DEVELOPMENT OF IMMUNOASSAYS FOR SPECIFIC CLASSICAL AND LECTIN PATHWAY ACTIVATION MARKERS

PhD Thesis

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Budapest 2023

1 INTRODUCTION

The complement system is a group of proteins that play a critical role in the innate immune response, and activation thereof is a defining feature of immune-mediated inflammation and infections. The complement system can be activated via three different pathways:
(1) the classical pathway (CP), mainly triggered by circulating antibody-antigen complexes,
(2) the lectin pathway (LP), activated by direct interaction between pattern recognition molecules and foreign carbohydrate structures or acetyl groups on the surface of pathogens, and
(3) the alternative pathway (AP), continuously activated at a low level by the presence of foreign substances and further acting as an amplification loop of the CP and LP.
Regardless of the pathway, once activated, the complement system triggers a cascade of reactions that result in the formation of a C3 convertase and ultimately in generation of the membrane attack complex, which can directly lyse pathogens, and the release of various inflammatory molecules to recruit and activate immune cells.

Assessing complement activation typically involves measurement of split products downstream of C3, indicating activation of all three complement pathways. Although C4d can distinguish CP/LP from AP activation, no validated assays were available commercially to differ between early classical and early lectin pathway activation.

C1 esterase inhibitor (C1-INH) is tightly regulating classical and lectin pathway activation via formation of covalent complexes with the respective activated serine proteases (C1r and C1s for the CP, MASP-1 and MASP-2 for the LP), making C1-INH complexes promising biomarkers to monitor early CP and LP activation.
Regulation of the pathways, resulting in formation of C1-INH complexes, is summarized in Figure 1.

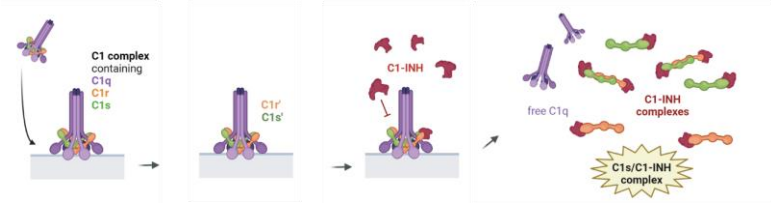

Figure 1: Proposed mechanism of C1-INH complex formation during regulation of complement activation by C1-INH, with CP regulation as an example.

Excessive complement activation via all three pathways was observed in COVID-19 and can lead to lung injury and severe disease outcome. Measurement of C1-INH complexes might therefore indicate how early complement activation occurs during an infection with the SARS-CoV-2 virus, and the extent of complement activation may be related to severity and mortality of COVID-19.

Another condition associated with complement activation is Sepsis, while early activation of the CP and LP were not investigated so far, due to missing validated tools to do so.

2 OBJECTIVES

Based on that, following objectives were determined for the PhD project, as part of the MSCA-ITN "CORVOS" (COmplement Regulation and Variation in Opportunistic infectionS) program:

1) **Development and Characterization of Immunoassays Detecting C1s/C1-INH and MASP-1/C1-INH complex concentrations**

2) **Validation of C1s/C1-INH and MASP-1/C1-INH complexes as markers for early classical and early lectin pathway activation**

3) **Investigation of C1-INH complexes in health and disease, focusing on**
    a. **healthy controls**
    b. **COVID-19 (focus on lectin pathway)**
    c. **Sepsis**

In addition to that, the program also included several trainings/secondments:

- Industrial training (6 months, Hycult Biotech, Uden, The Netherlands)
- Clinical training (1 month, Helsinki University Hospital, Helsinki, Finland)
- Entrepreneurship training (1 month, Hycult Biotech, Uden, The Netherlands)
- Academic secondment (6 months, Medical University, Innsbruck, Austria)

3 RESULTS

3.1 Development and Characterization of Immunoassays Measuring C1s/C1-INH and MASP-1/ C1-INH complexes

Covalent complexes between C1-INH and proteases (C1s and C1r for the CP, MASP-1 and MASP-2 for the LP), formed upon activation of the respective serine proteases, might be suitable markers to distinguish between early CP and early LP activation. Two immunoassays measuring C1s/C1-INH complex as a marker for early classical pathway activation, and MASP-1/C1-INH complex as an indicator for early lectin pathway activation were therefore developed and validated.

