

Inflammation and Hypercoagulability in Antineutrophil Cytoplasmic Antibody associated Vasculitis

Citation for published version (APA):

Busch, M. (2024). Inflammation and Hypercoagulability in Anti-neutrophil Cytoplasmic Antibody associated Vasculitis: Lessons learned from COVID-19. [Doctoral Thesis, Maastricht University]. Maastricht University. https://doi.org/10.26481/dis.20240508mb

Document status and date: Published: 01/01/2024

DOI: 10.26481/dis.20240508mb

Document Version: Publisher's PDF, also known as Version of record

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.

• The final author version and the galley proof are versions of the publication after peer review.

• The final published version features the final layout of the paper including the volume, issue and page numbers.

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Inflammation and Hypercoagulability in Anti-neutrophil Cytoplasmic Antibody associated Vasculitis

Lessons learned from COVID-19

AnxA

Matthias H. Busch

Inflammation and Hypercoagulability in Antineutrophil Cytoplasmic Antibody associated Vasculitis - Lessons learned from COVID-19

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Cover design: Wendy Schoneveld; wenz ID Layout: Tiny Wouters - Lenssen Printing: proefschriftmaken.nl

ISBN: 978-94-6469-820-6

Inflammation and Hypercoagulability in Antineutrophil Cytoplasmic Antibody associated Vasculitis - Lessons learned from COVID-19

DISSERTATION

To obtain the degree of Doctor at Maastricht University, on the authority of the Rector Magnificus, Prof. dr. P. Habibović, in accordance with the decision of the Board of Deans, to be defended in public on Wednesday May 8 2024, at 13:00 hours

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Table of contents

Chapter 1	General introduction	7
Chapter 2	Neutrophils and contact activation of coagulation as potential drivers of COVID-19	29
Chapter 3	Thrombin generation via the intrinsic coagulation pathway and von Willebrand factor reflect disease severity in COVID-19	45
Chapter 4	Vilobelimab treatment versus best supportive care for patients with severe COVID-19: an exploratory, open-label, phase 2 randomised controlled trial	65
Chapter 5	Annexin A1 is associated with adverse clinical outcomes in patients with COVID-19	83
Chapter 6	The intrinsic coagulation pathway plays a dominant role in driving hypercoagulability in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis	101
Chapter 7	General Discussion, Summary and Future Perspectives	119
Addendum	Samenvatting Impact Paragraph Dankwoord Curriculum Vitae	139 145 149 153
	List of publications	155



Chapter I

General introduction

GENERAL INTRODUCTION

The title of this thesis is "Inflammation and Hypercoagulability in Anti-neutrophil Cytoplasmic Antibody associated Vasculitis - Lessons learned from COVID-19" because, initially, the objective was to better understand hypercoagulability and its potential link to inflammation in antineutrophil cytoplasmic antibody-associated vasculitis (AAV). However, coronavirus 2019 (COVID-19) emerged and impacted all of society, including public health and research. Early findings pointed towards excessive activation of the immune system and an increased risk of thrombosis in patients with COVID-19. We hypothesized that the underlying mechanisms driving hypercoagulability and inflammation in COVID-19 might be similar to those in AAV. The rapid emergence of COVID-19 and the need for a better pathophysiological understanding offered the possibility to investigate the underlying mechanisms first in COVID-19 and to apply the lessons learned later in AAV. This chapter contains a brief overview of the complement system as an essential part of the innate immune system and the coagulation system. It then elaborates on the link between the immune and coagulation systems and introduces COVID-19 as well as AAV. The chapter ends with the aims and outline of the thesis.

Complement

The complement system is a critical component of the innate immune system, serving as a surveillance mechanism in the body's defense against pathogens. It consists of three pathways; the classical, alternative, and lectin pathways, that converge into the final pathway leading to the formation of the membrane-attack-complex¹.

In short, the alternative pathway is continuously active by cleavage of the plasma protein complement 3 (C3) into C3a and C3b by the serine esterase Bb. By doing so, C3b can form C3 convertase (C3bBb) of the alternative pathway. Under physiological conditions, C3b levels are tightly controlled by regulatory proteins leading to a rapid degradation of C3b in the plasma. However, danger signals can trigger monocytes, macrophages and neutrophils to secrete properdin. Properdin recognizes and binds to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs)². The binding leads to the attraction of more C3b and stabilizes C3bBb, thereby amplifying the generation of C3b in the fluid phase.

