to 36 hours at 4 °C.

Protein purification was also improved. By using a commercial ether-based kit together with the established ethanol washes, the sample quality was significantly improved at the cost of a minor in protein loss. Additionally, sample exposure to room temperature was minimised



(a) Comparison Between Fast and Slow Growing *P. aeruginosa* Isolates.

(b) Logarithmic Plot of *P. aeruginosa* Growth Data, Indicating Change into the Postexponential Growth Phase.

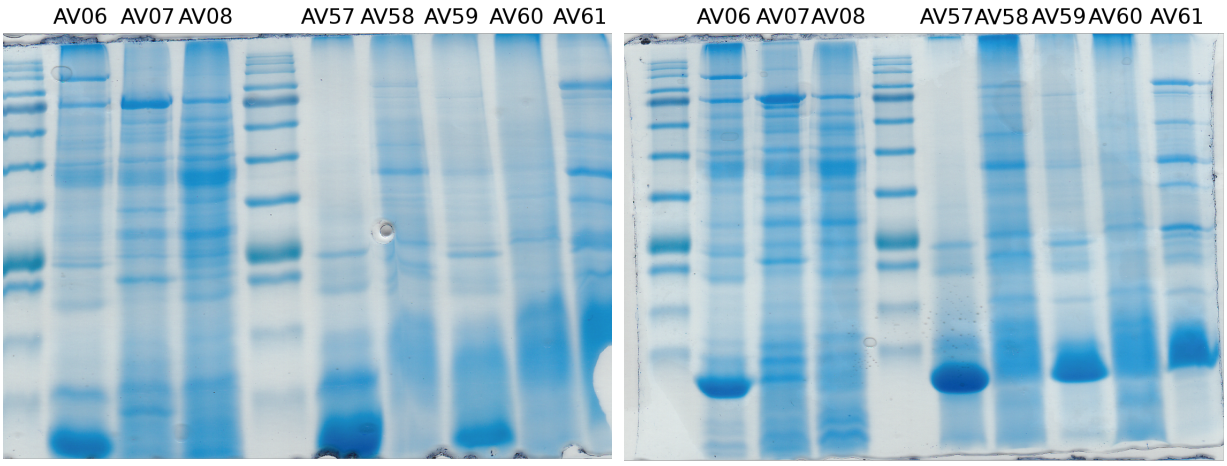
Figure 4.1.: Growth Behaviour of *P. aeruginosa* Isolates in TSB Medium. Graphs Display the Mean $OD_{595}/\log OD_{595}$ of Isolates of either the "Fast" or "Slow" Growing Group, Defined by an OD_{595} of ≥ 1 at 5 Hours Post-Inoculation.

by using a rotary vacuum evaporator to dessicate cleaned protein samples. Figure 4.2 shows a one-dimensional SDS-PAGE comparing the quality of protein specimens between the previously established and the improved protocol. The refined procedure made it possible to obtain enough protein of all isolates at acceptable quality. Only *P. aeruginosa* strains AV62 and AV63 predominantly produced alginate. This mucopolysaccharide interfered with the purification process. Therefore, the strains were excluded from further experiments. Finally, the visualisation of the protein bands after SDS-PAGE was improved by establishing a polyacrylamide gel staining procedure, which utilised a colloidal Coomassie staining solution based on a protocol by Dyballa et al.[52].

4.1.2. Improved Two-Dimensional Immunoblotting

Two-dimensional gel electrophoresis is dependent on two factors: the IP of the proteins contained in a sample, and the MW of these proteins. By using IEF strips with a broad pH range (3 – 11), an overview on the different IPs of all secreted proteins (see Figure 4.3) was obtained. Further experiments focused on the pH range of pH 4 – 7, as most spots were in this range, and a narrower pH range allowed better spatial resolution. However, other proteins with a more alkaline IP might prove to be interesting for future studies.

The original IEF protocol called for electrophoresis until 17,000 Vh were reached (limited to 4000 V). However, the resulting blots showed artefacts due to overfocusing caused by the oxidation of cysteine residues. This altered the IP and thus restarted the migration of



(a) Exemplary Secretomes of Different *P. aeruginosa* Strains Cleaned with the Original Protocol.

(b) Exemplary Secretomes of Different *P. aeruginosa* Strains Cleaned with the Improved Protocol.

Figure 4.2.: Comparison of Protein Purification Methods.
P. aeruginosa Secretomes Separated by One-Dimensional SDS-PAGE after Original Purification Protocol (Figure 4.2a) versus after Treatment with an Improved Method (figure 4.2b).

proteins, forming horizontal stripes on the blots[53]. Reducing the total voltage to 8000 Vh and a maximum of 3500 V lessened these artefacts while retaining sufficient separation.

Additionally, the total amount of protein was reduced from 125 µg to 100 µg, as some spots were overly pronounced in Coomassie stained gels. Furthermore, preliminary blots indicated good reactivity with the primary and secondary antibodies, allowing to adapt the dilutions from 1:500 to 1:1000 and 1:1000 to 1:10,000 for the primary and secondary antibody dilutions respectively. Changing the immunoblotting wash buffer and antibody diluent from TBS to TBS-T and TBS-T blocking buffer (see Appendix C) further reduced artefacts from unspecifically bound antibodies.

4.1.3. Improved Stripping and Reprobing of Immunoblots

Stripping immunoblots is the process of removing the primary and secondary antibodies from an immunoblot for repeated sampling. This is often done by exposing the immunoblot membranes to a detergent at low pH. Early attempts used a combination of SDS and a pH of 2.2. However, this only reliably removed the secondary antibodies. Primary antibodies often remained even after prolonged exposure to the stripping buffer for up to 3 hours instead of the standard 10 minutes.

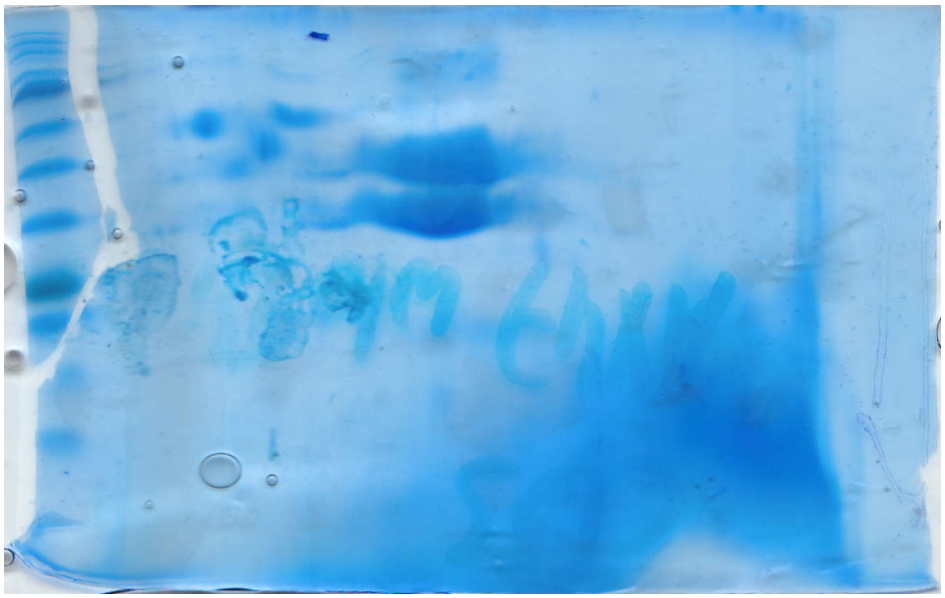


Figure 4.3.: Exemplary 2D SDS-PAGE Gel (Strain AX47).
pH Range from pH 3 – 11, Stained with Colloidal Coomassie Staining Solution. The Spots in the right Half of the Gel Indicate the Presence of Proteins with an IP > 7.

This problem was alleviated by adapting a protocol from Yeung et al.[51], in which a highly concentrated guanidine hydrochloride buffer is used (see Appendix C for more details). This consistently stripped all antibodies from the PVDF membranes within 30 minutes without losing a significant amount of blotted protein.

4.1.4. Summary of Changes

At a glance, all changes to protocols are complied into Table 4.1. The exact descriptions of all protocols are detailed in Chapter 3, and buffer compositions can be found in Appendix C.

4.2. One-Dimensional SDS-PAGE

Preliminary one-dimensional SDS-PAGEs indicated similar band patterns between some *P. aeruginosa* isolates from the same patient, with an example shown in Figure 4.4. This suggested possible duplicate isolates, or strains with similar expression patterns. For instance, all isolates from patient P01-01 (AX47 – AX50) showed reasonably similar band patterns in one-dimensional SDS-PAGEs. This suspicion was later confirmed by examining the PEGGY-Sue peak patterns of suspected strains. There, several strains showed matching patterns. For further experimentation, only one strain with the best growth characteristics

Table 4.1.: Adaptation of Employed Protocols.

Protocol	Initial Protocol	Improved Procedure
Growth curves	40 ml cultures, using three isolates as model	100 ml cultures, growth curves for all isolates
Precipitation	40 ml cultures 100% TCA 1:10 incubation for 12 h	100 ml or 600 ml cultures 99.6% TCA with 0.4% cholanoic acid 1:10 incubation for 36 h
Purification	5 washes in 70% Ethanol	5 washes in 70% Ethanol followed by commercial kit
Isoelectric focusing	Focusing to 17,000 Vh at 4,000 V total 125 µg of protein	Focusing to 8,000 Vh at 3,500 V total 100 µg of protein
SDS-PAGE staining	Coomassie G-250 solution	Colloidal Coomassie R-250
Immunoblotting	Primary antibody dilution 1:500 Secondary antibody diliution 1:1000 Dilutions in TBS	Primary antibody dilution 1:1000 Secondary antibody diliution 1:10000 Dilutions in TBS-T blocking buffer
Sripping and reprobing	SDS/low pH based protocol	Guanidine hydrochloride based protocol

(high yield and low exopolysachharide production) was chosen. Table 4.2 gives an overview of the selected strains, in conjuncture with their respective sera.

4.3. Semi-Automated One-Dimensional Immunoblotting

In order to focus the experimental effort on promising combinations of clinical isolate and patient serum, a screening assay for the quantitative detection of specific IgG4 and IgG1 was established. The resulting pairs were matched based on ECL detection intensity. Examplary datasets are shown in Figure 4.5 and 4.6 for AX47/D030 and AV59/D027, respectively. Complete data sets can be found in Appendix B.1.

Furthermore, the suspected duplicate isolates already identified in one-dimensional SDS-PAGEs were compared on the basis of their peak pattern. Isolates with congruent patterns, but comparatively low signal intensity were excluded from further experiments. Table 4.2 lists the studied strains and the respective sera used.

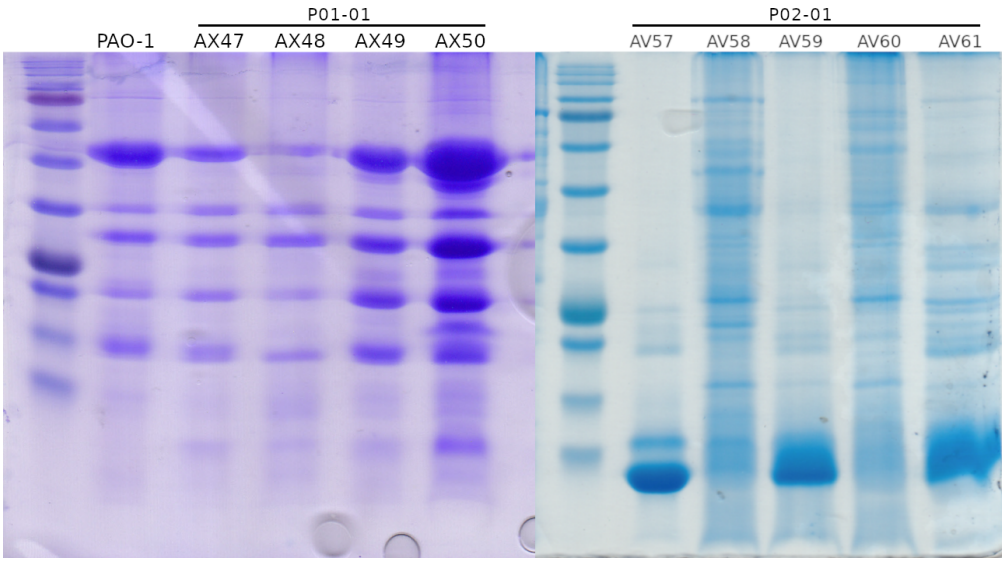


Figure 4.4.: Patient-Specific One-Dimensional SDS-PAGEs.
From left to right (FLTR): Protein Ladder, PAO-1 (Reference Strain), Strains from P01-01 (AX47, AX48, AX49, AX50), Protein Ladder, Strains from P02-01 (AV57, AV58, AV59, AV60, AV61). Left Gel: Coomassie Brilliant Blue Staining, Right Gel: Colloidal Coomassie Staining. Composed Picture.

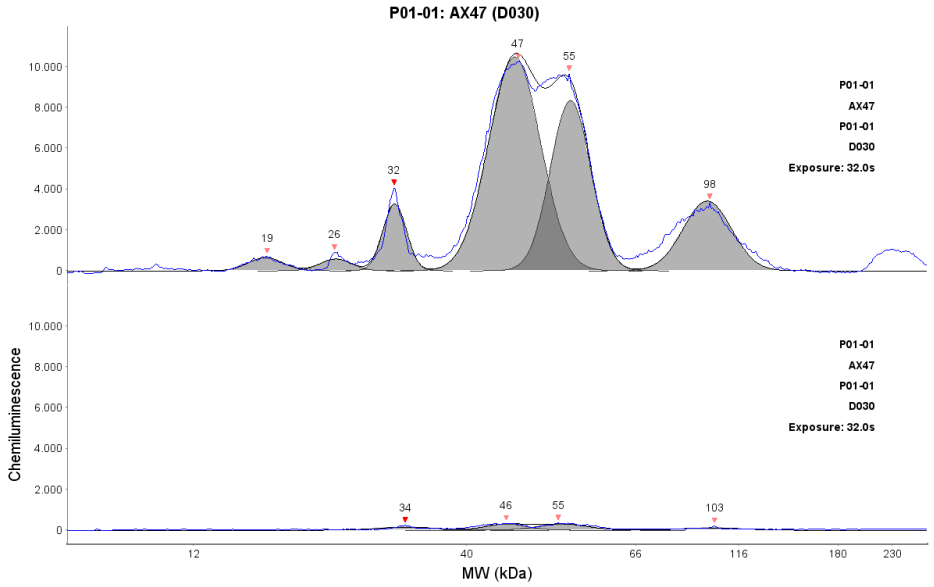


Figure 4.5.: PEGGY-Sue Blot Patient P01-01 (Strain AX47, Serum D030).
Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

4.4. 2D-Immunoblotting

Although previous blots showed proteins with IPs < 4 and > 7 (see Figure 4.3), only the range between pH $4 - 7$ was examined further by immunoblotting, because the majority of proteins were localised in this range. Two two-dimensional SDS-PAGEs gels were prepared

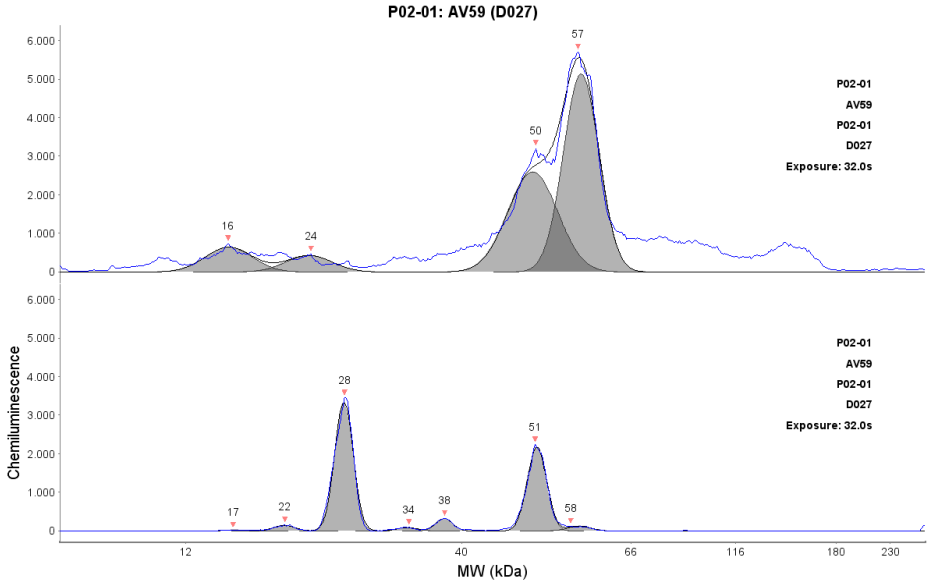


Figure 4.6.: PEGGY-Sue Blot Patient P02-01 (Strain AV59, Serum D027).
Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

Table 4.2.: Selected Strains and Serum Samples.
If the Protein Peak Patterns in the Semi-Automated Immunoblots Differed Substantially, one Strain per Expression Pattern was Used for further Experiments. All Isolates from Patient P02-05 were Removed from further Study due to low Secretion of Proteins and Excessive Polysaccharide Production.

Patient	Selected Strains	Selected Sera
P01-01	AX47	D030
P01-03	AW24	D006/D025
P01-05	AV17/AV18	D009
P02-01	AV59/AV61	D018/D027
P02-04	AV06/AV07	D019
P02-05	—	—
P02-06	AV79	D013

for each isolate/serum pair: one for immunoblotting and one for total protein staining with colloidal Coomassie. The immunoblots were then analysed for IgG4 and IgG1 binding. This was followed by colorimetric total IgG detection. Between the assays, bound antibodies were removed using the GnHCl stripping protocol, detailed in Chapter 3.

Figure 4.7 shows a side-by-side comparison of a total protein stain with colloidal Coomassie and an anti-IgG1, as well as an anti-IgG4 immunoblot for secreted proteins from strain AX47 that were probed with serum D030. There is a clear difference in reactivity between IgG1

and IgG4, especially visible in the lowest pH on the left and the highest pH on the right of the blots. The leftmost IgG4-reactive protein spot is also visible on blots of PAO-1 and AV18, and because of its high intensity, it was of key interest for further analysis. A different example is given in figure 4.8. While the results from the PEGGY-Sue experiments seemed promising for this combination, only few proteins were detectable on the two-dimensional blots, with similar spot patterns in the IgG1 and IgG4 blots.

Figure 4.7.: Patient P01-01: Secreted Proteins of *P. aeruginosa* Strain AX47, pH 4 – 7 Probed with Serum D030.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

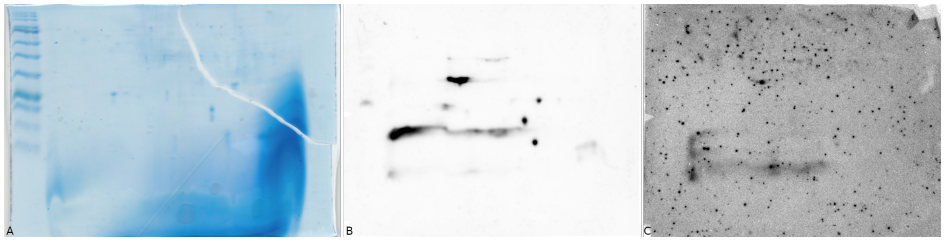


Figure 4.8.: Patient P02-01: Secreted Proteins of *P. aeruginosa* Strain AV59, pH 4 – 7 Probed with Serum D027.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

4.5. LC-MS/MS

4.5.1. Protein Identification from LC-MS/MS Data

To identify reactive secreted proteins of *P. aeruginosa*, the generated peptide fragment spectra were compared against the entries of the peptide database described in Section 3.9.3. The latter contained a total of 452 data sets from annotated *P. aeruginosa* proteomes. The exclusion process for entry into the database is depicted in Figure 4.9.