3.1.1 Monoclonal antibodies are specific for C1-INH complex components

The antibodies chosen for assay development are specific for either C1s, MASP-1 or C1-INH, and do not give signals for other complement proteins of the respective pathways, as determined in direct ELISAs, as well as Western Blots.

3.1.2 Assay development

Assay development and optimization was performed in cooperation with Hycult Biotech, and resulted in two immunoassays, measuring C1s/C1-INH and MASP-1/C1-INH complex levels in human serum and plasma. Both assays are based on basic sandwich ELISAs and are now commercialized by Hycult Biotech.

3.1.3 Technical Validation of Immunoassays

The newly developed immunoassays were technically validated through performance of several experiments (inter-/intra-assay variation, recovery, cross-reactivity with non-complexed components or animal species, testing of different sample matrices (citrate plasma, heparin plasma, EDTA plasma, serum)).
The experiments showed that C1-INH complex assays work in a reliable way and are very specific for human C1s/C1-INH and MASP-1/C1-INH complex without giving false-positive signals by the non-complexed human proteins. C1-INH complexes can be measured in all sample matrices tested, while dilution needs to be adapted dependend on the sample type used (summarized in Table 1).

Table 1: Summary of newly developed C1-INH complex assays.

	C1s/C1-INH complex assay	**MASP-1/C1-INH complex assay**
Standard range	100-1.6 ng/mL	25-0.4 ng/mL
cross-reactivity (CP/LP proteins)	no	no
cross-reactivity (animals)	orang uta, spider monkey, (mouse)	orang utan, spider monkey
inter-assay variation	< 10%	< 10%
intra-assay variation	< 10%	< 10%
recovery in EDTA plasma	96%	91%
appropriate sample types	EDTA plasma, citrate plasma, Heparin plasma, serum	EDTA plasma, citrate plasma, Heparin plasma, serum

3.1.4 Stability Testing of C1-INH complexes

Stability of C1s/C1-INH and MASP-1/C1-INH complexes in plasma and in purified form was tested during multiple freeze-thaw cycles, as well as during sample handling at room temperature and on ice.
Stability testing revealed that samples should be stored in aliquots, that EDTA plasma is the preferred sample type in order to avoid aritificial complement activation, and that samples should be kept on ice after thawing until the meausrement of C1-INH complexes for most reliable test results.

3.2 C1s/C1-INH and MASP-1/C1-INH complexes are suitable markers for early classical and early lectin pathway activation

In vitro activation experiments in human serum were performed using zymosan (activation of all three complement pathways), IgM- (specific CP activation) or mannan- (specific LP activation) coated wells.
While concentrations of both complexes increased in a time-dependent manner during activation with zymosan (Figure 2A+B), specific activation of either the CP or the LP confirmed that C1s/C1-INH and MASP-1/C1-INH complexes are specific markers for early classical or early lectin pathway activation, respectively (see Figure 2C+D).

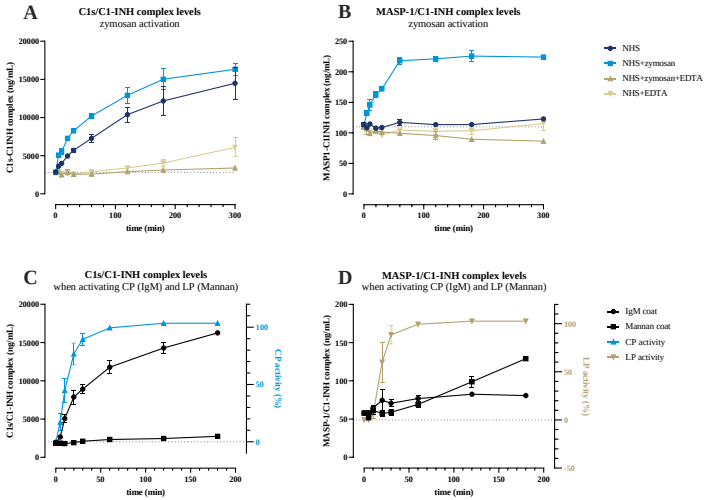

Figure 2: Validation of C1-INH complexes as markers for classical and lectin pathway activation. Complement in NHS was either activated with zymosan, IgM or Mannan and levlels of C1s/C1-INH complex (A: zymosan, C: IgM and Mannan) and MASP-1/C1-INH complex (B: zymosan, D: IgM and Mannan) were determined in the complex assays. Besides that, CP and LP activity, measured by the formation of the Terminal complement complex (TCC/C5b-9) was further determined in the experiments specifically activating the respective pathways (C: CP, D: LP). Auto-activation was determined in NHS without additions, while EDTA served as a negative control (A+B).