The classical pathway is initiated by C1q mediated recognition and binding of PAMPs and DAMPs on pathogens or apoptotic cells and by immunoglobulins. C1q binding activates C1r and C1s, C1s in turn cleaves C2 and C4, thereby generating C3 convertase (C4bC2a) in the classical pathway. C3 convertase is also formed in the lectin pathway upon recognition of carbohydrate patterns by mannose-binding lectin (MBL) and ficolins. They form complexes and activate MBL-associated serine proteases (MASPs) leading to

the cleavage of C2 and C4 and the formation of C4bC2a. In both pathways, C4bC2a cleaves C3 into C3a and C3b leading to amplification of C3b generation.

The gradual increase of C3b fragments in the alternative, classical, and lectin pathways leads to the binding of C3b to C3 convertases. As a result, C5 convertase is generated because the C3 convertase loses its affinity to cleave C3, cleaving C5 instead. Cleavage of C5 generates C5b and the potent anaphylatoxin C5a. C5b associates with C6, C7, C8, and C9 to ultimately form C5b-9, the membrane-attack-complex³.

Activation of the complement system enables the recognition and elimination of pathogens, the release of proinflammatory stimuli and the cross-talk with the coagulation system. For instance, opsonization by C1q and C3b induces phagocytosis of targets and membrane attack complex mediated lyses of susceptible microorganisms. C3a and C5a levels steadily increase during complement activation and are potent proinflammatory signals. C3a and C5a attract neutrophils, monocytes and macrophages via C3a and C5a receptors to the site of inflammation. Moreover, C5a primes and activates neutrophils, causing the generation of more C5a via the alternative pathway of complement and thus a feedback amplification of neutrophil activation⁴.

Coagulation

Blood coagulation, also known as hemostasis, is maintained by a delicate balance between platelets, the coagulation pathway and fibrinolysis. Thrombus formation can be triggered by the extrinsic and intrinsic (or contact) pathways of coagulation that unite into the common pathway^{5,6}.

The extrinsic pathway is initiated upon tissue factor (TF) exposure to blood after vessel trauma occurred. TF binds circulating factor VII (FVII) to form the TF-FVIIa complex that catalyzes and activates FIX and FX. The intrinsic pathway is initiated when anionic compounds (i.e., collagens, DNA or histones) come in contact with circulating prekallikrein leading to its conversion into kallikrein⁷⁻⁹. Kallikrein activates FXII into activated FXII(a) and cleaves high-molecular-weight kininogen into bradykinin. FXII is also directly activated by collagens⁸. FXIIa activates FXI into FXIa, subsequently activating FIX to cleave FX into FXa. FXIIa positively amplifies this cascade by converting prekallikrein into kallikrein. In the common pathway, FXa forms with the cofactor FVa the prothrombinase complex that cleaves prothrombin into thrombin¹⁰. Thrombus formation is achieved by thrombin, which converts fibrinogen into fibrin and activates platelets and FXIII, initiating fibrin polymerization. Thrombin also amplifies the coagulation pathway by directly activating FV, FVIII and FXI. Under normal conditions, FVIII remains stable when it forms a complex with von Willebrand factor (vWF). However, thrombin cleaves this complex, resulting in the dissociation of FVIII from vWF. FVIII then interacts with FIXa to increase the generation of FXa. Activated platelets contribute to this activation loop by releasing TF, FV and FXIII¹¹ and by enhancing FXI

Τ

and FIX activation. Globally, inactivation of the coagulation cascade is achieved by antithrombin and C1 inhibitor which bind to coagulation factors and thrombin, by tissue factor pathway inhibitor (TFPI) that forms inactivating complexes with TF-FVIIa complex and FXa, and by a protein C dependent proteolysis of FVa and FVIIIa⁵.

Crosstalk between Complement and Coagulation

Due to their significant evolutionary homology, the complement and coagulation systems exhibit extensive interactions at various levels.