17 unique proteins were identified by searching the resulting master database for the linked qualifiers “secreted” and “identified”. Here, “secreted” meant that the cellular location

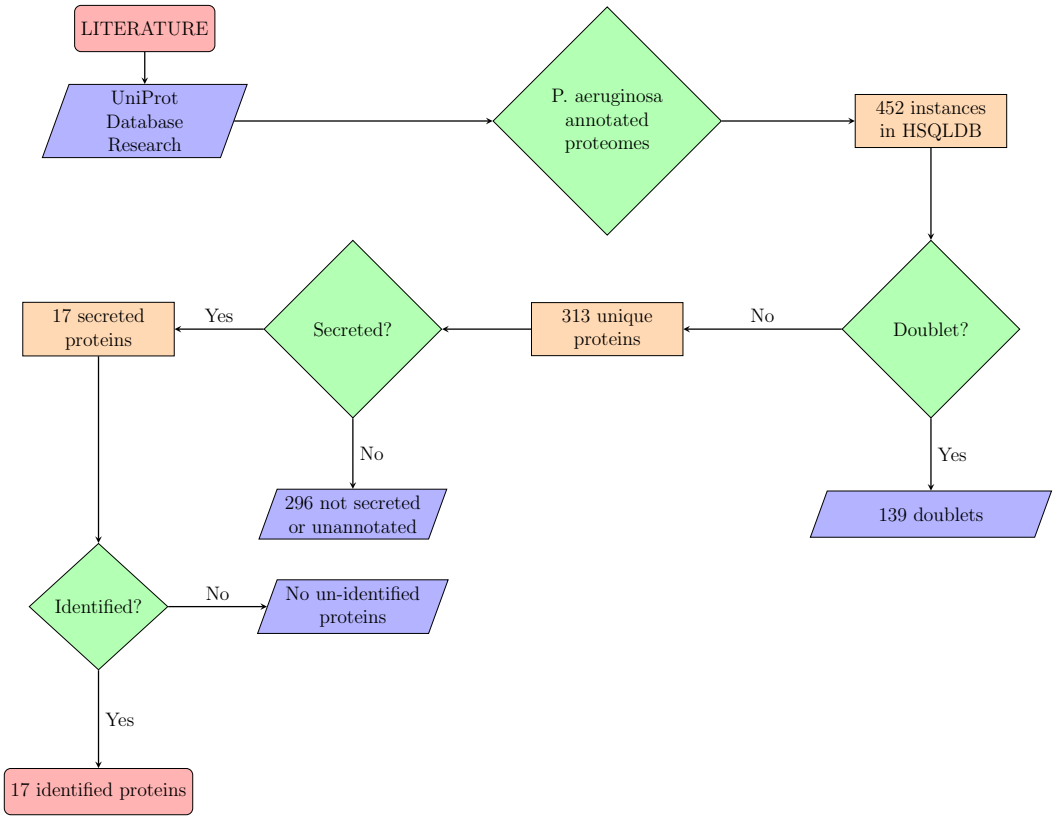


Figure 4.9.: Flowchart Showing the Process for Database Construction and Protein Identification.
In total, only 17 out of 452 Datasets were Annotated as "Secreted" and thus Recognised for Spectrum Searches.

of the protein was annotated as experimentally confirmed "extracellular" in the UniProt database. This allowed for a higher discriminatory power between definitive and possible extracellular proteins. "Identified" related to protein fragments with a peptide sequence match of over 95% in any LC-MS/MS experiment. These identified proteins make up the entire known secretome of *P. aeruginosa*. Section 4.5.2 gives an overview of the proteins identified from immunoblots, describing their MW and IP. A cumulative description of all identified proteins and their reactivity is given in Section 4.5.3. Furthermore, as the LC-MS/MS showed, protein expression varied between different strains and even their mode of identification (comparing blot-digests and supernatant-digests), as is shown in Section 4.5.4.

4.5.2. Identifications and Protein Distribution on Immunoblots

After ECL detection of IgG1 and IgG4 reactive proteins, the immunoblots were stripped once more and then incubated with an anti-human-IgG-AP conjugate. This made it possible to visualise all bound specific IgG from the patient sera on the blots. The AP blots were

scanned and digitally superimposed with the IgG4 and IgG1 ECL blots. This digital map was used as the template to select samples for trypsin digestion. In the following, Figures 4.10 and 4.11 show examples of the overlays. Blue labels show spots with bound IgG4, and red marks indicate bound IgG1. Spots with an orange circle indicate reactivity to both IgG subclasses. Green labels indicate spots without bound IgG, but large amounts of protein stained with Ponceau-red. These spots were also selected for identification to identify possible unreactive proteins. Further blots from all strains are depicted in Appendix B.3.

All labelled spots were excised, the proteins extracted and digested with trypsin for identification by LC-MS/MS. Figures 4.12 – 4.28 below accumulate all identifications of a single protein on a "virtual membrane". The theoretical MW/IP is denoted in red with the matching UniProt accession. Spots showing IgG1 reactivity are yellow, IgG4 binding is green. Blue marks indicate spots that bound both IgG subclasses. Furthermore, pie charts provide an overview of the quantitative split between reactivity types.

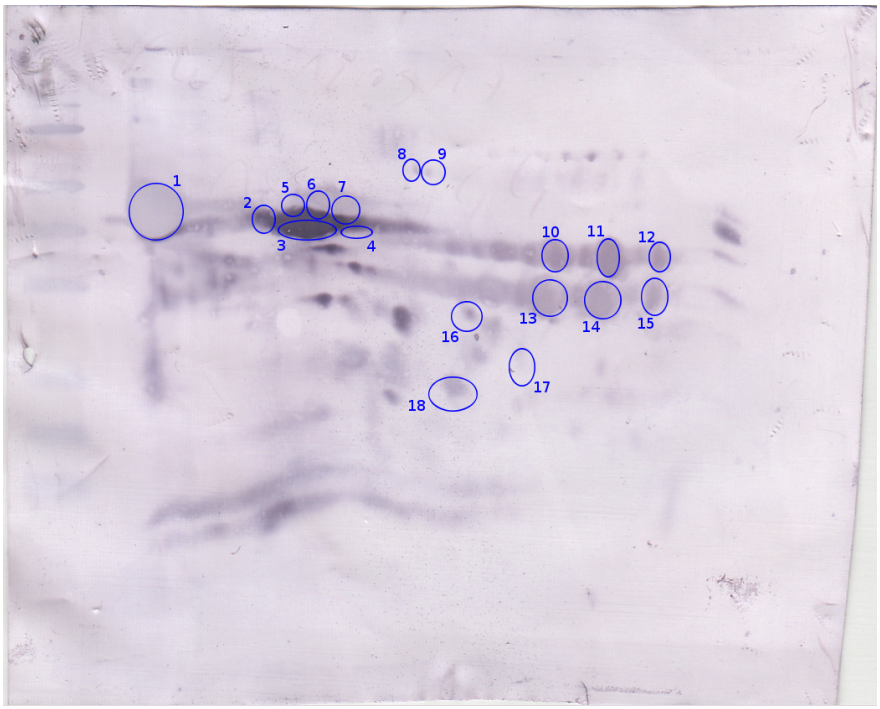


Figure 4.10.: AP-Blot Overlay, Strain AX47 against Serum D030 (P01-01). pH 4 – 7. Only IgG4-Reactive Spots were Excised from this Blot.

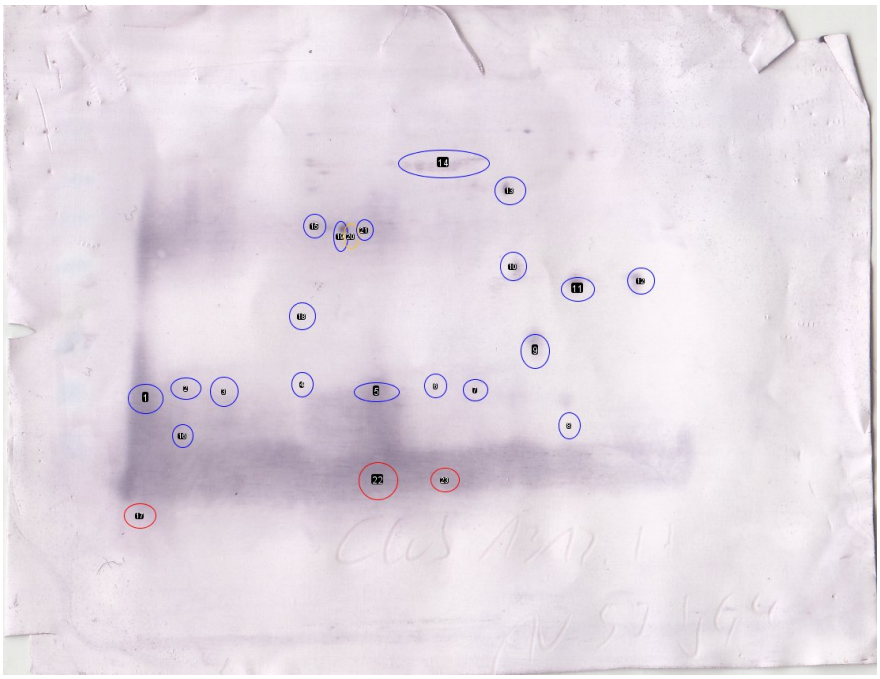
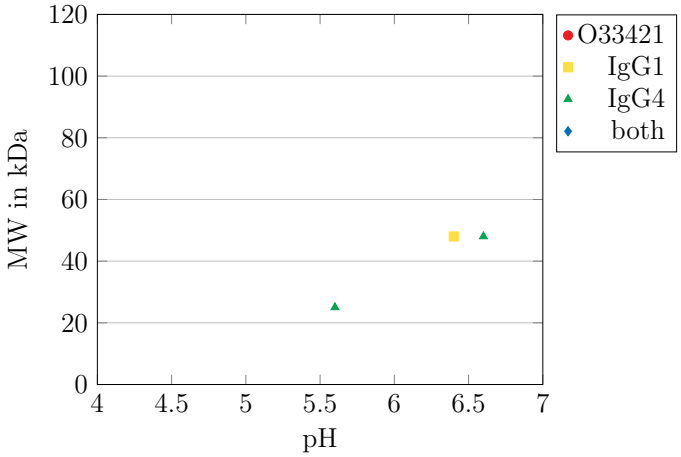
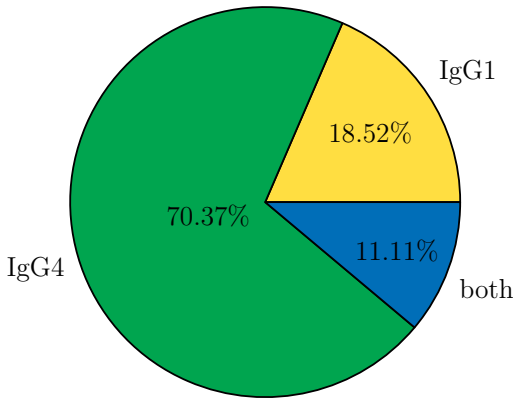


Figure 4.11.: AP-Blot Overlay, Strain AV59 against Serum D018 (P02-01). pH 4 – 7. Overlay Shows Spots in which Signal from IgG1 (red, Spots 17, 22, 23) or IgG4 (blue, Spots 1 – 16, 18, 19, 21) and from Both (spot 20) was Detected.

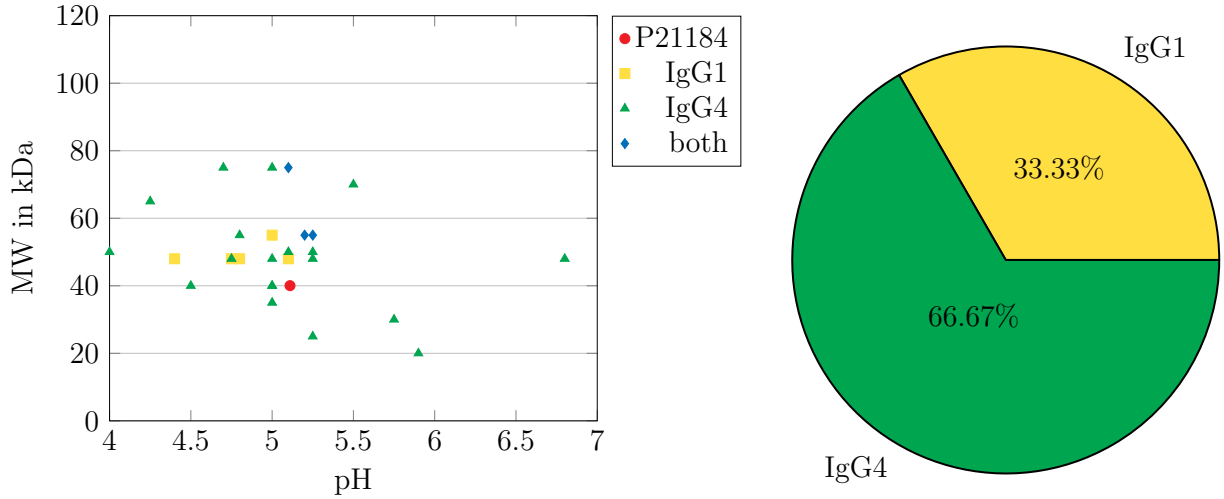


(a) Spatial Distribution from Ig-Binding to Spots Identified as A-Type Flagellar Hook-Associated Protein 2 Fragments on Immunoblots. Theoretical MW/IP Denoted in Red, in this Case out of Bounds of the Virtual Blot.



(b) Qualitative Assessment of the Antibody Binding from Spots Identified as A-Type Flagellar Hook-Associated Protein 2.

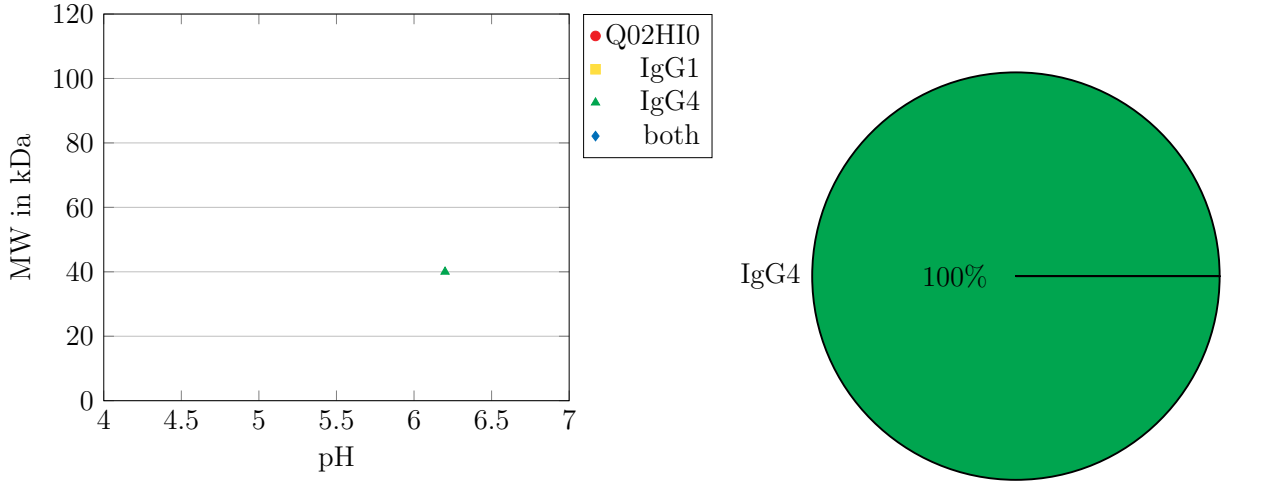
Figure 4.12.: Cumulative Visualisation of all LC-MS/MS Identifications of A-Type Flagellar Hook-Associated Protein 2.



(a) Spatial Distribution of Ig-Binding from Spots Identified as A-Type Flagellin Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assesment of the Antibody Binding from Spots Identified as A-Type Flagellin.

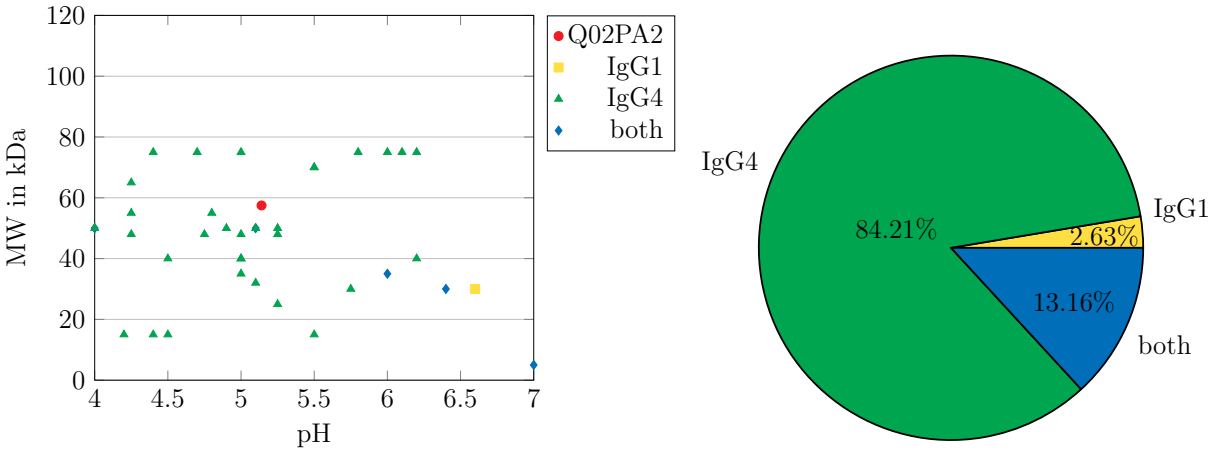
Figure 4.13.: Cumulative Visualisation of all LC-MS/MS Identifications of A-Type Flagellin.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Alkaline Phosphatase Fragments on Immunoblots. Theoretical MW/IP denoted in Red, in this Case out of Bounds of the Virtual Blot.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Identified Alkaline Phosphatase.

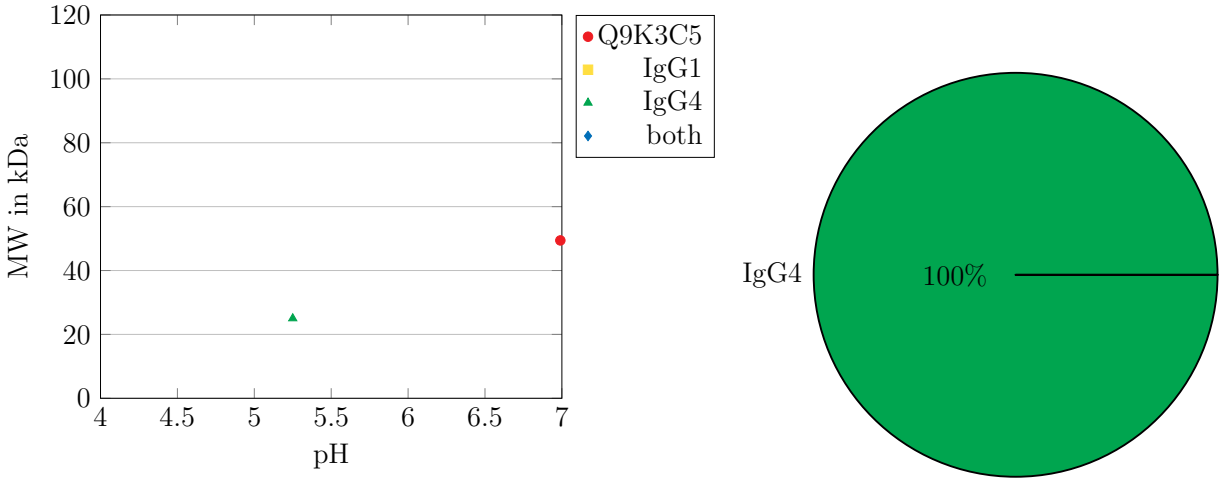
Figure 4.14.: Cumulative Visualisation of all LC-MS/MS Identifications of Alkaline Phosphatase.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Aminopeptidase Fragments on Immunoblots by. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Aminopeptidase.