3.3 Measurements of C1-INH complexes in healthy individuals

To establish reference values for both C1-INH complexes, C1s/C1-INH and MASP-1/C1-INH complex levels were measured in the newly developed immunoassays in a total of 96 healthy adult individuals. Measurements revealed first reference ranges of 1846 ± 1060 ng/mL C1s/C1-INH complex (mean ± 2SD) and median levels of 36.9 (13.18 - 87.89) ng/mL MASP-1/C1-INH complex [median (2.5 percentile range – 97.5 percentile range)] in human EDTA plasma (Figure 3), while correction for gender or age does not seem to be necessary when investigating the two complexes.

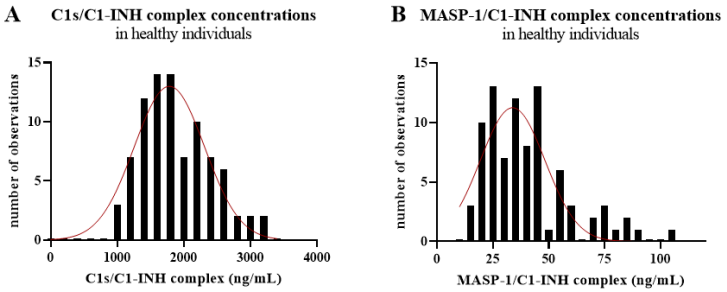

Figure 3: Levels of C1-INH complexes in healthy adults. C1-INH complex levels were measured in 96 healthy adult individuals, while distribution of C1s/C1-INH complex (A) and MASP-1/C1-INH complex levels (B) are illustrated in histograms.

3.4 Investigation of complement activation and C1-INH complexes in COVID-19

3.4.1 C1-INH complex levels are increased in COVID-19 and are associated with disease severity

Measurement of C1-INH complexes, as well as several other complement and laboratory parameters, in two cohorts with SARS-CoV-2 infected patients revealed increased C1s/C1-INH and MASP-1/C1-INH complex levels in COVID-19 ($p<0.0001$), indicating activation of the classical and the lectin pathway. Besides that, both complex levels were associated with disease severity, with higher C1-INH complex levels in more severe cases.

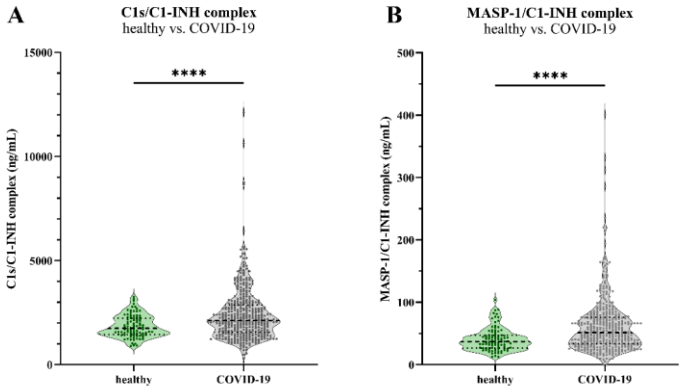

Figure 4: C1-INH complex levels in healthy individuals and COVID-19 patients. C1s/C1-INH complex (A) and MASP-1/C1-INH complex levels (B) were measured in COVID-19 patients (n = 414) and healthy controls (n = 96), utilizing the newly developed C1-INH complex assays in a first proof-of-concept study.