For instance, MASP-1, C5a and the membrane attack complex can directly activate proteins from the coagulation system. MASP-1 shares homology with thrombin¹² and it can cleave fibrinogen and activate thrombin by cleavage of prothrombin in vitro^{13,14}. However, its clinical relevance in diseases is currently limited. C5a stimulates TF expression on endothelial cells and monocytes^{15,16}. Mast cells and basophils upregulate plasminogen-activator inhibitor-1 upon stimulation with C5a¹⁷. Furthermore, the observation that C5 and C3 double knockout mice had longer bleeding times and fewer thrombotic events suggests that the complement system plays a role in hemostasis¹⁸. The membrane attack complex can disrupt the integrity of cell membranes from pathogens, endothelial cells and platelets. The subsequent exposure of phosphatidylserines provides a scaffold for the prothrombinase complex to bind¹⁹.

Coagulation factors and thrombin can also directly interact with compounds of the complement system. In vitro, FIXa, FXIa, FXa, and thrombin act as C3 convertase to generate C3a and C5a and this effect is reduced by low molecular weight heparin (LMWH)^{20,21}. FXIIa and kallikrein from the intrinsic pathway of coagulation are both linked to C3 and C5 cleavage²². It is also postulated that activity in the alternative complement pathway is associated with exposure of vWF²³. Finally, TFPI not only inactivates the extrinsic coagulation pathway but it also reduces MBL activity by interacting with MASP-2²⁴.

Immunothrombosis

The complex interplay between the immune and coagulation systems plays a crucial role in defending against pathogens. Immunothrombosis refers to this intricate process, wherein the innate immune system cross-talks with the coagulation system to protect against the dissemination of pathogens²⁵.

The local exposure of pathogens to immune cells triggers adjacent endothelial cells to express selectins, integrins and intracellular adhesion molecule 1. In conjunction with chemokine and C5a signaling, these proteins facilitate leukocyte trafficking, adhesion, and migration toward the site of infection^{26,27}. Attracted leucocytes, in turn, interact with both the intrinsic and extrinsic coagulation pathways. For example, activated neutrophils and monocytes express TF and release TF loaded microvesicles that contribute to the

activation of the extrinsic coagulation pathway²⁸⁻³⁰. TF expression is enhanced further by platelet-monocyte interactions and activated platelets. Neutrophils release serine proteases, such as cathepsin G and neutrophil elastase, that can degrade TFPI thus preventing the inactivation of TF-VIIa complex driven thrombin formation³¹. Proresolving coagulation factors like thrombomodulin can also be rendered inactive by other compounds released from neutrophils³². In this line, neutrophil extracellular traps (NETs) formation is an essential process in which neutrophils prevent pathogen dissemination and activate the coagulation system. The exact mechanisms that initiate NETs formation remain to be elucidated, but it has been shown that interferon alpha can prime neutrophils to induce NETs formation upon stimulation with C5a in vitro³³. These findings indicate an important link between neutrophils and complement activation in the formation of NETs. Interleukin-8, lipopolysaccharide, phorbol myristate acetate (PMA), and Toll-like receptor 4 (TLR-4) depended platelet-neutrophil interactions also induce NETs formation^{34,35}. During NETs formation, intracellular products from neutrophils protrude in a spider-webbed pattern into the extracellular space³⁴. Structurally these webs consist of DNA and chromatin fragments that trap pathogens. Proteolytic enzymes like neutrophil elastase, myeloperoxidase and histones are released, generating a highly cytotoxic environment for pathogens and the surrounding structures (i.e., blood vessels or tissues). Indeed, NETs-derived extracellular histones exhibit adverse effects on the endothelium and are linked to endothelial damage and the release of vWF³⁶. Extracellular histones exposed to blood are highly thrombogenic; intravenous injection of histones into mice caused death, with neutrophil migration to the lungs, alveolar fibrin deposition, and microvascular thrombosis^{37,38}. In this context, histones and DNA fragments from NETs can result in thrombin generation through the activation of the intrinsic coagulation pathway^{7,39}. NETs decorated with vWF also promote trapping and activation of platelets and microvesicles, leading to TF dependent activity of the extrinsic coagulation pathway⁴⁰.

Taken together, immunothrombosis helps to contain and antagonize the dissemination and the adverse effects of pathogens. However, dysregulation of these intricate interactions can disrupt homeostasis, leading to disease under certain conditions. In the following sections, we explore how excessive inflammation driven by complement activation, neutrophils and NETs formation is linked to hypercoagulability and unfavorable outcomes in COVD-19 and AAV.