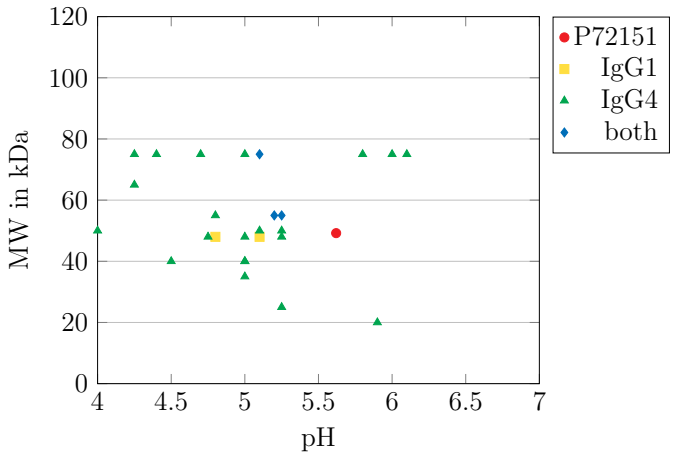
Figure 4.15.: Cumulative Visualisation of all LC-MS/MS Identifications of Aminopeptidase.



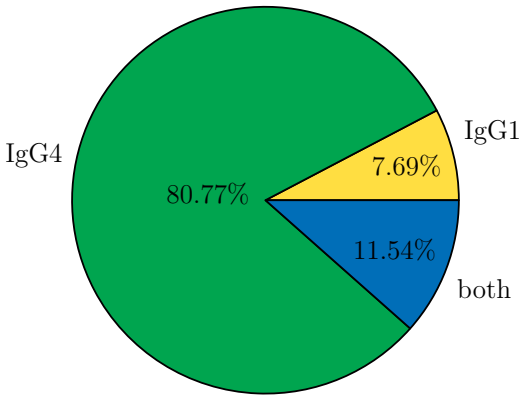
(a) Spatial Distribution of Ig-Binding from Spots Identified as B-type Flagellar Hook-Associated Protein 2 Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Identified B-Type Flagellar Hook-Associated Protein 2.

Figure 4.16.: Cumulative Visualisation of all LC-MS/MS Identifications of B-Type Flagellar Hook-Associated Protein 2.

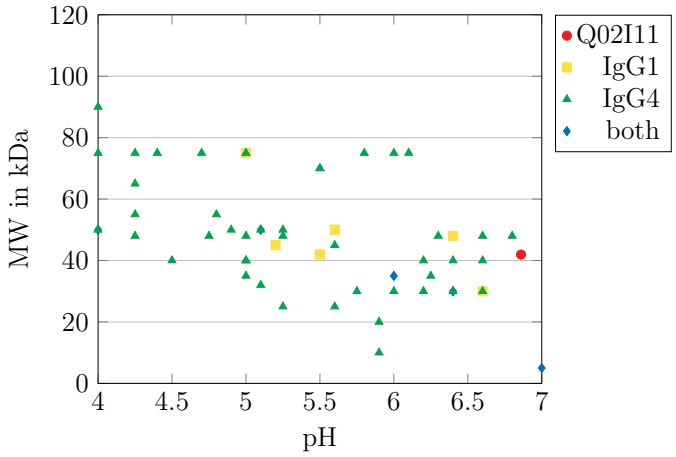


(a) Spatial Distribution of Ig-Binding from Spots Identified as B-Type Flagellin Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

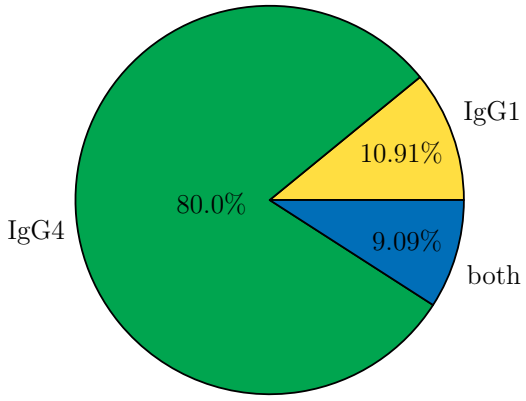


(b) Qualitative Assessment of the Antibody Binding from Spots Identified as B-Type Flagellin.

Figure 4.17.: Cumulative Visualisation of all LC-MS/MS Identifications of B-Type Flagellin.

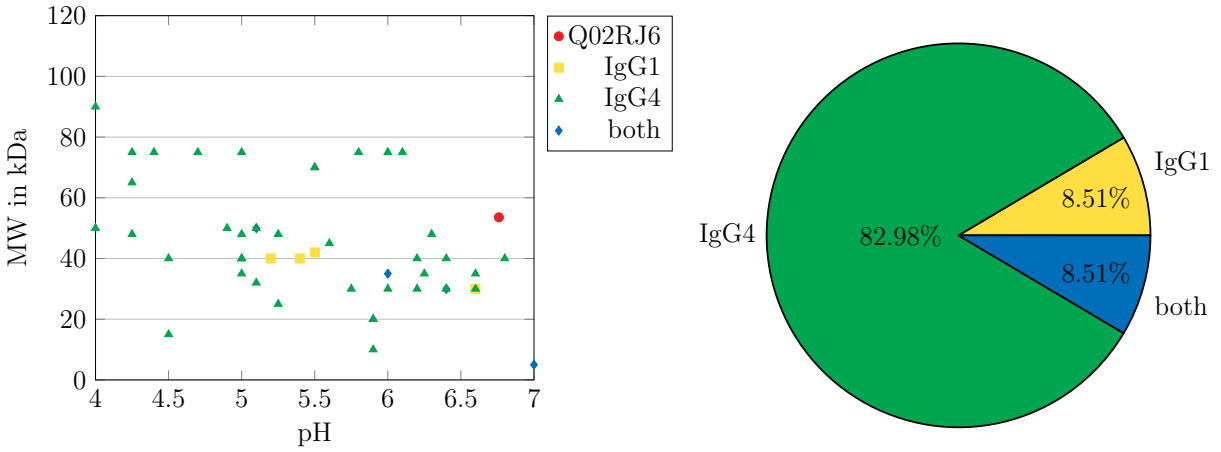


(a) Spatial Distribution of Ig-Binding from Spots Identified as Chitin-Binding Protein Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.



(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Chitin-Binding Protein.

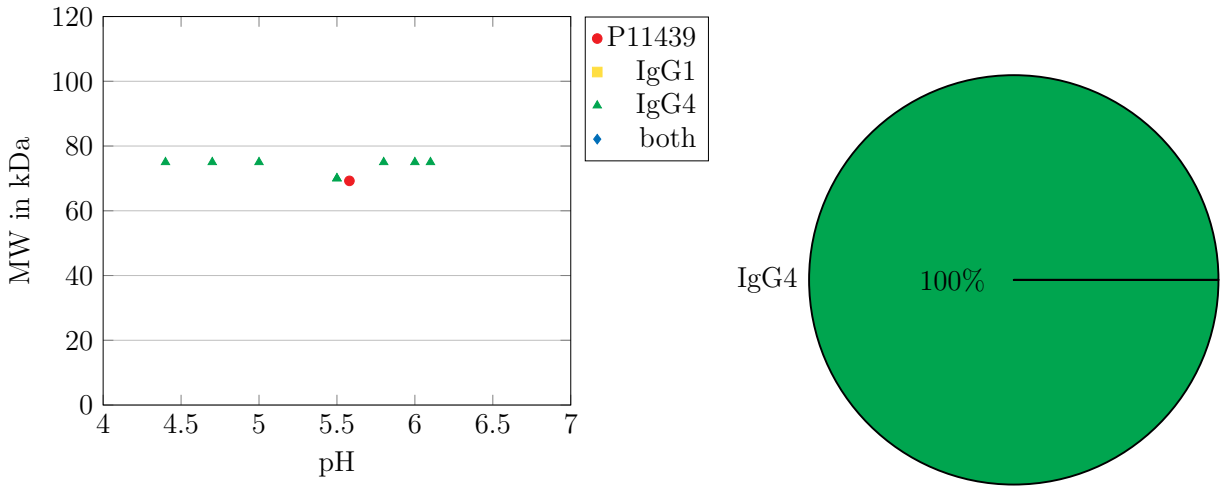
Figure 4.18.: Cumulative Visualisation of all LC-MS/MS Identifications of Chitin-Binding Protein.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Elastase Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Elastase.

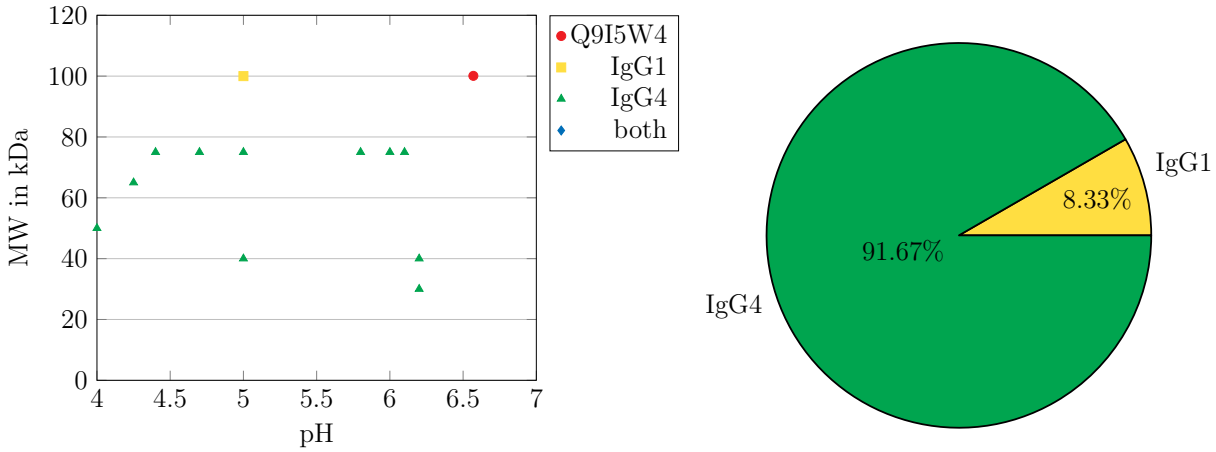
Figure 4.19.: Cumulative Visualisation of all LC-MS/MS Identifications of Elastase.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Exotoxin A Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Exotoxin A.

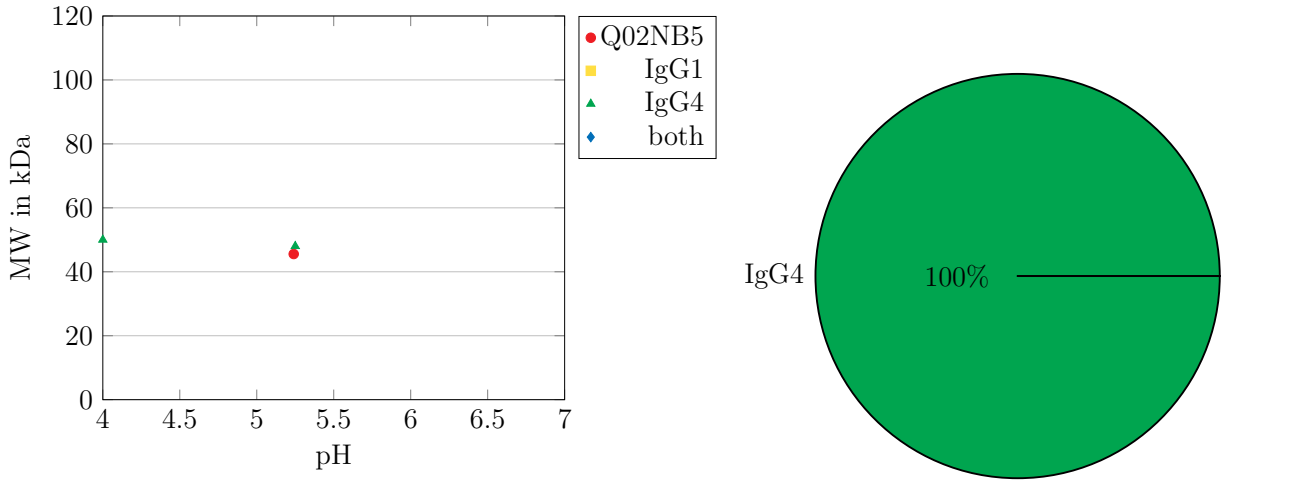
Figure 4.20.: Cumulative Visualisation of all LC-MS/MS Identifications of Exotoxin A.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Immunomodulating Metalloproteinase Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Immunomodulating Metalloproteinase.

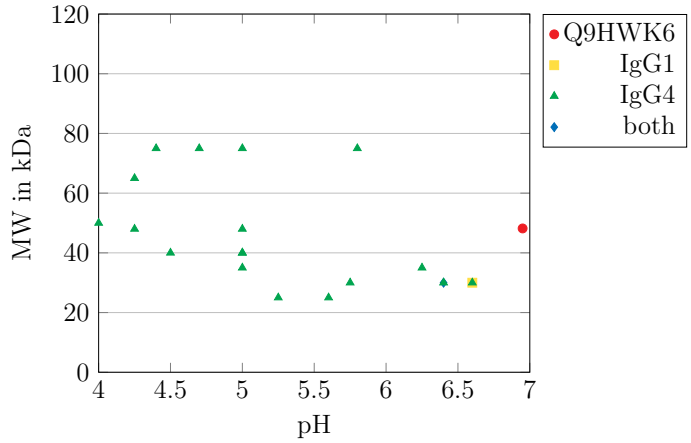
Figure 4.21.: Cumulative Visualisation of all LC-MS/MS Identifications of Immunomodulating Metalloproteinase.



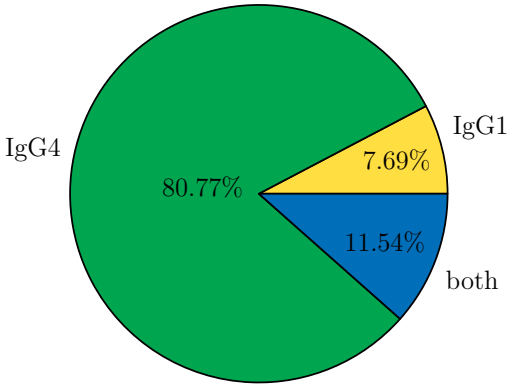
(a) Spatial Distribution of Ig-Binding from Spots Identified as Isocitrate Dehydrogenase Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Isocitrate Dehydrogenase.

Figure 4.22.: Cumulative Visualisation of all LC-MS/MS Identifications of Isocitrate Dehydrogenase.

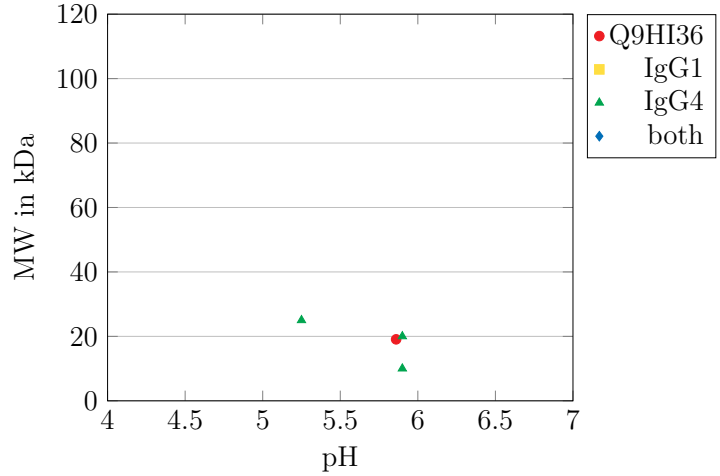


(a) Spatial Distribution of Ig-Binding from Spots Identified as Lysyl Endopeptidase Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

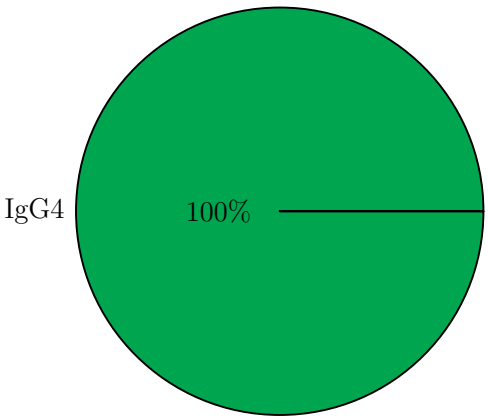


(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Lysyl Endopeptidase.

Figure 4.23.: Cumulative Visualisation of all LC-MS/MS Identifications of Lysyl Endopeptidase.

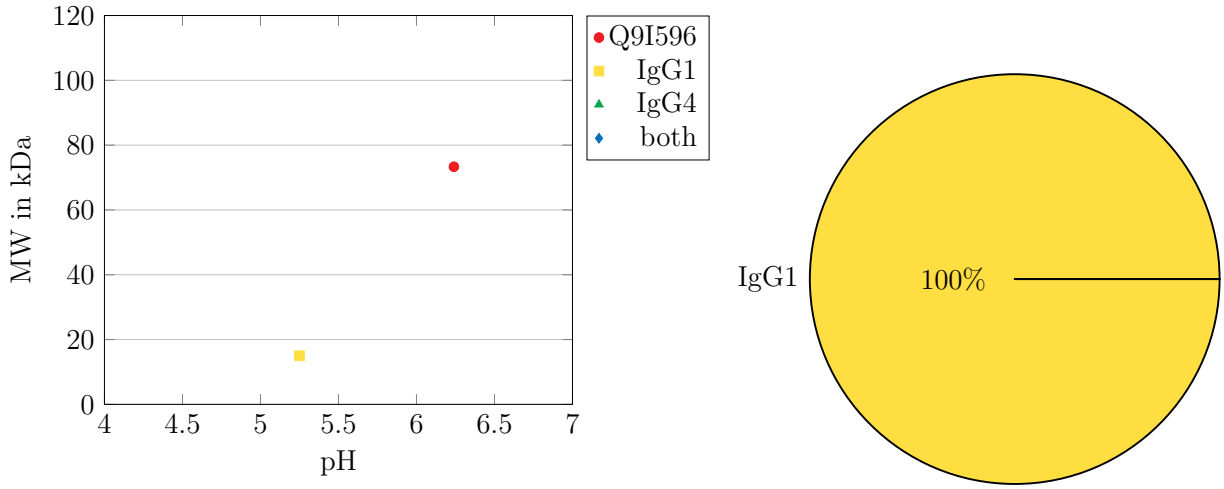


(a) Spatial Distribution of Ig-Binding from Spots Identified as Major Exported Protein Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.



(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Major Exported Protein.

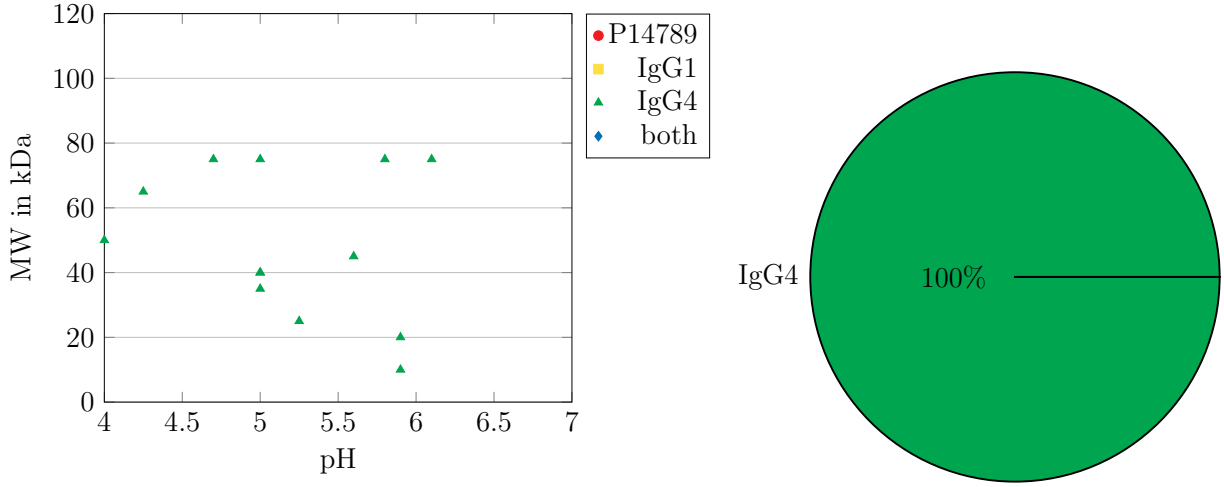
Figure 4.24.: Cumulative Visualisation of all LC-MS/MS Identifications of Major Exported Protein.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Neutral Ceramidase Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Identified Neutral Ceramidase.