3.4.2 Lectin pathway activation is independent of *MBL2* genotype groups

Lectin pathway activation does highly rely on its key pattern recognition molecule MBL. Binding of MBL to the SARS-CoV-2 spike protein was shown in a concentration-dependent manner *in vitro*, using serum samples of healthy individuals with different *MBL2* genotypes.
However, genetic variations in the six common polymorphisms of the *MBL2* gene were not found to be associated with either susceptibility to SARS-CoV-2, disease severity, or outcomes like COVID-19 related mortality and the development of Long COVID.

3.4.3 Classical pathway activation is associated with COVID-19 disease severity, and C1s/C1-INH complex levels are associated with CP activators

Measurement of C1s/C1-INH complex concentrations in COVID-19 showed associations with disease severity, while levels also correlated with the presence of anti SARS-CoV-2 antibodies and acute phase proteins C-reactive protein (CRP) and Long Pentraxin 3 (PTX3), both known to trigger activation of the classical pathway.

3.4.4 C1-INH complex levels of hospitalized COVID-19 patients change in the presence of co-infections

C1-INH complex concentrations were measured in follow-up samples of 12 hospitalized COVID-19 patients, while 7 patients were additionally suffering from fungal or bacterial co-infections (*Candida spp.*: n=4, *Escherichia coli*: n=2, *Aspergillus spp.*: n=2). Experiments showed that co-infections can further influence C1-INH complex levels that are already altered due to the SARS-CoV-2 infection, while changes are dependent on the underlying co-infection.

3.5 C1-INH complexes in Sepsis patients

C1-INH complex levels were further determined in a cohort of 70 sepsis patients.
Levels of C1s/C1-INH as well as MASP-1/C1-INH complexes were significantly increased in the patients when compared to healthy controls ($p<0.0001$), indicating strong activation of both, the classical and the lectin pathway in sepsis. However, levels did not differ between survivors and non-survivors, but can be influenced by the underlying infection (e.g. bacterial infection or SARS-CoV-2).

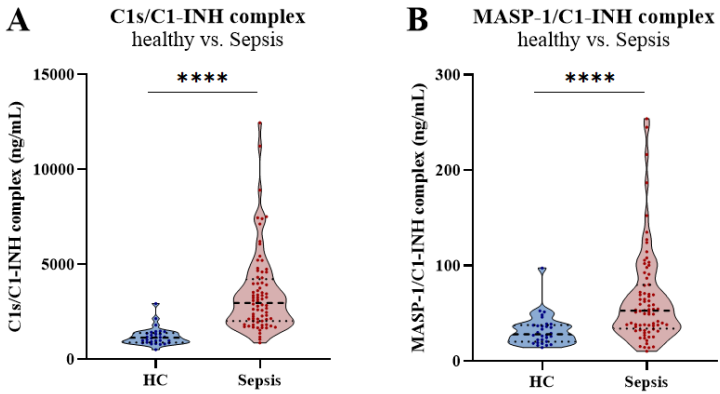

Figure 5: C1-INH complex levels in healthy controls (HC) and Sepsis patients. C1s/C1-INH complex (A) and MASP-1/C1-INH complex levels (B) were measured in healthy controls and Sepsis patients, using the newly developed immunoassays.

4 CONCLUSION

During the doctoral project, two immunoassays, allowing measurement of C1s/C1-INH complex and MASP-1/C1-INH complex levels in human plasma and serum samples, were developed. The assays were thoroughly optimized and validated, and are now commercialized by Hycult Biotech.

Besides that, it was shown that C1-INH complexes are appropriate markers to monitor early activation of the classical (C1s/C1-INH complex) and the lectin pathway (MASP-1/C1-INH complex) *in vitro*.

A first reference range in healthy humans was set, and measurements in patients suffering from infectious diseases (COVID-19, Sepsis) showed increased C1-INH complex concentrations in line with other complement activation products and inflammatory parameters, supporting the application of C1-INH complex measurements as early complement activation markers also *in vivo*.

In conclusion, measurement of C1s/C1-INH and MASP-1/C1-INH complex levels can thereby help to unravel early complement activation in human diseases and allows to distinguish between CP and LP activation, which was not possible with commercially available assays in the past.

In addition, the C1-INH complex assays might be helpful tools to investigate CP/LP activation of new therapeutics in clinical studies, and to monitor treatment with complement-targeting drugs, such as C1-INH or the recently approved classical pathway inhibitor anti C1s (Sutimlimab).

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