COVID-19

The COVID-19 pandemic that resulted from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection led to mortality rates of >2% in adult patients. Mortality rates were highest in patients admitted to the intensive care unit (ICU) due to acute respiratory distress syndrome (ARDS)⁴¹⁻⁴⁴.

Т

In the pandemic's first months, little was known about the pathophysiological mechanisms behind COVID-19. Emerging evidence suggested that patients with COVID-19 and ARDS presented with hyperinflammation as demonstrated by high levels of proinflammatory cytokines^{42,45}. ARDS is characterized by neutrophil and monocyte recruitment and their infiltration into the extravascular compartments of the lungs⁴⁶. Primed neutrophils release oxidants, proteases and form NETs, exposing the environment to toxic substances such as histones. Subsequent microvascular and endothelial cell damage imposes alveolar endothelial and epithelial barrier disruption as well as alveolar edema⁴⁷. These adverse changes can ultimately lead to progressive respiratory insufficiency, the need for ICU admission, and high mortality rates.

Besides systemic hyperinflammation, a high incidence of thrombotic events was observed in COVID-19, particularly in those patients admitted to the ICU⁴⁸. Coagulation abnormalities were characterized by high D-dimer levels, mild thrombocytopenia and prolongation of the prothrombin time, without evidence for disseminated intravascular coagulation^{42,49-51}. This COVID-19 induced hypercoagulability may be the consequence of hyperinflammation and pulmonary vasculature damage. Indeed, early in the pandemic a study demonstrated increased levels of surrogate markers for NETs in patients with COVID-19^{52,53}. A small study showed that NETs and neutrophil-platelet aggregates colocalized with microthrombi in lung biopsies of patients with COVID-19⁵², pointing towards the activation of the intrinsic and extrinsic pathways of coagulation as a result of immunothrombosis.

Targeting hyperinflammation, and thus hypercoagulability, by inhibiting IL-1 or IL-6 was therefore suggested as a potential treatment early on⁴⁵. Before dexamethasone had been proven effective in COVID-19⁵⁴, glucocorticosteroids, although effective in dampening hyperinflammation, were not routinely given due to safety concerns. Neutrophil recruitment is guided, in part, via the complement protein C5a-C5a receptor axis, pointing to a potential therapeutical target given the low burden of viral and bacterial infections in patients with a complement protein C5 deficiency⁵⁵. Importantly, SARS-CoV-2 shares significant genetic homology with SARS-CoV-1 and Middle East respiratory syndrome (MERS)-CoV, respectively⁵⁶. *In vivo* studies demonstrated the efficacy of complement inhibition to attenuate lung damage in SARS-CoV-1 and MERS-CoV infected mice^{57,58}. C5 inhibition attenuated inflammation and provided organ protection, including for the lungs, in sepsis⁵⁹. Therefore, inhibition of complement at the level of C5 could potentially prevent disease progression into severe COVID-19.

Taken together, hyperinflammation and hypercoagulability were rapidly identified as hallmarks of a severe course of COVID-19. These features share striking homology with those of AAV. A dysregulated neutrophilic driven immune response triggered by either SARS-CoV-2 in COVID-19 or ANCA positivity in AAV may lead in both diseases to hyperinflammation, endothelial cell disruption, hypercoagulability and ultimately to organ damage (**Figure 1.1**).



Figure 1.1 Linking hyperinflammation to hypercoagulability in COVID-19 and AAV. The hypothesis is that COVID-19 and AAV are associated with adverse inflammatory responses characterized by complement activation, neutrophil activation, NETs formation, endothelial damage, and tissue damage. The excessive inflammation results in the activation of the coagulation system, leading to hypercoagulability and adverse clinical outcomes. The underlying mechanisms and potential targets for intervention have yet to be discovered (*question marks*) and are therefore studied in this thesis. *Abbreviations*: COVID-19, coronavirus 2019. AAV, antineutrophil cytoplasmic antibody-associated vasculitis. NETs, neutrophil extracellular traps (Figure created with BioRender.com).