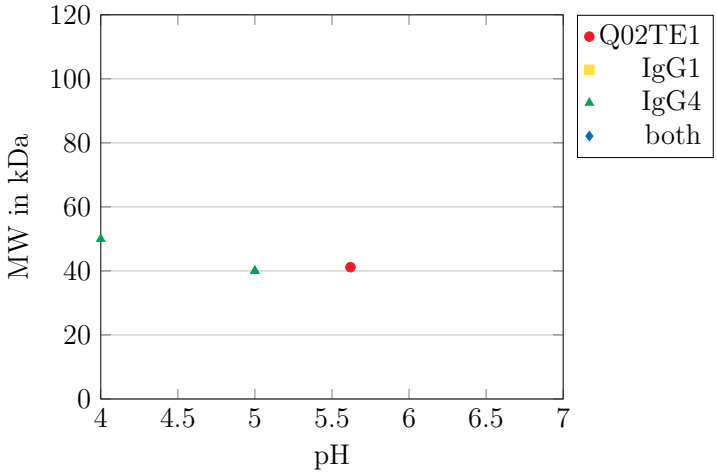
Figure 4.25.: Cumulative Visualisation of all LC-MS/MS Identifications of Neutral Ceramidase.



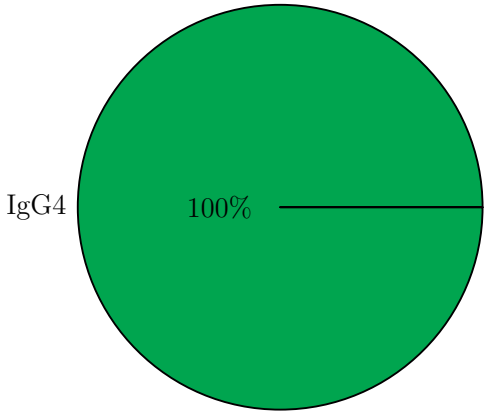
(a) Spatial Distribution of Ig-Binding to Spots Identified as Protease LasA Fragments on Immunoblots. Theoretical MW/IP Denoted in Red, in this Case out of Bounds of the Virtual Blot.

(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Protease LasA.

Figure 4.26.: Cumulative Visualisation of all LC-MS/MS Identifications of Protease LasA.

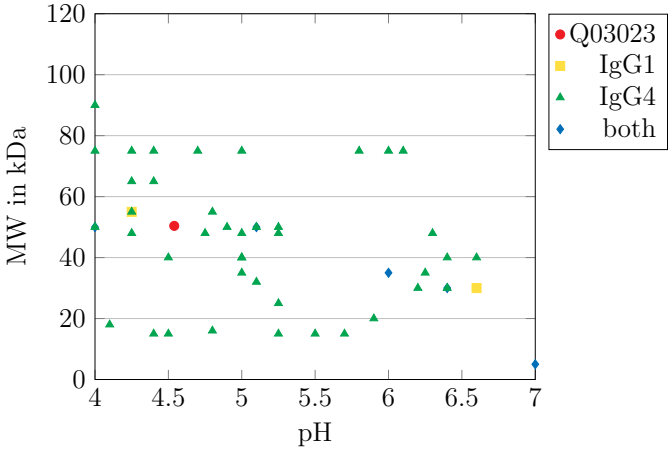


(a) Spatial Distribution of Ig-Binding from Spots Identified as Putative Prophage Major Tail Sheath Protein Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.

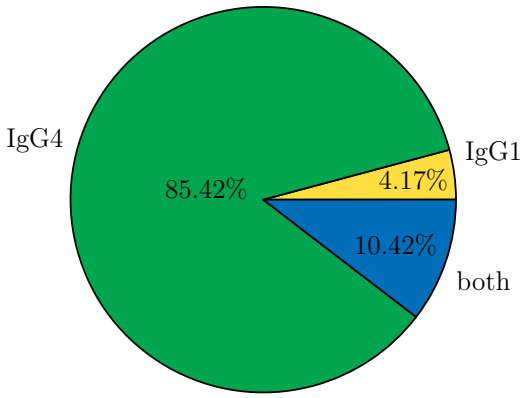


(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Putative Prophage Major Tail Sheath Protein.

Figure 4.27.: Cumulative Visualisation of all LC-MS/MS Identifications of Putative Prophage Major Tail Sheath Protein.



(a) Spatial Distribution of Ig-Binding from Spots Identified as Serralysin Fragments on Immunoblots. Theoretical MW/IP Denoted in Red.



(b) Qualitative Assessment of the Antibody Binding from Spots Identified as Serralysin.

Figure 4.28.: Cumulative Visualisation of all LC-MS/MS Identifications of Serralysin.

4.5.3. Quantification of Immunoglobulin Binding to Bacterial Proteins

Of key interest was a quantitative assessment of the observed antibody binding. This was measured by querying the constructed master database for all spots that contained a given protein. Following this, the registered antibody binding for each of these spots was recorded and tallied up. It was also possible to calculate averaged statistics such as the percentage ratio of IgG4 and IgG1 reactive spots. These results are summarised in Table 4.3 and furthermore broken down for each protein and contextualised with further literature data in the discussion.

Table 4.3.: Tabular Overview of all Identified Proteins.
Listed are Name, UniProt Accession and Gene as Protein Qualifiers, Total Number of Identifications from Western Blot Membranes and the Distribution Concerning the Specific Immune Reaction. Numbers Left to 100 were either Reactive to both Antibodies, or Showed no Reactivity.

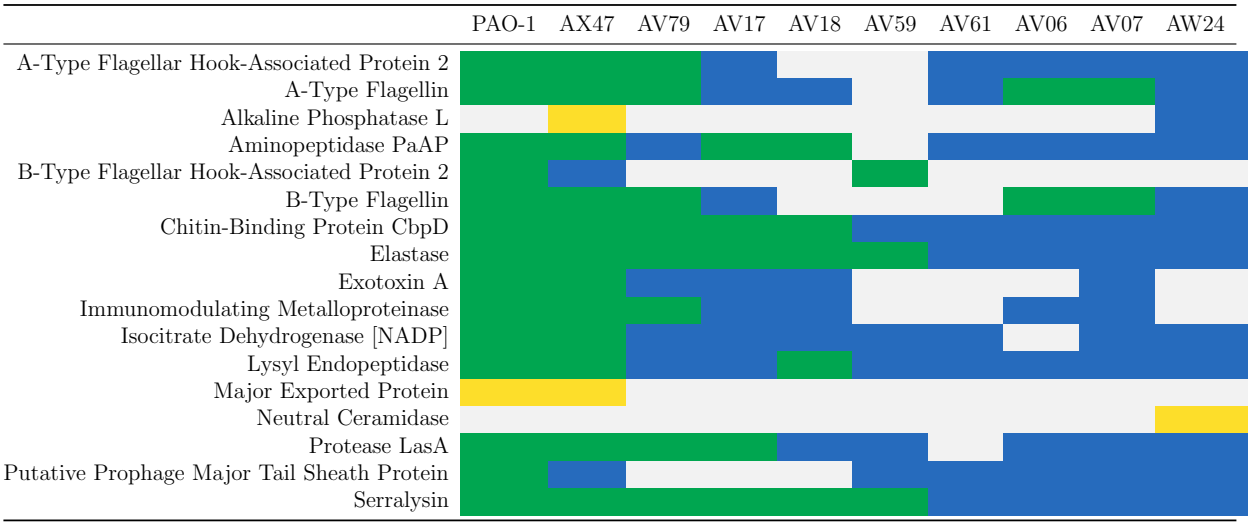
Protein Name	Accession	Gene	Identifications	IgG1	% IgG1	IgG4	% IgG4
A-Type Flagellar Hook-Associated Protein 2	O33421	*fliD*	3	1	25.00	3	75.00
A-Type Flagellin	P21184	*fliC*	27	5	18.52	19	70.37
Alkaline Phosphatase	Q02HI0	*phoA2*	1	0	0.00	1	100.00
Aminopeptidase	Q02PA2	*lap*	38	1	2.63	5	13.16
B-Type Fagellar Hook-Associated Protein 2	Q9K3C5	*fliD*	1	0	0.00	1	100.00
B-Type Flagellin	P72151	*fliC*	26	2	7.69	21	80.77
Chitin-Binding Protein	Q02I11	*cbpD*	55	6	10.91	44	80.00
Elastase	Q02RJ6	*lasB*	47	4	8.51	39	82.98
Exotoxin A	P11439	*eta*	8	0	0.00	8	100.00
Immunomodulating Metalloproteinase	Q9I5W4	*impA*	12	1	8.33	11	91.67
Isocitrate Dehydrogenase	Q02NB5	*icd*	2	0	0.00	2	100.00
Lysyl Endopeptidase	Q9HWK6	*prpL*	20	1	5.00	18	90.00
Major Exported Protein	Q9HI36	*hcpA*	3	0	0.00	3	100.00
Neutral Ceramidase	Q9I596	*PA0845*	1	1	100.00	0	0.00
Protease lasA	P14789	*lasA*	13	0	0.00	13	100.00
Putative Prophage Major Tail Sheath Protein	Q02TE1	*gpFI*	3	0	0.00	3	100.00
Serralysin	Q03023	*aprA*	48	2	4.17	41	85.42
Total Number of Identifications			308	24	7.79	232	75.32

4.5.4. Inter-Strain Differences in Protein Expression

Of further interest were the differences in protein expression between *P. aeruginosa* strains. Such differences were obvious because protein distribution patterns differed on the Western blots (E.g. compare Figures 4.7 and 4.8). To objectify these findings and contextualise them with the results of identifications from individual spots, full culture supernatant digests were prepared for identification by LC-MS/MS.

Table 4.4 was constructed on this data basis. All strains examined are shown, together with the method that was able to identify the proteins of the strain in question. However, this comparison does not contain any information about the quality or quantity of the identification or the associated bound antibody subtypes. Possible reasons for differences in protein expression are discussed in Chapter 5.

Table 4.4.: Heat Map Showing Protein Identification Methods.
Blue-Labelled Proteins were Identified only by Direct Mass Spectrometry of the Bacterial Supernatants. Yellow-Labelled Proteins were Identified in Spots on the Immunoblots, and a Green Label Means that the Protein was Identified in both Methods. Grey Labels Indicate Proteins that were not Found in a given *P. aeruginosa* Strain, but that are Part of *P. aeruginosa's* Known Exoproteome.



5. Discussion

5.1. Adapted Protocols

Many protocols established for *S. aureus* were not compatible with *P. aeruginosa*. This is most likely due to the striking difference between organisms. Fristly, *S. aureus* is a Gram-positive bacterial species, while *P. aeruginosa* is Gram-negative. Secondly, while *S. aureus* is a highly adapted commensal of the human microbiome, *P. aeruginosa* usually lives in abiotic environments, and is not a typical member of the human flora. *P. aeruginosa* was more difficult to grow than *S. aureus*. However, by modifying culture times, precipitation procedures and purification protocols, protein samples of sufficient quality with an adequate yield were obtained. Furthermore, optimising immunoblotting protocols and an improving stripping procedure reduced the required protein amounts and permitted better comparison between samples.

5.2. Identified Proteins

This Section alphabetically lists all exoproteins of *P. aeruginosa* identified by LC-MS/MS and discusses their functions and possible roles as putative virulence factors. The specified accession number is representative for all synonymous accessions. In the UniProt database, several accession numbers refer to the same protein (in the sense of the product of a specific gene). This is due to the fact that proteins are usually entered as strain-specific. The accession numbers used in this thesis refer to the protein with the best sequence match in LC-MS/MS. Further details on the construction of the database and the exclusion procedure are described in Section 3.9.3.

5.2.1. A-Type Flagellar Hook-Associated Protein 2

Table 5.1.: Overview of *P. aeruginosa* A-Type Flagellar Hook-Associated Protein 2.

Name	A-type flagellar hook-associated protein 2	**Gene**	*fliD*
Synonyms	Flagellar or Filament cap protein	**Type**	Auxilliary protein
Accession	O33421	**Function**	Flagellar growth, adhesion to mucin

The A-type flagellar hook-associated protein 2 has two main functions. Primarily, it forms a cap-like structure at the end of the bacterial flagella. This prevents the outflow of not yet polymerised flagelin monomers from inside the flagella into the extracellular space[54]. Secondarily, the A-type flagellar hook-associated protein 2 binds to extracellular polysaccharides such as mucin[55]. Thus, the protein plays an essential role in the colonisation of the intrapulmonary environment, for instance in CF patients.

The A-type flagellar hook-associated protein 2 has been identified much more frequently than its B-type counterpart (see Table 4.4).

5.2.2. A-Type Flagellin

Table 5.2.: Overview of *P. aeruginosa* A-Type Flagellin.

Name	A-type flagellin	**Gene**	*fliC*
Synonyms	None recorded	**Type**	Structure protein
Accession	P21184	**Function**	Monomer, forms bacterial flagellum

The A-type flagellin is one of the two flagella-forming monomeric structural proteins of *P. aeruginosa*. These flagella give *P. aeruginosa* its motility. The expression of flagella is a double-edged sword for bacteria. On the one hand, flagellins allow movement in the environment, and also play a role as an anchor to Muc1 mucin[56]. As such, they facilitate colonisation. On the other hand, the evolutionary highly conserved flagella are among the most potent activators of the innate and adaptive immune system[57]. Past research has evaluated *P. aeruginosa* flagellin and flagellin-associated proteins as potential targets for vaccines[58]. Therefore, a frequently observed adaptation of colonising *P. aeruginosa* strains

is the absence or altered expression of flagellins[59].

This dichotomy is also evident in the identifications. Although A-type flagellin is identified about as frequently as its B-type counterpart (see Table 4.4), some strains show reduced or completely ceased production. Compared to the type B flagellin, there was a less pronounced IgG4 bias, as can be seen in Table 4.3.

5.2.3. Alkaline Phosphatase

Table 5.3.: Overview of *P. aeruginosa* Alkaline Phosphatase.

Name	Alkaline phosphatase	**Gene**	*phoA2*
Synonyms	Low molecular weight phosphatase, Protein DING	**Type**	Hydrolase
Accession	Q02HI0	**Function**	Phosphomono- and di-esterase

The alkaline phosphatase belongs to the protein family *PstS*, the members of which are an integral part of the phosphate metabolism of *P. aeruginosa*. The Alkaline phosphatase is a hydrolase with both phosphomono- and phosphodiesterase activity[60]. Thus, it provides vital organophosphates for the bacterial cell.

The protein has only been identified in two strains (see Table 4.4). Of these, one came from the direct supernatant digestion and one from the immunoblots (see Figure 4.14). Therefore, only very limited statements can be made about the immunogenicity of the protein.

5.2.4. Aminopeptidase

Table 5.4.: Overview of *P. aeruginosa* Aminopeptidase.

Name	Aminopeptidase	**Gene**	*lap*
Synonyms	Leucine aminopeptidase, PaAP	**Type**	Metalloproteinase
Accession	Q02PA2	**Function**	N-terminal proteinase, leucine preference

The *P. aeruginosa* aminopeptidase is a secreted, N-terminal specific exometalloproteinase. It preferentially releases leucine, but can also target other amino acids non-specifically[61]. It requires zinc ions (Zn^{2+}) as a cofactor for activation[62]. The aminopeptidase works best

in an alkaline environment, with a pH optimum of 8.5.

The protein was identified in all strains but one. Therefore, a broad expression and essential function in the metabolism of *P. aeruginosa* can be assumed (see Figure 4.4). However, its significance as a virulence factor remains to be determined. Nonetheless, the high identification rates and almost absent IgG1 binding capacity (see Figure 4.15) indicate Th_2 cell-stimulating properties.

5.2.5. B-Type Flagellar Hook-Associated Protein 2

Table 5.5.: Overview of *P. aeruginosa* B-type Flagellar Hook-Associated Protein 2.

Name	B-type flagellar hook-associated protein 2	**Gene**	*fliD*
Synonyms	Flagellar or Filament cap protein	**Type**	Auxilliary protein
Accession	Q9K3C5	**Function**	Flagellar growth, adhesion to mucin

The B-type flagellar hook-associated protein 2 is essential for the assembly of flagella by preventing the efflux of flagellin monomers from inside unfinished flagella[63]. Furthermore, it also enables adhesion to mucin in the hosts airways, contributing to virulence[55].

Although this protein appears to be indispensable for cell survival, it was only identified in three strains (see Table 4.4). The most likely explanation seems, that this protein is usually not released by *P. aeruginosa* but is assembled directly into the mentioned flagellar cap. Bacterial lysis may explain that the protein was identified in three strains.

5.2.6. B-Type Flagellin

Table 5.6.: Overview of *P. aeruginosa* B-Type Flagellin.

Name	B-type flagellin	**Gene**	*fliC*
Synonyms	None recorded	**Type**	Structure protein
Accession	P72151	**Function**	Monomer, forms bacterial flagellum

The B-type flagellin is a structural component of flagella. It is responsible for motility and possibly adherence to host cells and matrix proteins[59], and shares most of its properties with its type A counterpart.

It was observed as frequent as the A-type flagellin, however it showed a more pronunced IgG4 bias (as displayed in Table 4.3).

5.2.7. Chitin-Binding Protein

Table 5.7.: Overview of *P. aeruginosa* Chitin-Binding Protein.

Name	Chitin-binding protein	**Gene**	*cbpD*
Synonyms	CbpD	**Type**	Adhesion
Accession	Q02I11	**Function**	Binds chitin without modification

This protein is secreted by *P. aeruginosa* and binds chitin without modifying it. It belongs to a family of other chitin-binding proteins called *Carbohydrate Binding Module Family 73*[64]. Other members of this family are known to be important virulence factors in other bacteria and often have peptidohydrolytic properties, and are stapholytic or chitinolytic, respectively[65].

The *P. aeruginosa* chitin-binding protein has been detected in every strain and is a very prevalent secreted protein. However, its significance as a virulence factor in respiratory infections remains elusive. Further research is needed to determine possible additional ligands and potential enzymatic activity.

5.2.8. Elastase

Table 5.8.: Overview of *P. aeruginosa* Elastase.

Name	Elastase	**Gene**	*lasB*
Synonyms	None recorded	**Type**	Metalloproteinase
Accession	Q02RJ6	**Function**	Hydrolysis of elastin, collagen III/IV, fibronectin, IgA

The *P. aeruginosa* elastase is encoded by the lasB gene. It is a member of the family of metalloendoproteinase enzymes. Accordingly, it can cleave proteins. The enzyme uses both zinc (Zn^{2+}) and calcium ions (Ca^{2+}) as cofactors[66]. The elastase is secreted as a pre-pro enzyme[67]. The pre-peptide is cleaved off during transport across the inner cell membrane[68], while the pro-peptide sequence is cleaved off extracellularly. In the latter process, completely processed elastase molecules play the dominant role in a manner of autocatalysis. There is also experimental support for a direct secretion of already active enzymes[67]. Additionally, there is evidence of a stimulatory relationship between the elastase and the protease lasA (This is further discussed in Section 5.2.15).

The elastases' primary target are collagens III and IV, which primarily make up the basal membrane. This allows *P. aeruginosa* to breach the host barriers. Thus, the progression from superficial colonisation into an invasive infection is facilitated, which is especially true for the hosts airways[69]. A secondary target is immunoglobulin A (IgA), which protects mucosal surfaces from bacterial invasion. The elastase is able to cleave IgA, allowing *P. aeruginosa* to make contact with the underlying matrix proteins. This immune evasion mechanism provides elastase-positive bacteria with a significant advantage as a major driver of virulence in pulmonary infection models[70].

The heat map in Table 4.4 shows, that all tested isolates express and secrete the elastase in some manner, indicating a widespread expression in both laboratory strains and clinical isolates. Most spots on the immunoblots were bound by IgG4. This is consistent with previous research, indicating a link between proteolytic activity and Th_2 cell stimulating antigenic proteins[15].

5.2.9. Exotoxin A

Table 5.9.: Overview of *P. aeruginosa* Exotoxin A.

Name	Exotoxin A	**Gene**	*eta*
Synonyms	ETA, PE	**Type**	Toxin
Accession	P11439	**Function**	Transferase, inhibition of protein synthesis

Exotoxin A is a secreted transferase of *P. aeruginosa*, responsible for transferring ribosylated adenosine diphosphate (ADP) from nicotinamide adenine dinucleotide (NAD^+) onto the eukaryotic elongation factor 2, thus arresting protein synthesis in host cells[71]. This is remarkably similar to other ADP-transferring toxins from bacteria like *Vibrio cholerae*, *Corynebacterium diphteriae* and *Bordetella pertussis*[72]. The exotoxin A is discussed as the most important toxin and a crucial virulence factor of *P. aeruginosa*[73].

However, despite its importance for virulence, the protein was identified in only a few strains (see Table 4.4). All spots were bound by IgG4. This might indicate a missing environmental stimulant for production of the toxin.

5.2.10. Immunomodulating Metalloproteinase

Table 5.10.: Overview of *P. aeruginosa* Immunomodulating Metalloproteinase.

Name	Immunomodulating metalloprotease	**Gene**	*impA*
Synonyms	IMPa	**Type**	Metalloproteinase
Accession	Q9I5W4	**Function**	Disruption of innate immune response

The immunomodulating metalloproteinase (IMPa) is a secreted enzyme. As a metalloproteinase, metal ions are required as a co-factor, in this case zinc ions (Zn^{2+}). The primary human target of the proteinase is the P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophil granulocytes. This ligand is essential for binding to P-selectin, and thus for the extravasation of leukocytes. Therefore, the homing of activated immune cells to the site of an infection is impaired by IMPa[74]. In addition, the IMPa inhibits CD55, a glycoprotein present on all host cells in contact with the humoral immune system. In homeostasis, CD55 protects host cells from the damaging effects of activated complement proteins by inhibiting the complement cascade[75].

These functions characterise IMPa as an important virulence factor of *P. aeruginosa*. The altered leukocyte migration disrupts an initial cellular response of the immune system. In addition, due to the disinhibition of the complement system, local tissue destruction may be the result of IMPa action. This could create a niche for *P. aeruginosa* proliferation.

IMPa was identified in almost all isolates (see Table 4.4). This suggests a highly conserved expression pattern and high significance for the survival of *P. aeruginosa* in vivo. All spots identified by immunoblotting were exclusively bound by IgG4, comparable to other proteases examined (see Figure 4.21).

5.2.11. Isocitrate Dehydrogenase [NADP]

Table 5.11.: Overview of *P. aeruginosa* NADP-Dependent Isocitrate Dehydrogenase.

Name	NADP-dependent Isocitrate dehydrogenase	**Gene**	*icd*
Synonyms	IDH/IDP/Oxalosuccinate decarboxylase	**Type**	Metabolic enzyme
Accession	Q02NB5	**Function**	Decarboxylation of isocitrate

The NADP-dependent isocitrate dehydrogenase is an oxidoreductase present in *P. aeruginosa*. It is part of the tricarboxylic acid cycle, and thus of the energy generation system. This dehydrogenase decarboxylates isocitrate in presence of $NADP$ into 2-oxoglutarate, CO_2 and $NADPH$[76]. It requires both Zn^{2+} and Mg^{2+} to function. While not directly involved in virulence, this enzyme is essential for metabolism, and, hence, bacterial fitness. It is possible, that this enzyme is only found extracellularly due to cell lysis, but it has been annotated as "secreted" in the reviewed literature and thus was included.

The dehydrogenase was identified in all but one strain, albeit most of the time only in mass spectrometry of direct culture supernatants (see Table 4.4). This limits statements about its immunogenicity, as only two identifications could be obtained from immunoblots. Both of these were bound by IgG4.

5.2.12. Lysyl Endopeptidase

Table 5.12.: Overview of *P. aeruginosa* Lysyl Endopeptidase.

Name	Lysyl Endopeptidase	**Gene**	*prpL*
Synonyms	Protease IV	**Type**	Protease
Accession	Q9HWK6	**Function**	Lysine-specific protease

The lysyl endopeptidase is a peptidase preferentially cleaving lysine residues[77]. It is produced as an inactive pro-enzyme and is autocatalytically activated. The peptidase is deemed to play a crucial role in corneal infections[78].

This protein was expressed by all examined strains, indicating non-environment dependant secretion. 80.77% of all identifications came from spots that showed bound IgG4, which is again concordant with previous findings in literature.

5.2.13. Major Exported Protein

Table 5.13.: Overview of *P. aeruginosa* Major Exported Protein.

Name	Major exported protein	**Gene**	*hcpA*
Synonyms	Secreted protein hcp	**Type**	Other
Accession	Q9HI36	**Function**	Not detailed

The major exported protein is part of the *hcp1* protein family[79][80]. These proteins are secreted *P. aeruginosa* in the context of chronic infection in CF patients.[81] Typically, they form extracellular hexameric structures. These proteins are thought to play a supporting role as virulence factors in chronic infections. The encoding gene locus (HCI-I) is also widespread among other bacterial species. However, the exact function of the proteins produced has not yet been adequately researched.

The major exported protein could only be detected on the immunoblots of two isolates, but not in the supernatant (see Table 4.4). This suggests an environment-dependent expression, which is also discussed in literature[81]. All identified instances were bound by IgG4. However, since only a small number of fragments were found, no conclusive assessment of a potential Th_2 cell bias can be given.

5.2.14. Neutral Ceramidase

The neutral ceramidase is a hydrolase that catalyses the cleavage of ceramides to sphingosine and free fatty acids and the reverse reaction[82]. Its pH optimum is between 7.5 and 9.5. Zn^{2+} and Mg^{2+} are required as co-factors for the reaction. The neutral ceramidase is an essential part of the lipid metabolism of *P. aeruginosa*. In addition, a role as a virulence factor

Table 5.14.: Overview of *P. aeruginosa* Neutral Ceramidase.

Name	Neutral Ceramidase	**Gene**	*PA0845*
Synonyms	Acylsphingosine deacylase, NC-Dase	**Type**	Hydrolase
Accession	Q9I596	**Function**	Synthesis and degradation of ceramides

is discussed. The C-terminal end of the protein has structural similarity to both cadherin and invasin (a bacterial integrin binding protein[83]). Therefore, the neutral ceramidase could also serve as a binding partner for integrins. Thus, a possible membrane-bound production of the protein is also being discussed. This would facilitate *P. aeruginosa* binding to cells of the host organism.[84].

Only one immunoblot spot containing the neutral ceramidase could be identified. This could indicate a membrane-bound expression. However, no reliable assessment of a possible immune bias can be discussed with this limited number of samples.

5.2.15. Protease LasA

Table 5.15.: Overview of *P. aeruginosa* Protease LasA.

Name	Protease LasA	**Gene**	*lasA*
Synonyms	Stapholytic protease	**Type**	Metalloproteinase
Accession	P14789	**Function**	Elastolysis, stapholysis

The LasA protein is a zinc (Zn^{2+}) dependent metalloproteinase[85]. The main targets of the protease are elastins, but non-specific proteolysis is also possible. It preferentially targets triglycine segments[86]. In addition, the protease can lyse staphylococci. This occurs through the disruption of the pentaglycine cross-links in staphylococcal cell walls[87]. Furthermore, previous research has demonstrated that the LasA protease enhances the activity of the *P. aeruginosa* elastase lasB, as well as, other elastases, like the enzyme expressed by human neutrophils. This is hypothesised to function by exposing vulnerable peptide sequences in the target through proteolysis[86].

As a virulence factor, the protease has two functions. Primarily, by disrupting the barrier

function of tissue, it allows *P. aeruginosa* to create niches in which it is easier for microorganisms to multiply. Secondarily, with its stapholytic properties the protease contributes to the displacement of possible competitors from the shared habitat. This gives a clear advantage to LasA-expressing strains, especially in polymicrobially colonised areas such as the upper respiratory tract. It remains to be determined what role this protease plays in the known dynamics of airway colonisation in CF patients. Here, a progressive displacement of *S. aureus* by *P. aeruginosa* with age is highly characteristic[45].

The protein has been identified in all but one strain (see Table 4.4). This underlines its importance as a virulence factor of *P. aeruginosa*. Similar to other proteases, all spots identified on the immunoblots were bound by IgG4 (see Figure 4.26).

5.2.16. Putative Prophage Major Tail Sheath Protein

Table 5.16.: Overview of *P. aeruginosa* Putative Prophage Major Tail Sheath Protein.

Name	Putative prophage major tail sheath protein	**Gene**	*gpFI*
Synonyms	GpFI	**Type**	Other
Accession	Q02TE1	**Function**	Not detailed

The Putative prophage major tail sheath protein (GpFI) is part of a cluster of possible phage proteins that have been integrated into the genome of *P. aeruginosa*[88]. No benefit for the survival or virulence of *P. aeruginosa* has yet been determined.

Overall, the protein was detected in 7 of 10 isolates. Only the reference strain showed sufficient expression for immunoblot identification (see Figure 4.27). Both of these were bound by IgG4. However, a statement on the immunogenicity of the protein is difficult to derive with an insufficient sample size.

5.2.17. Serralysin

Serralysin is a zinc- (Zn^{2+}) and calcium- (Ca^{2+}) dependent metalloproteinase secreted by *P. aeruginosa*[89]. Its preferred targets are amino acids with hydrophobic side chains. Serralysin works with considerably less specificity than comparable proteinases from other bac-

Table 5.17.: Overview of *P. aeruginosa* Serralysin.

Name	Serralysin	**Gene**	*aprA*
Synonyms	Alkaline metalloproteinase	**Type**	Metalloproteinase
Accession	Q03023	**Function**	Negative influence on complement activation

terial species such as *B. subtilis*[90].

Serralysin plays a leading role in the inhibition of the innate immune response. In particular, the proteinase inactivates the complement protein C2, which prevents the formation of the C3 convertase C4b2b. This is essential for two of the three complement pathways. Thus, the formation of the opsonin C3b and the chemoattractant anaphylatoxin C5a is prevented. Accordingly, neutrophil function is also impaired[91].

Serralysin was identified in all *P. aeruginosa* strains. The majority (85.42%) of all spots from which serralysin was identified were bound by IgG4. This suggests a strong Th_2 cell stimulating property, in conjunction with previous research on the immunomodulatory properties of proteases.

5.3. Evaluation of Immune Reactions

As shown in the comparison between identifications from immunoblots and from direct digestion of culture supernatants, all identified proteins are bound by specific immunoglobulins to varying degrees. Thus, they must be targets of the hosts immune system. However, as the distribution graphs in Section 4.5.2 show, the proportions of bound IgG4 versus IgG1 were differing considerably. As Table 4.3 illustrates, more spots were bound by IgG4 (75.32%) compared to IgG1 (7.79%). In the following Section, possible explanations for this are discussed.

There are large inter-individual differences in the composition of the specific Igs between CF patients. However, the aim of this work was the discovery of bacterial proteins that are able to elicit a type 2 immune response. Therefore, to assess the overall immune reactivity of a given bacterial protein, all spots were considered, disregarding with which sera they

reacted. This allowed for generalisation, but obscured, for example, that some sera were dominated by *P. aeruginosa* specific IgG1 but bound only a few spots of the protein under discussion. This effect is amplified by the use of donor-specific sera/strain pairs. Thus, only the most reactive sera from a patient in the screening assay were tested against the most productive *P. aeruginosa* isolate of a given expression profile. Another problem lies in the way proteins are recognised by the immune system. Igs do not recognise a protein in its entirety, but only a fraction (their epitope). This epitope is specific to a clonal group of B- and T-cells. Accordingly, a protein can be recognised by a number of a patients T- and B-cells and thus can be bound by Igs of different subclasses. Despite this intra- and inter-individual variance, a drastic difference between IgG4- and IgG1-binding emerged. IgG4-binding was considered as a proxy of IgE-binding, as both Ig subclasses are generated under similar immunological conditions. Thus in many cases IgG4 indicates a type 2 immune response that is orchestrated by Th_2 cell cells. This has been directly demonstrated in *S. aureus*[15, 14, 16]. IgG4-binding and, hence, a presumed Th_2 cell bias was particularly pronounced in proteins with proteolytic activity, which corresponds to reports in the literature[8].

This exploratory study did not test control sera from healthy volunteers. Many healthy people lack will lack specific antibodies against *P. aeruginosa*. Therefore, CF patients with known long-term exposure to the bacterial species were selected for study. Further experiments are necessary to test whether the identified proteins can skew the immune response to a Th_2 cell dominated reaction in healthy individuals. Moreover, a general propensity for a Th_2 cell bias in the immune system of patients with CF can not be excluded. However, it is clear that the proteins identified and discussed here are bound by IgG4 and can therefore elicit a biased immune response that is probably driven by Th_2 cells.

5.4. Differences in Protein Expression and Distribution

By comparing the proteins identified on immunoblots with those identified in bacterial culture supernatants, some assumptions can be made about the expression patterns of different strains. On one hand, some proteins such as serralysin and elastase were expressed by almost all strains and were found in both detection methods. On the other hand, the neutral ceramidase or the major exported protein were only found in a few strains, sometimes only

in one of the two detection methods. Clearly, not all proteins encoded in the genome are produced by *P. aeruginosa* under all conditions. The culture conditions used in this work may have been more favourable for some proteins than for others. Another possible reason is the adaptation of a *P. aeruginosa* strain to the specific environment of its host. A frequently documented form of this adaptation through suppression is the loss of flagellar proteins. Flagella are evolutionarily highly conserved proteins between bacterial species. The innate immune system recognises them very easily, for example via Toll-like Receptors[57]. Therefore, the loss of flagellar motility can be an advantage for some strains. Moreover, some strains actively suppress the production of flagella when colonising the respiratory tract[92]. The mechanism of suppression can be a lack of transcription factors, the specific suppression of a gene, or even its permanent deactivation by mutation. An opposite example would be proteins that are only necessary in specific circumstances, like infection, such as the neutral ceramidase. This protein has a strong association with invasive infections[84], and is most likely only expressed in such a setting. Proteins identified in all strains, on the other hand, are probably essential for *P. aeruginosa*, such as serralysin and the chitin-binding protein.

As discussed before, all known secreted proteins of *P. aeruginosa* have been identified in this study. However, some proteins have a theoretical IP outside the pH range of 4 – 7. These likely make up the spots outside of the tested pH range, that can be seen on Figure 4.3. Some of the proteins with an IP >7 could be the A-type flagellar hook-associated protein 2 (calculated IP at pH = 8.41), the alkaline phosphatase (calculated IP at pH = 9.03) or the LasA protease (calculated IP at pH = 8.88). Nevertheless, these proteins were also found in spots between pH 4 – 7. This may be due to proteolytic cleavage resulting in several protein spots after gel electrophoresis. The protein fragments or isoforms usually have an IP that differs from the complete protein. Accordingly, they show a different horizontal migration behaviour, as well as an accelerated vertical migration due to smaller molecular weight in SDS-PAGE.

6. Conclusions

The results discussed and the findings of the literature review allow three important conclusions to be drawn.

Firstly, it has been shown, that the expression patterns of some proteins were strongly strain-dependent. While some proteins were detected in all strains, the production of other proteins was much more restricted. Strain-specific genetic adaptations to the host environment might account for this. Possible positive effects for *P. aeruginosa* could range from increased invasiveness to reduced detectability by the host's immune system. However, a lack of required transcription factors or unfavourable culture conditions might also play a role.

Secondly, it was shown that the tested *P. aeruginosa* isolates secrete proteins that were recognised by the immune system of the donors suffering from CF. The identified proteins comprise the entire recorded secretome of *P. aeruginosa*. Of these, 8 proteins show proteolytic activity and 5 are structural proteins. Two proteins are known to be exotoxins, one is a metabolic enzyme and one a phage protein. Some of the identified proteins, especially the proteases, may be essential for the virulence of *P. aeruginosa*, for example by creating survival niches for the bacteria or by directly and indirectly inhibiting an anti-bacterial immune response[66, 71, 75, 91].

Lastly, a clear IgG4 bias was observed in Ig-binding to the peptides identified by immunoblotting. Around three quarters of all spots were selectively IgG4-reactive compared to 7.8% that were bound only by IgG1. The remaining spots were bound by either both IgG1 and IgG4, or by no IgG at all. This was unexpected, as a protective immune response against extracellular bacteria relies on a type 1 or type 3 immune response, that is orchestrated by

Th_1 cells or Th_{17} cells, respectively, and which is dominated by IgG1 in humans. In contrast, IgG4 is generated in chronic exposure to an antigen, and – like IgE – depends on Th_2 cell derived cytokines[19]. Deviating the immune response from Th_1 cells to Th_2 cells could allow bacterial pathogens to flourish. Thus, it has been proposed, that enforcing a Th_2 cell dominated response could be a mechanism of immune evasion[14]. It could give *P. aeruginosa* an advantage in colonisation and persistence in the lungs of CF patients. Therefore, protracted respiratory illnesses and frequent hospital visits could become more common.

In summary, this work has shed light on *P. aeruginosa* host-pathogen-interactions. It shows, that *P. aeruginosa* secretes immunogenic proteins that are recognised with a strong IgG4 bias in CF. This might indicate an ineffective immune response to the pathogen. These findings open a door toward a better understanding of type 2 immune responses to bacteria and their role in the pathogenesis of CF. However, further research is needed. It is still unclear whether the proteins themselves or the way they are exposed to the immune system promote a Th_2 cell dominated response, or whether CF patients per se have a higher propensity for a dysregulated type 2 immune response. Furthermore, the changed pulmonary environment due to a sustained inflammatory reaction in CF patients creates surroundings not accounted for by in-vitro methods.

7. Abstract

The study of host-pathogen interactions is central to a better understanding of the human microbiome, infections and the inner workings of immune cells. One focal point of this research is how the human immune system recognises both harmful and harmless antigens, integrates the resulting signals and forms a response, and how, conversely, microbes can manipulate this reaction.

In this thesis, *Pseudomonas aeruginosa* (*P. aeruginosa*), a critical pathogen in chronic and nosocomial infections, was in the focus. The aim was to search for bacterial proteins that favour a type 2 immune response, as it is orchestrated by $CD4^+$ type 2 T helper cells (Th_2 cells). The humoral arm of a type 2 response is dominated by IgG4 and IgE. Such immune responses are typically directed against multicellular pathogens like helminths and other parasites. However, type 2 immune responses are suboptimal for the defence against extracellular bacteria like *P. aeruginosa*. Previous research suggests that some bacterial proteins may promote a switch to such an insufficient immune response as a mechanism of immune evasion.

To optimise the sensitivity of the search for type 2 response inducing proteins of *P. aeruginosa*, cystic fibrosis (CF) patients were studied, as many are exposed to the pathogen in their airways over prolonged time periods. As such, the humoral immune response of 9 CF patients to their own *P. aeruginosa* strain was examined. For this, the secretomes of 9 clinical *P. aeruginosa* isolates from CF patients and the *P. aeruginosa* reference strain PAO-1 were studied by 2D-immunoblotting for their ability to be bound by IgG4 and IgG1 from respective patient sera. IgG4 served as a proxy for IgE, as assays analysing IgE binding suffer from low sensitivity because of low serum concentrations of IgE. Antibody reactive *P. aeruginosa* proteins were then identified by liquid chromatography tandem mass spectro-

metry and the results were compared with proteomics data from literature.

In total, 308 distinct protein spots were analysed. These belonged to 17 bacterial proteins, which comprise the entire known *P. aeruginosa* secretome. Of these spots, 232 were bound by IgG4, and 24 by IgG1 only. Notably proteases like serralysin and *P. aeruginosa* elastase presented with an IgG4 bias. This is concordant with previous research linking proteases to a type 2 immune response. Moreover, structural proteins like flagellins were also immunodominant. Flagellins are known as common targets of immune detection in bacteria. These proteins also demonstrated a clear IgG4 bias.

Thus, the search for secreted *P. aeruginosa* proteins that elicit an IgG4-dominated antibody response was successful. It remains to be shown whether these bacterial proteins are also recognized by IgE and Th_2 cells, meaning whether they are truly driving a type 2 immune response in CF patients. It is also an open question whether the observed IgG4 bias in the antibody response to the exoproteome of *P. aeruginosa* is specific to CF or a general feature of the human immune response to the pathogen.

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Appendices

A. Supplementary Data

Table A.1.: Overview of all *P. aeruginosa* Isolates and Respective Patient/Sera.

Patient	Strain Name	Associated Sera
Reference	PAO-1	—
P01-01	AX47, AX48, AX49, AX50	D001, D017, D030
P01-03	AV79, AV80	D006, D025
P01-05	AV17, AV18, AV19	D009
P02-01	AV57, AV58, AV59, AV60, AV61	D002, D018, D027
P02-04	AV06, AV07, AV08	D007, D015, D019
P02-05	AV62, AV63	D012, D028
P02-06	AW21, AW22, AW23, AW24	D013

Table A.2.: Growth Data on Examined *P. aeruginosa* Isolates.
An $OD_{595} > 1$ at $t = 300\ min$ was Determined as the Cut-Off to Distinguish between Slow/Fast Replicating Isolates. t_{stat} = Time to Stationary Growth Phase. t_{prec} = Time to Precipitation. *n.o.* = Not Observed. Preliminary Experiments Indicated Similar Growth of AX48 – AX50 to AX47.

Patient	Isolate	OD_{595} at t=300 min	t_{stat} in min	t_{prec} in min
Reference strain	PAO-1	1.380	240	420
P01-01	AX47	1.170	330	510
	AX48			
	AX49		*n.o.*	
	AX50			
P01-03	AV79	0.672	585	765
	AV80	0.802	480	660
P01-05	AV17	1.000	450	630
	AV18	2.470	330	510
	AV19	0.940	490	670
P02-01	AV57	2.510	330	510
	AV58	1.400	405	585
	AV59	1.180	435	615
	AV60	1.350	390	570
	AV61	1.330	420	600
P02-04	AV06	2.150	330	510
	AV07	1.220	420	600
	AV08	1.430	360	540
P02-05	AV62	0.553	510	690
	AV63	0.444	570	750
P02-06	AW21	0.376	420	600
	AW22	0.730	450	630
	AW23	0.573	510	690
	AW24	0.730	420	600

B. Supplementary Images

B.1. PEGGY-Sue Monodimensional Immunoblots

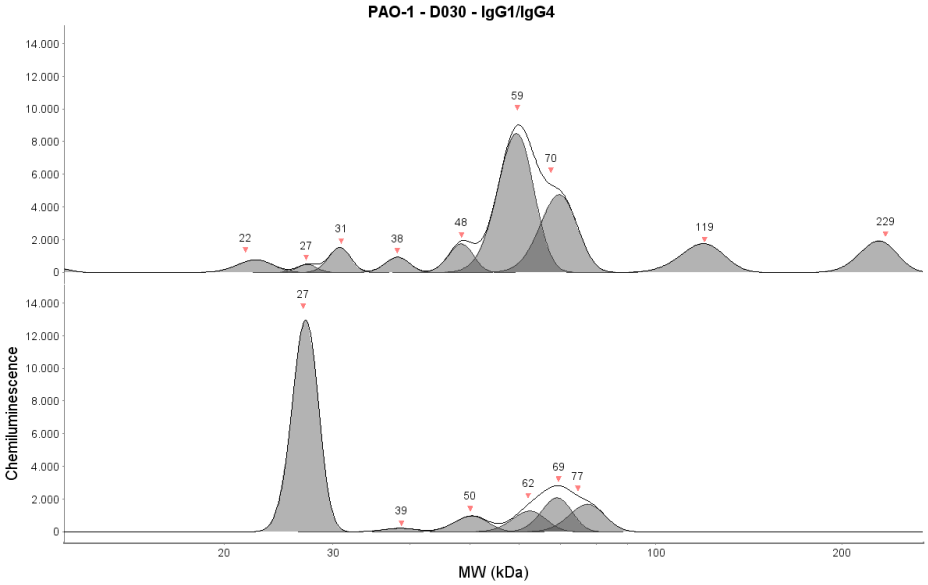

Figure B.1.: PEGGY-Sue Blot (Strain PAO-1, Serum D030).
Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

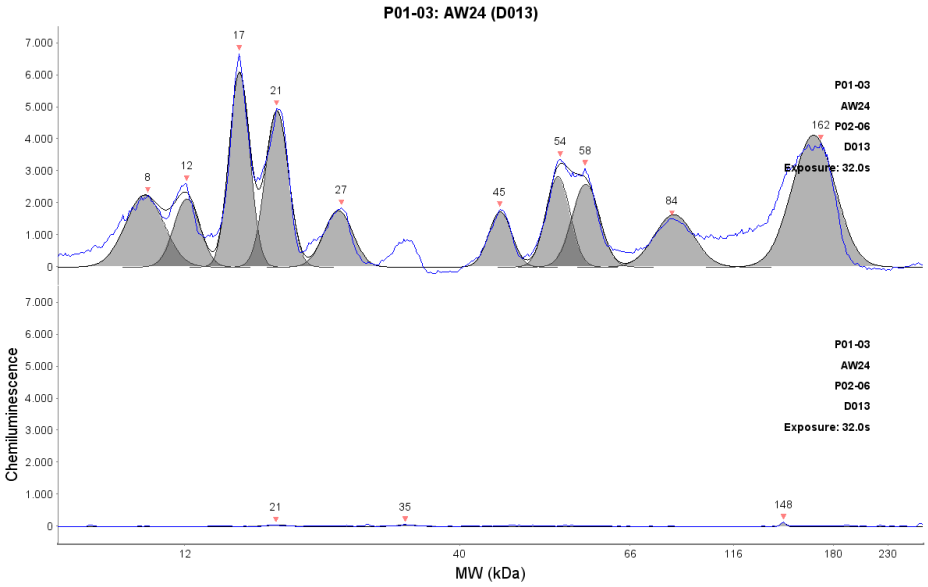

Figure B.2.: PEGGY-Sue Blot Patient (Strain AW24, Serum D013).
Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

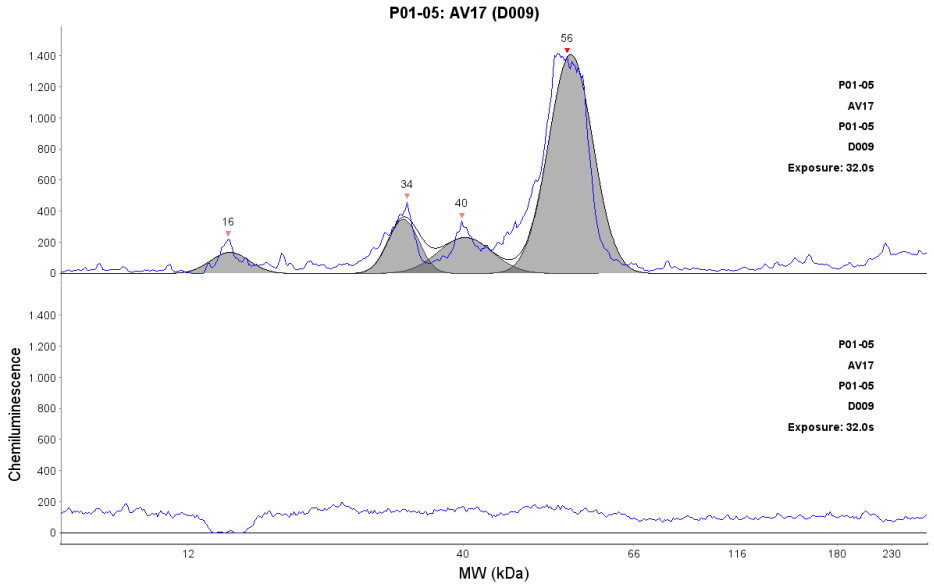

Figure B.3.: PEGGY-Sue Blot Patient P01-05 (Strain AV17, Serum D009). Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

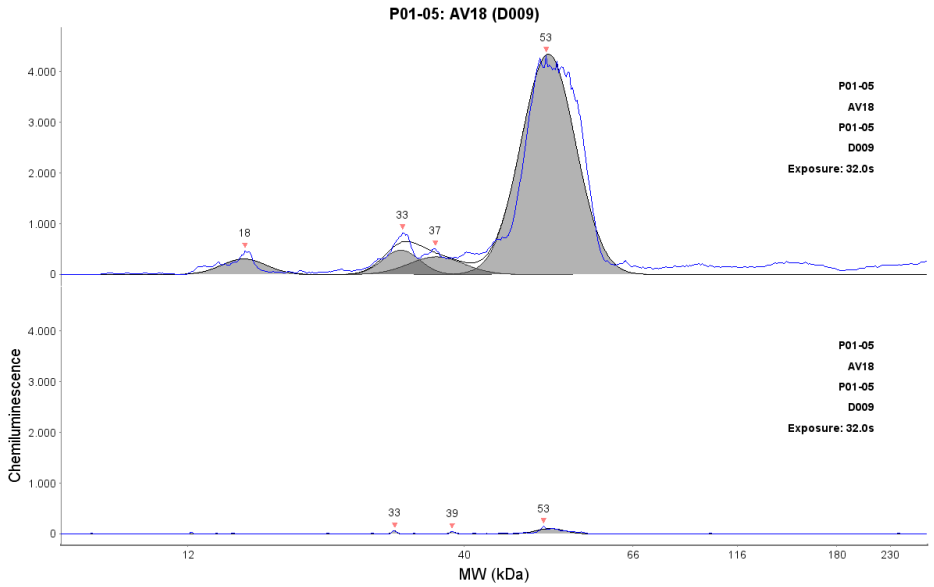

Figure B.4.: PEGGY-Sue Blot Patient P01-05 (Strain AV18, Serum D009). Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

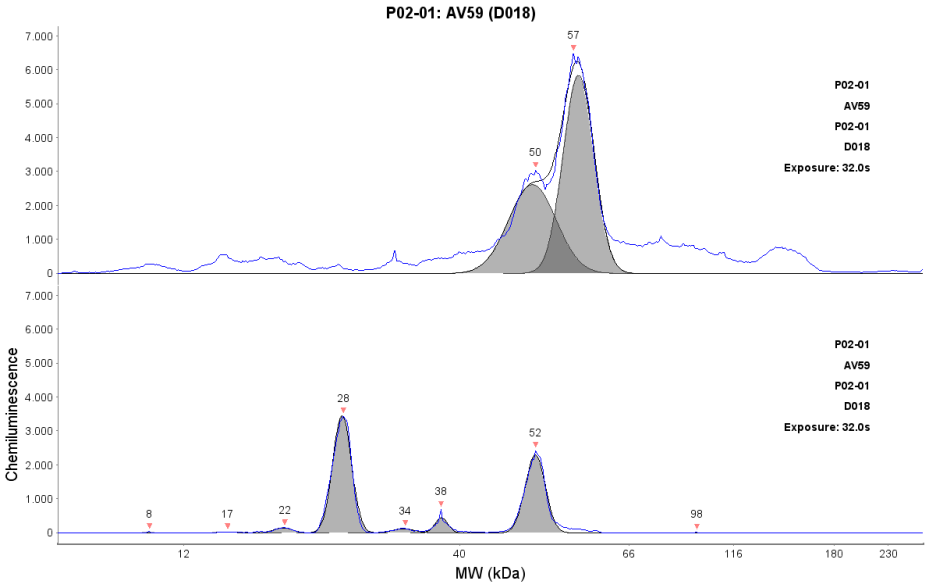

Figure B.5.: PEGGY-Sue Blot Patient P02-01 (Strain AV59, Serum D018). Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

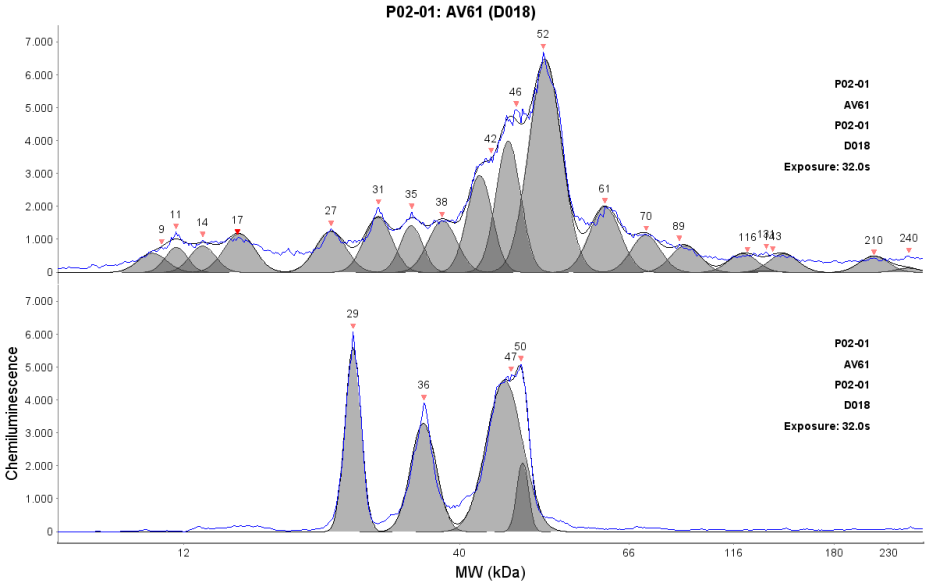

Figure B.6.: PEGGY-Sue Blot Patient P2-01 (Strain AV61, Serum D018). Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

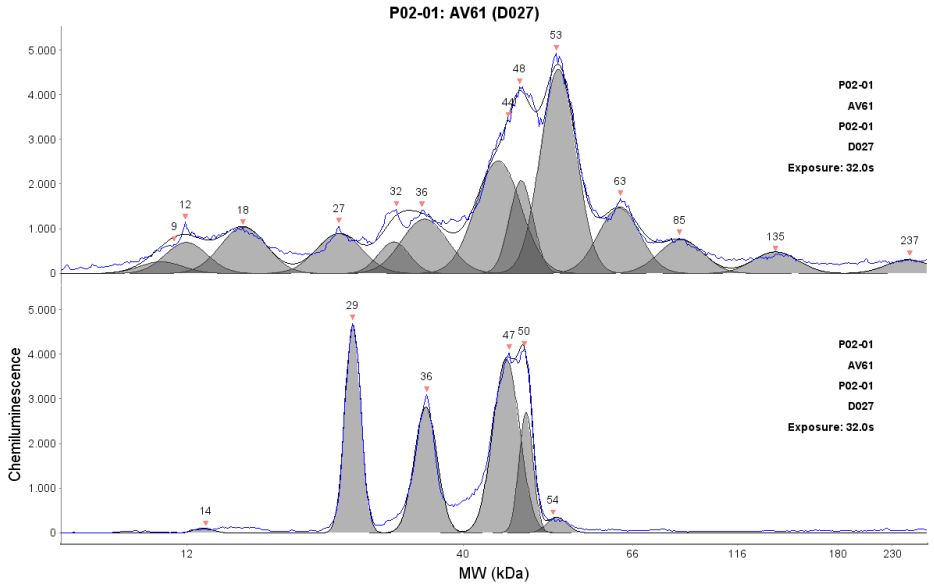

Figure B.7.: PEGGY-Sue Blot Patient P02-01 (Strain AV61, Serum D027). Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

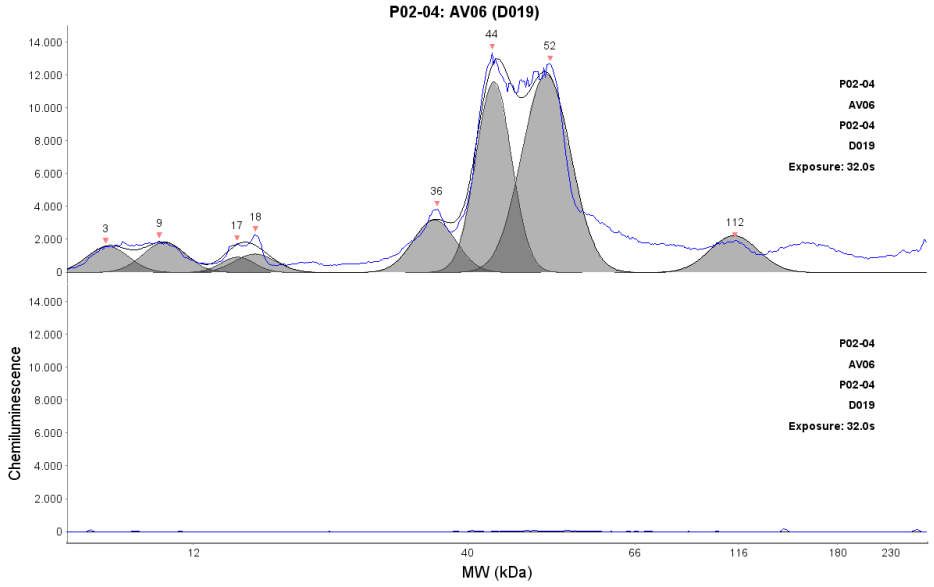

Figure B.8.: PEGGY-Sue Blot Patient P02-04 (Strain AV06, Serum D019). Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

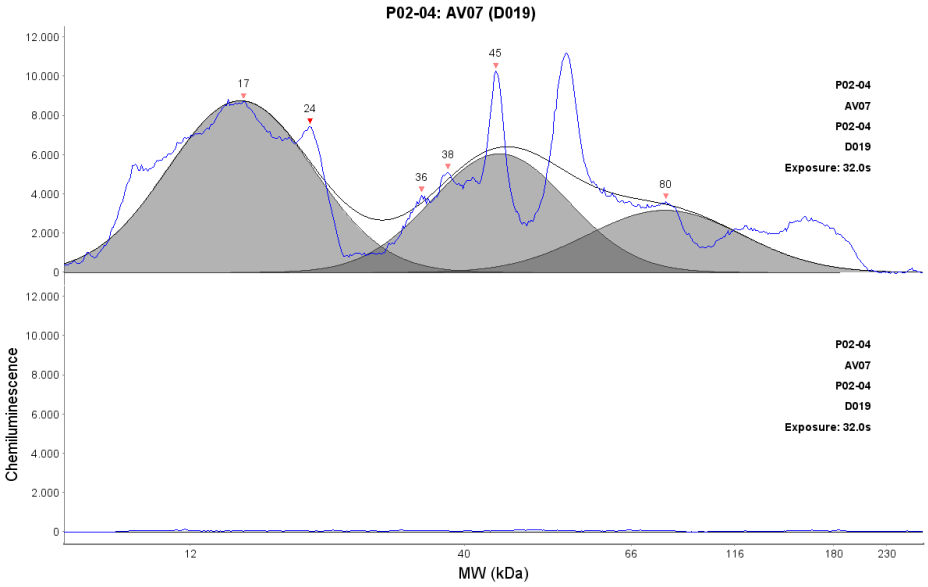

Figure B.9.: PEGGY-Sue Blot Patient P02-04 (Strain AV07, Serum D019).
Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

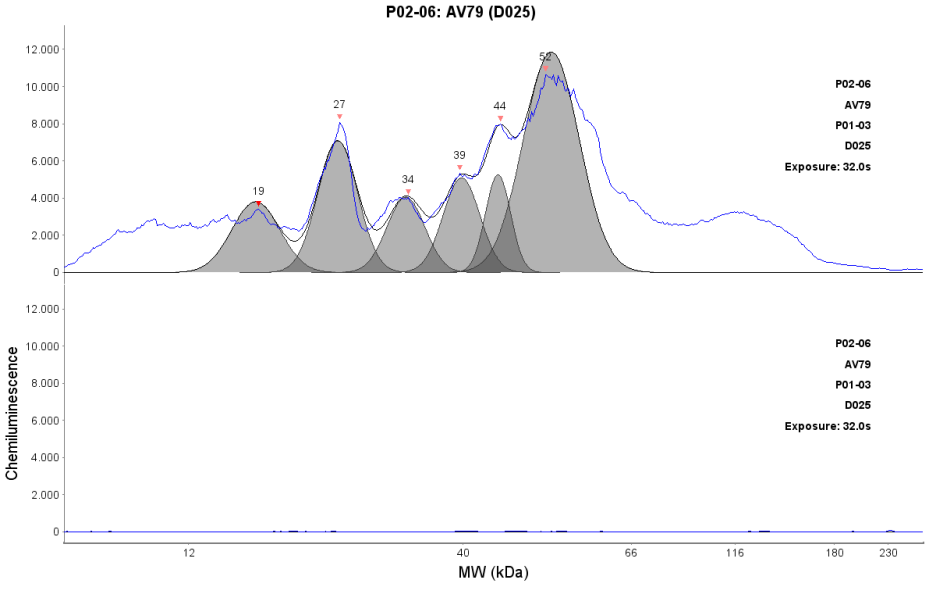

Figure B.10.: PEGGY-Sue Blot (Strain AV79, Serum D025).
Upper Graph Shows IgG1-Reactivity Measured by ECL, Lower Graph Shows IgG4-Reactivity. Exposure 32 s.

B.2. 2D-Immunoblots

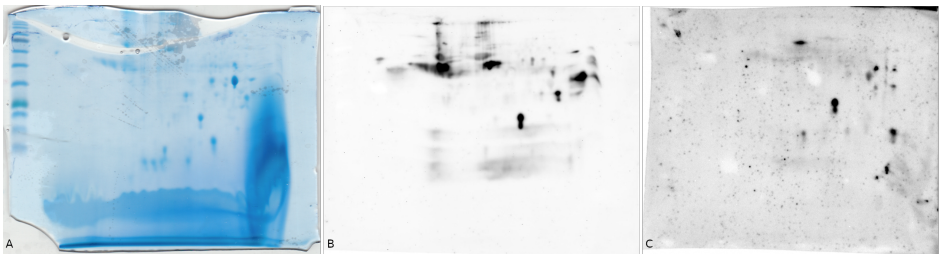

Figure B.11.: Patient P01-03: Secreted Proteins of *P. aeruginosa* Strain AW24, pH 4 – 7 Probed with Serum D025.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

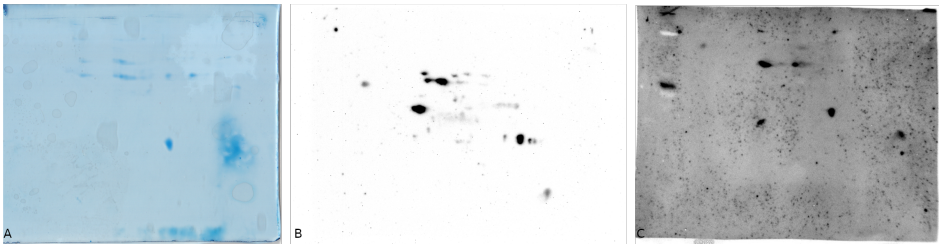

Figure B.12.: Patient P01-05: Secreted Proteins of *P. aeruginosa* Strain AV17, pH 4 – 7 Probed with Serum D009.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

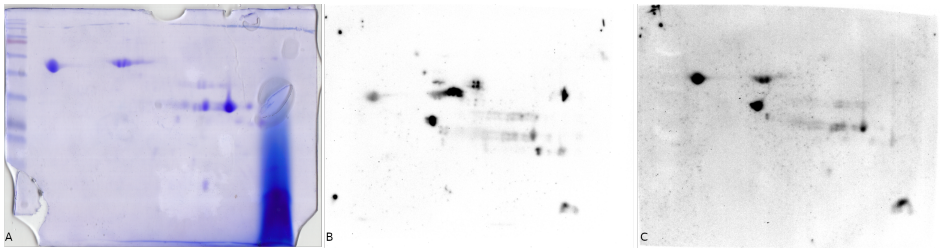

Figure B.13.: Patient P01-05: Secreted Proteins of *P. aeruginosa* Strain AV18, pH 4 – 7 Probed with Serum D009.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

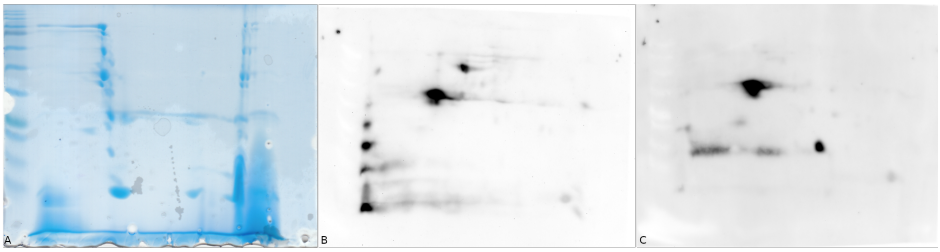

Figure B.14.: Patient P02-01: Secreted Proteins of *P. aeruginosa* Strain AV61, pH 4 – 7 Probed with Serum D027.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

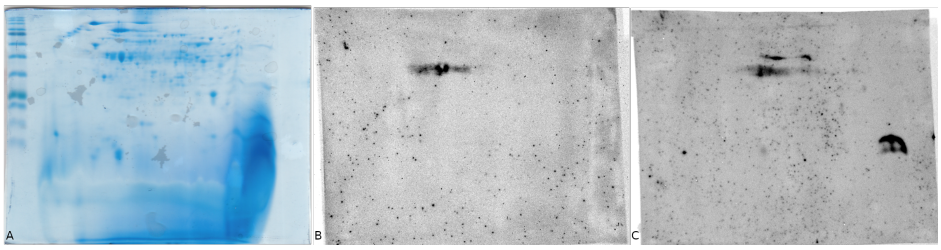

Figure B.15.: Patient P02-04: Secreted Proteins of *P. aeruginosa* Strain AV06, pH 4 – 7 Probed with Serum D019.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

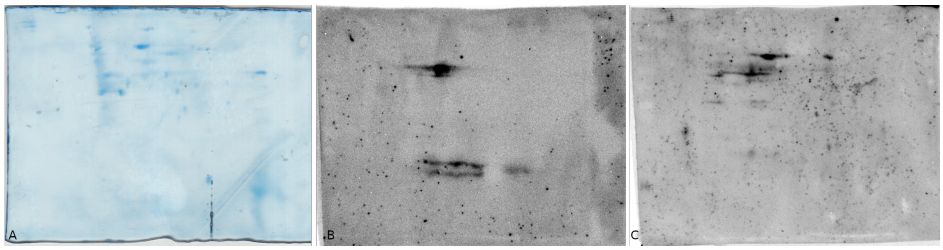

Figure B.16.: Patient P02-04: Secreted Proteins of *P. aeruginosa* Strain AV07, pH 4 – 7 Probed with Serum D019.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

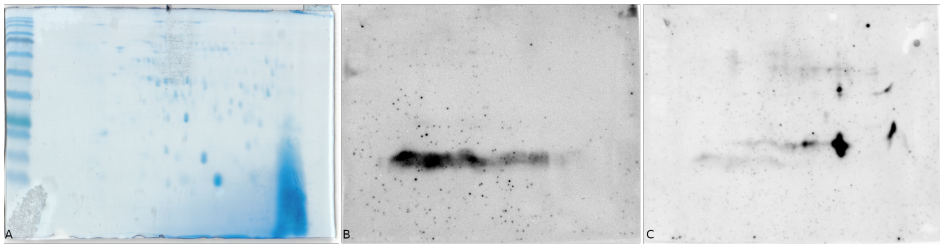

Figure B.17.: Patient P02-06: Secreted Proteins of *P. aeruginosa* Strain AV79, pH 4 – 7 Probed with Serum D013.
(A) *P. aeruginosa* Proteins. (B) IgG1 Binding. (C) IgG4 Binding.

B.3. Colorimetric Assay Blots for Membrane Digestion

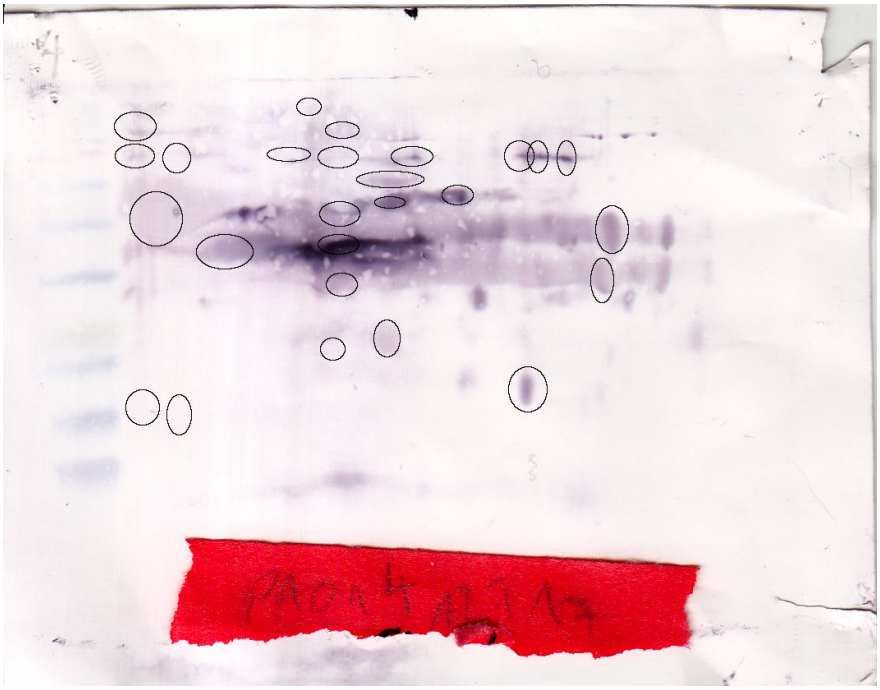

Figure B.18.: AP-Blot ECL-Overlay (Strain PAO-1, Serum D027), pH 4 – 7.
As this was Used for Preliminary Analysis, all Cut Spots were Identified as IgG4 Reactive in Previous Experiments.

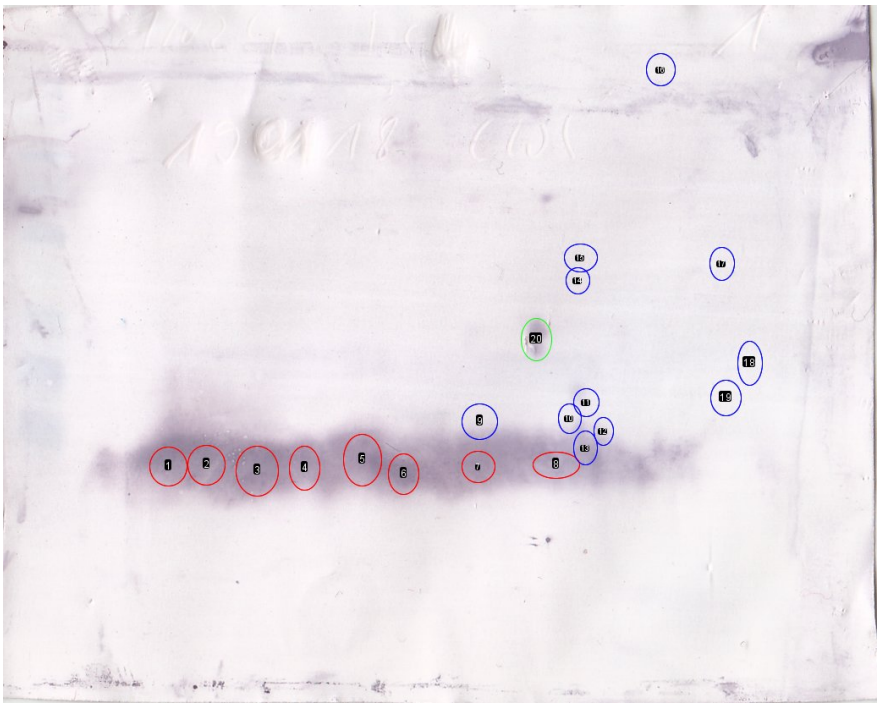

Figure B.19.: AP-Blot ECL-Overlay (Strain AW24, Serum D006), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 1 – 8), IgG4 (Blue, Spots 9 – 19) or neither (Green, Spot 20) was Detected.

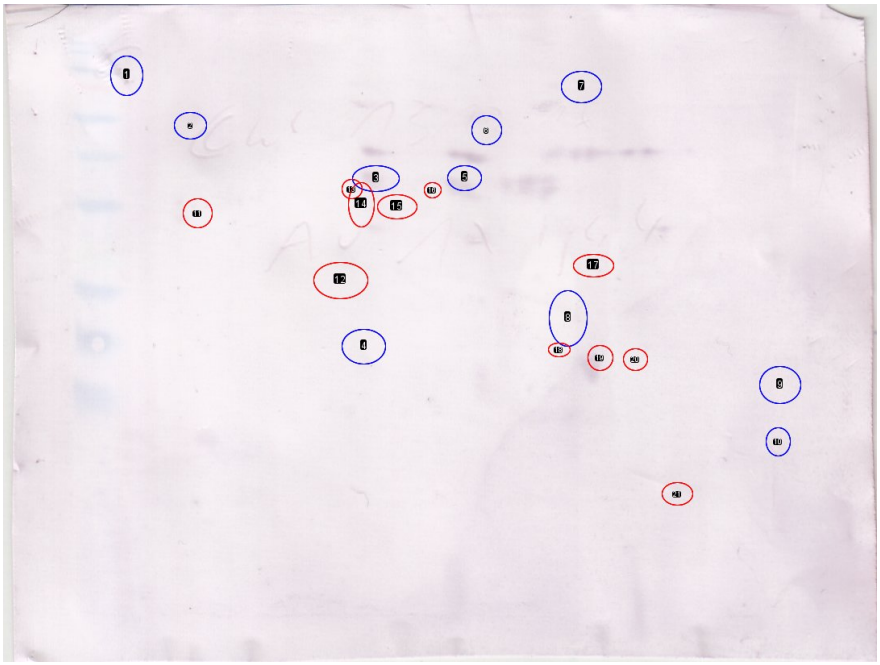

Figure B.20.: AP-Blot ECL-Overlay (Strain AV17, Serum D009), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 1 – 8) or IgG4 (Blue, Spots 9 – 19) was Detected.

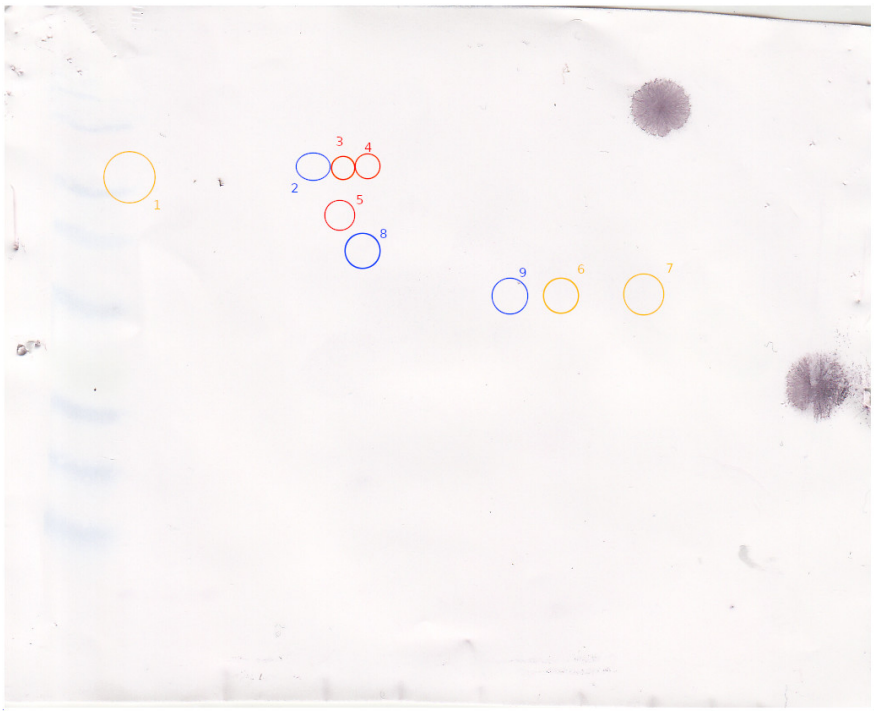

Figure B.21.: AP-Blot ECL-Overlay (Strain AV18, Serum D009), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 3 – 5) or IgG4 (Blue, Spots 2, 8, 9) and both (Orange, Spots 1, 6, 7) was Detected. Because of Poor AP Blot Quality, Overlay was Created Digitally from SDS-PAGE Gel.

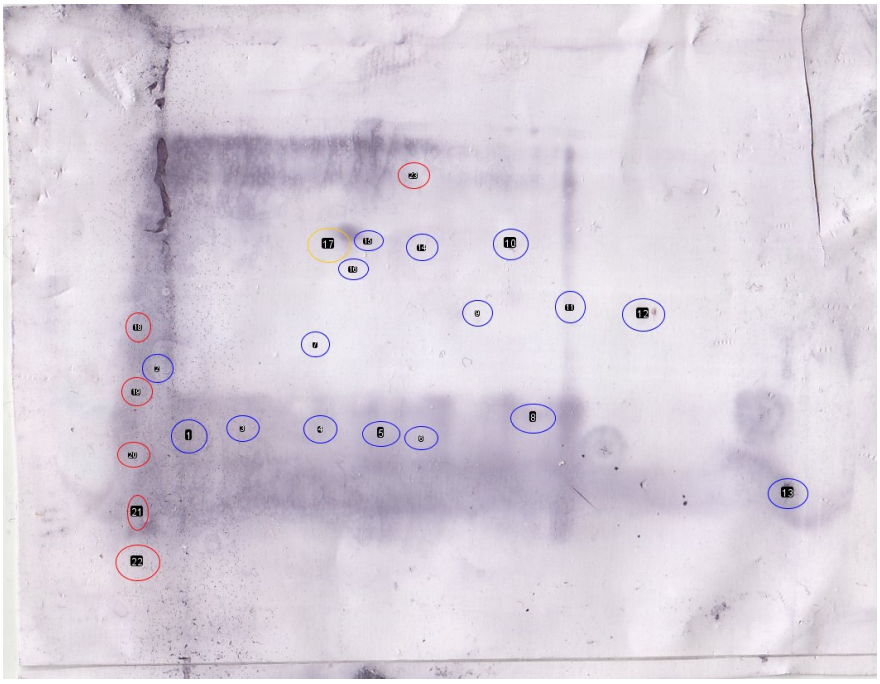

Figure B.22.: AP-Blot ECL-Overlay (Strain AV61, Serum D018), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 18 – 22) or IgG4 (Blue, Spots 1 – 16), or both (Spot 17) was Detected.

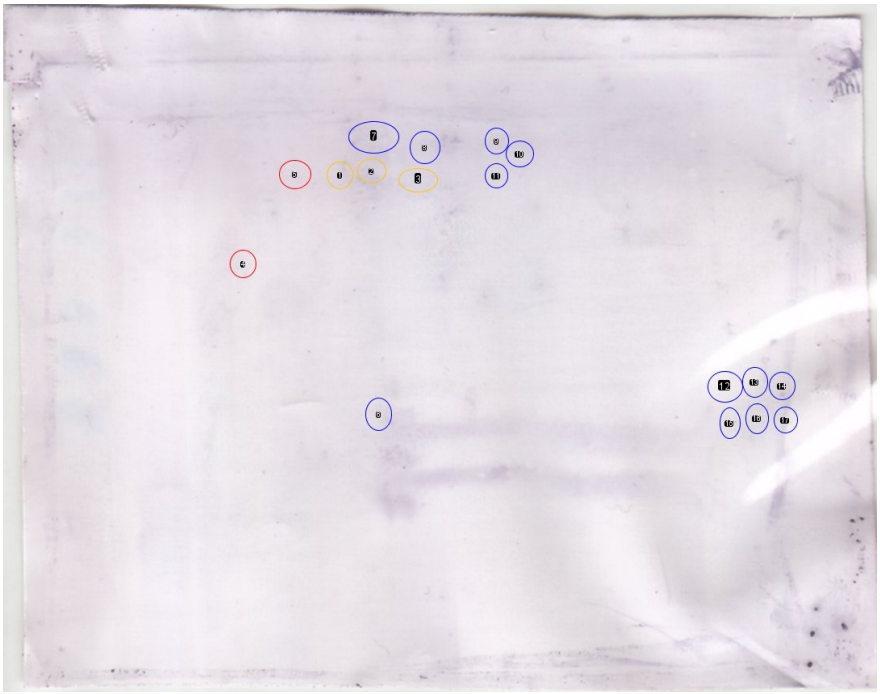

Figure B.23.: AP-Blot ECL-Overlay (Strain AV06, Serum D019), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 4,5) or IgG4 (Blue, Spots 6 – 17) or both (Spots 1 – 3) was Detected.

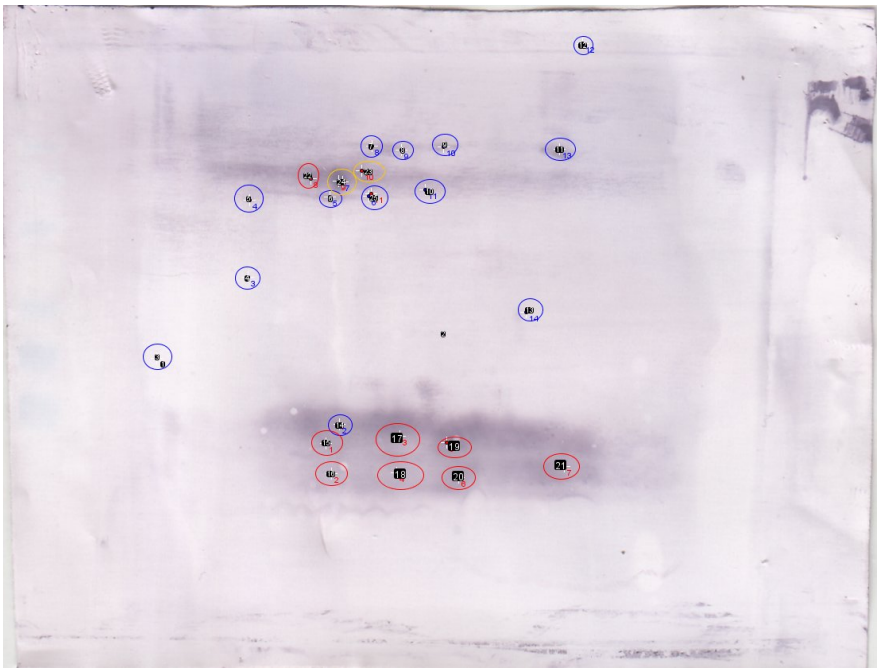

Figure B.24.: AP-Blot ECL-Overlay (Strain AV07, Serum D019), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 15 – 22) or IgG4 (Blue, Spots 1 – 14) or both (Spots 23, 24) was Detected.

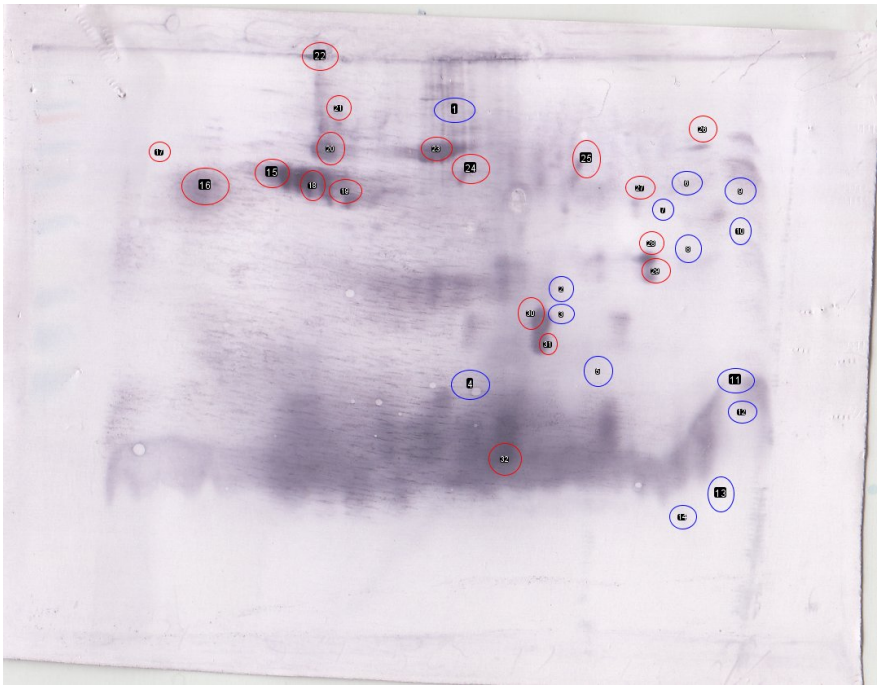

Figure B.25.: AP-Blot ECL-Overlay (Strain AV79, Serum D013), pH 4 – 7.
Overlay Shows Spots in which Signal from IgG1 (Red, Spots 15 – 32) or IgG4 (Blue, Spots 1 – 14) was Detected.

C. Materials and Equipment

C.1. Composition of Non-Standard Chemicals and Buffers

Table C.1.: TSB Medium Composition.

Tryptic soy broth (TSB)	
30 g	TSB dry medium
Ad 1000 ml	Aqua dest.
	Sterilise by autoclaving at 121 °C for 15 min

Table C.2.: RHB Composition.

Rehydration buffer (RHB)	
8M	Urea
2M	Thiourea
Ad	Aqua dest. to desired volume
(opt.) 2% v/v	CHAPS for non-LC-MS/MS samples

Table C.3.: Composition of Utilised TRIS-Buffers.

	TRIS pH 8.8	TRIS pH 6.8
TRIS Base	153.9 g	60.6
TRIS HCl	36.9 g	–
SDS	4 g	4 g
MiliQ Aqua/HPLC-H_2O	1000 ml	1000 ml
Adjust pH	8.8	6.8

Table C.4.: Composition of SDS-PAGE Gels.

Separation Gel		Stacking Gel	
Directions for 1 gel, thickness: 0.75 mm; total volume 6 ml.			
2.7 ml	Aqua dest.	1.6 ml	Aqua dest.
1.5 ml	1.5 M TRIS (pH 8.8) with 0.4% v/v SDS	0.625 ml	0.5 M solution of TRIS (pH 6.8) with 0.4% v/v SDS
1.8 ml	40% Acrylamide solution	1.8 ml	40% Acrylamide solution
5 µl	TEMED	3.5 µl	TEMED
	Mix well		Mix well
50 µl	10% Ammonium persulfate (APS) solution	15 µl	10% APS solution
	Shake quickly and cast immediately		Shake quickly and cast immediately on-top of SDS-gel Insert comb with desired well number

Table C.5.: SDS Loading Buffer/Running Buffer Compostion.

SDS-Loading Buffer (5× Concentrate)		SDS-Running Buffer (10× Concentrate)	
0.6 ml	1 M TRIS HCl (pH 6.8)	144 g	Glycine
1 ml	20% SDS solution	30 g	TRIS base
5 ml	1% w/v Bromophenol blue solution	1000 ml	HPLC-H_2O
0.5 ml	β-Mercaptoethanol		
1.9 ml	HPLC-H_2O		

Table C.6.: Coomassie Staining Solution Composition.

Coomassie Brilliant Blue R250		Colloidal Coomassie G250	
0.3% (w/v)	Coomassie Brilliant Blue R250	5% (w/v)	Aluminium sulfate-(14-18)-hydrate
45% (v/v)	Methanol		HPLC-H_2O until dissolved
10% (v/v)	Acetic acid puriss.	10% (v/v)	Ethanol (96%)
45% (v/v)	HPLC-H_2O	0.02% (w/v)	Coomassie Brilliant Blue G-250
		2% (v/v)	Orthophosphoric acid (85%)
		Ad	HPLC-H_2O to final volume

Table C.7.: Equilibration Buffer/Rehydration Buffer Composition.

DTT Equilibration Buffer		IAA Rehydration Buffer	
50 mM	TRIS HCl	50 mM	TRIS HCl
	Dissolve in HPLC-H_2O, adjust pH to 8.8		Dissolve in HPLC-H_2O, adjust pH to 8.8
6 M	Urea	6 M	Urea
2% v/v	20% SDS solution	2% v/v	20% SDS solution
20% v/v	Glycerol	20% v/v	Glycerol
130 mM	DTT	135 mM	IAA

Table C.8.: Agarose Gel Composition.
For Fixing IEF-Strips on SDS-PAGE-Gels.

Agarose Gel	
25 mM	TRIS HCl
192 mM	Glycine
Ad	Aqua dest. until dissolved
0.1% v/v	SDS
0.5% w/v	Low-melting Agarose
	Bromophenol blue until desired tinge
	Heat until Agarose is melted

Table C.9.: TBS Concentrate/TBS-T Solution/Blocking Buffer Composition.

TBS 10× Concentrate		TBS-T 1× Solution		TBS-T Blocking Buffer	
24.7 g	TRIS HCl	100 ml	TBS 10× concentrate		TBS-T as base
80 g	Sodium chloride	Ad 1000 ml	HPLC-H_2O	5% w/v	Fat-free milk powder
Ad 1000 ml	HPLC-H_2O	0.5 ml	Tween 20		Shake at room temperature
	Adjust pH < 7.6				

Table C.10.: Western Blot Transfer Buffer/Ponceau S Stain Composition.

Transfer Buffer		Ponceau S	
3.025 g	TRIS Base		Aqua dest. as base
15 g	Glycine	5% v/v	Acetic acid puriss.
Ad 1000 ml	HPLC-H_2O	0.1% v/v	Ponceau S stock solution
	Mix until dissolved		
20% v/v	Methanol (> 99.5%) prior to use		

Table C.11.: GnHCl Stripping Buffer/Wash Buffer Composition.

Stripping Buffer		Wash Buffer	
6 M	GnHCl	0.14 M	Sodium chloride
20 mM	TRIS HCl	10 mM	TRIS HCl
0.1 M	DTT		Dissolve in HPLC-H_2O
	Dissolve in HPLC-H_2O	0.05% v/v	Triton X-100
0.2% v/v	Triton X-100		Adjust pH to 7.2
	Adjust pH to 7.25		

Table C.12.: Phosphatase Buffer.

Phosphatase Buffer	
12.1 g	TRIS base
5.8 g	Sodium Chloride
3.3 g	Magnesium chloride hexahydrate ($MgCl \cdot 6H_2O$)
Ad 1000 ml	Aqua dest.
	Adjust pH to 9.5

Table C.13.: LC-MS/MS Buffer Composition.

Buffer A		Buffer B	
	HPLC-H_2O as base		ACN as base
0.5% v/v	Dimethyl sulfoxide	5% v/v	Dimethyl sulfoxide
0.1% v/v	Acetic acid puriss.	0.1% v/v	Acetic acid puriss.

C.2. Antibodies, Chemicals and Equipment

Table C.14.: Antibodies Used for Experiments in this Project.

Host	Species	Target	Conjugate	Catalog-No.	Distributor	Preparation
Goat	Anti-Human	IgG Fab H+L	AP	31310	Invitrogen	Added Glycerol to 50% concentration
Mouse	Anti-Human	IgG1 Fc	HRP	A-10648	Invitrogen	Reconstituted in 200 µL of PBS, pH 7.2
Mouse	Anti-Human	IgG4 Fc	HRP	A-10654	Invitrogen	Reconstituted in 200 µL of PBS, pH 7.2, Thiomersal added to 0.02%

Table C.15.: Chemicals used for Experiments in this Project.

Name	Catalog Name	Catalog Number	Distributor
Acetic acid	Acetic acid puriss.	27225-1L-M	Sigma-Aldrich
Acrylamide solution (40%)	Acrylamide solution (40 %) Mix 37.5 : 1 for molecular biology	A4989,1000	PanReac AppliChem
Acetonitrile (ACN)	N.N.	N.N.	N.N
Agarose	Universal Agarose	BS20.46.500	Bio&Sell
Aluminum sulfate hydrate Al2(SO4)3 · 16H2O	Aluminium sulfate hydrate	A7523	Sigma-Aldrich
APS	Ammonium persulfate (analytical grade)	13375.03	SERVA Electrophoresis
BCIP/NBT substrate	1 Step NBT/BCIP substrate solution	34042	ThermoScientific
Bipyridyl	2,2-Bipyridyl	D216305	Sigma-Aldrich
Bromophenol blue	N.N.	N.N.	N.N.
CHAPS	CHAPS hydrate	C9426	Sigma
Cholanoic acid	Sodium deoxycholate	D6750	Sigma-Aldrich
Coomassie G250	Coomassie G250	42655	Fluka Chemie AG
Coomassie R250	Coomassie R250	12553	Merck
Cover Fluid	Immobiline DryStrip Cover Fluid	17133501	GE Healthcare Life Sciences
Dithiothreitol	Dithiothreitol (electrophoresis grade)	20711.02	SERVA Electrophoresis
ECL substrate	SuperSignal West Femto Maximum Sensitivity Substrate	34096	ThermoFisher Scientific
Ethanol (96%)	Ethanol, extra pure	P075.5	Carl Roth
Fat-free Milk Powder	Blotting-grade Blocker	#1706404	Bio-Rad Laboratories
Glycerol	Glycerol	3783.3	Carl Roth
Glycine	Glycine	G7126	Sigma
Guanidine hydrochloride	Guanidine hydrochloride	G4505	Sigma-Aldrich
Iodoacetamide	Iodoacetamide (NMR-grade)	I6125-10G	Sigma-Aldrich
Magnesium Chloride	Magnesium chloride	M-9272	Sigma

Table C.16.: Chemicals used for Experiments in this Project (Continuation).

Name	Catalog Name	Catalog Number	Distributor
Methanol (>99,5)	Methanol, extra pure	CP43.3	Carl Roth
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine (GC-grade)	35925.01	SERVA Electrophoresis
PBS	DPBS, w/o: Ca and Mg	P04-361000	PAN-Biotech
Phosphoric acid	ortho-Phosphoric acid 85 %	6366.1	Carl Roth
Ponceau S	Ponceau S	P-3504	Sigma-Aldrich
Propanol	2-Propanol (UV/IR-grade)	T910.1	Carl Roth
Protein Marker VI (10-245 kDa)	Protein Marker VI (10 - 245) prestained	A8889,0500	PanReac/AppliChem
Sodium chloride	Sodium chloride	3957.1	Carl Roth
Sodium dodecyl sulfate (20% solution)	Roti-Stock 20 % SDS	1057.1	Carl Roth
Sodium dodecyl sulfate (solid)	Sodium dodecyl sulfate (GC grade)	L4390	Sigma-Aldrich
Thiomersal	Thimerosal (HPLC-grade)	T5125-10G	Sigma-Aldrich
Thiourea	Thiourea	T7875	Sigma-Aldrich
Trichloroacetic acid	Trichloroacetic acid	8789.1	Carl Roth
TRIS Base	TRIS (blotting grade)	0188.4	Carl Roth
TRIS HCl	Trizma hydrochloride	T3253-1KG	Sigma-Aldrich
Triton X-100	Triton X-100	5050200	Ferak Laborat
Trypsine	Trypsine Stock (10 ng/µl)	N.N.	Dept. of Otolaryngology
Tryptic soy broth	Tryptone soy broth	CM0129	Oxoid
Tween 20	Tween 20	9127.2	Carl Roth
Urea	Urea for molecular biology	A1049,5000	PanReac AppliChem
β-Mercaptoethanol	β-Mercaptoethanol	M3148	Sigma-Aldrich

Table C.17.: Equipment Used for Experiments in this Project.

Type	Catalog Name	Catalog Number	Distributor
2-D clean-up kit	2-D Clean-Up Kit	80648451	GE Healthcare Life Sciences
Biopsy punch	Standard Biopsiestanze 2 mm	48201	pfm medical AG
Centrifugal vaccuum concentrator	Eppendorf speed-vac	Discontinued	Eppendorf
Centrifuge	Heraeus™ Multifuge™ X3 Universalzentrifuge	10325804	Thermo Fisher Scientific
ECL imager	NH DyeAGNOSTICS Octoplus QPLEX	PR435	NH DyeAGNOSTICS
Eppendorf tube shaker	Eppendorf ThermoMixer comfort	Discontinued	Eppendorf
Gel electrophoresis chamber	Mini-PROTEAN®Tetra Handcast Systems	165800XFC	Bio-Rad
IEF-cell	PROTEAN IEF Cell	#1654001	Bio-Rad
Immobilized pH gradient strips	Immobiline® Drystrip pH 4-7, 7 cm	GE17-6001-10	GE Healthcare Life Sciences
Linear shaking water bath	OLS Aqua Pro Shaking Water Bath	OLS200	Grant Instruments
Low binding microcentrifuge tube	1.7 ml Low Binding MCT	39640T	Sorenson BioScience
LTQ-Orbitrap Velos	LTQ-Orbitrap Velos	N.N.	Thermo Electron Corparation
Micro centrifuge	Hettich™ MIKRO 220 Mikroliterzentrifuge	10501914	Thermo Fisher Scientific
nanoAcquity UPLC	nanoAcquity UPLC	N.N.	Waters Corporation

Table C.18.: Equipment Used for Experiments in this Project (Continuation).

Type	Catalog Name	Catalog Number	Distributor
PEGGY-SUE	Peggy Sue	N.N.	Protein Simple
PEGGY-SUE detection kit	Biotin Detection Module for Jess, Wes, Peggy Sue or Sally Sue	DM-004	Protein Simple
PEGGY-SUE separation kit	12-230 kDa Peggy Sue or Sally Sue Separation Module	SM-S001	Protein Simple
Picotip emitter	N.N.	N.N.	New Objective Inc.
PVDF-membrane	Immobilon-P PVDF Membrane	IPVH00010	Merck
UPLC pre-column	nanoACQUITY UPLC Symmetry C18 Trap Column, 100Å, 5 µm, 180 µm x 20 mm, 2G, V/M	186006527	MZ-Analysentechnik GmbH, Waters Corporation
UV-Spectrometer	Gene Ray UV Photometer	123483850544	Biometra
Water jet filter	Steritop Threaded Bottle Top Filter	SCGPT02RE	Sigma-Aldritch
Western Blotting Equipment	TE 77XP Semi-Dry Transfereinheit	TE77XP.01	SERVA; Hoefer, Inc.
Whatman paper	N.N.	N.N.	N.N.
ZipTip	ZipTip®Pipette Tips	ZTC04SXXX	Merck

D. Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Greifswald, den 25. Oktober 2022.

Christopher Wirks

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