COVID-19 and Annexin A1

Little is known about the actual role of AnxA1 in COVID-19. It belongs to the annexin protein superfamily and is a pro-resolving mediator of inflammation in humans. AnxA1 is endogenously stored in immune cells like macrophages, neutrophils and monocytes^{60,61}. Cell activation and leukocyte adhesion result in the translocation and secretion of AnxA160,61. Extracellular AnxA1 has effects on immune cells to resolve proinflammatory states. In particular, adhesion and recruitment of circulating immune cells to endothelial cells is reduced by AnxA162-64. AnxA1 signaling is also associated with a reduced expression of pro-inflammatory cytokines like interleukin-6 (IL-6)⁴⁴. Contrarily, AnxA1 promotes T-cell activation but the consequences of this action are insufficiently understood⁶⁵. AnxA1 has also a role in the regulation of pathogen clearance⁶⁶, apoptosis of innate immune cells⁶⁷, and polarization towards M2 phenotype macrophages⁶⁸. AnxA1 mediates its effects via the formyl peptide receptor 2 (FPR2), a seven-membranespanning G-protein-coupled receptor that is expressed by a variety of cells. The Nterminal region is the bioactive part of AnxA1 that binds to and activates FPR2. Interestingly, AnxA1 dependent FPR2 signaling is induced by glucocorticosteroids, which augment AnxA1 and FPR2 gene expression in innate immune cells⁶⁹⁻⁷¹. The exact underlying mechanisms of the glucocorticosteroids mediated AnxA1 increase are not yet understood. Interestingly, proteomics of neutrophils from patients with AAV revealed an altered regulation of apoptotic proteins like AnxA172. Plasma AnxA1 levels were increased and correlated with glomerular crescents in kidney biopsies from patients with

active AAV⁷³. Since AnxA1 actions are mediated downstream of glucocorticosteroids, AnxA1 and its mimetic peptide Ac2-26 may therefore be useful as biomarkers and therapeutic targets for inflammatory conditions. In this line, excessive activation of the innate immunity, particularly neutrophils, is important in the progression to a severe course of COVID-19. A deregulated homeostasis of AnxA1 may play a role in the pathogenesis considering its widespread effects on macrophages, neutrophils, and monocytes in humans and is in fact targetable.

AAV

AAV is according to the 2012 revised international Chapel Hill consensus conference nomenclature of vasculitides defined as "a form of auto-immune mediated vasculitis predominantly affecting the small sized vessels on the background of myeloperoxidase (MPO)-ANCA or proteinase-3 (PR3)-ANCA positivity in most patients74,75." Based on the clinical presentation, AAV is subdivided into granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA). The course of disease varies from mild symptoms to life-threatening disease in organs like the lungs or kidneys as a result of a fulminant inflammatory response in the affected vessels. Massive hemoptysis due to pulmonary capillaritis or rapidly progressive glomerulonephritis are clinical features requiring immediate medical attention and initiation of immunosuppressants to prevent further tissue damage⁷⁶. Remissioninduction strategies consist of either cyclophosphamide (CYC) and/or rituximab (RTX) based schemes combined with glucocorticosteroids (GCS)77-80. Recently, the C5a receptor 1 inhibitor avacopan has also been approved as a glucocorticosteroids sparing agent during remission-induction for selected patients with AAV⁸¹. After remission is achieved, it is maintained for at least two years with either azathioprine (AZA) or RTX^{82,83}.

Ever since the first discovery of autoantibodies directed against neutrophils and monocytes in patients with AAV by *Fokko van der Woude* in 1985⁸⁴, the understanding of the pathogenesis of AAV has rapidly improved in the past decades. Neutrophils are primed by tumor necrosis factor α (TNF α), interleukin (IL)-1 β or C5a during proinflammatory conditions like infections^{85,86}. Upon priming, the proteases MPO and PR3 are expressed on the membrane surface of neutrophils. In the presence of MPO- or PR3-ANCA, these autoantibodies bind to the exposed MPO or PR3 resulting in a strong activation of the neutrophil⁸⁷. Cytotoxic compounds, like neutrophil elastase, matrix metalloproteinase (MMP) and reactive oxygen species, are released by neutrophils, thereby damaging the endothelial layer of the vasculature⁸⁸. The inflammatory response is further driven by neutrophils forming NETs to increase further the concentration of lytic compounds like citrullinated histones and proteases⁸⁹. Indeed, NETs complexes have been shown to circulate during active AAV^{90,91} and deposit in kidney tissue⁹², leading to necrotizing vascular and extravascular inflammation. The inflammatory reaction is maintained and augmented by subsequent interactions of the immune system. For instance, NETs fixate C5a and activate the alternative complement pathway⁹³. MPO decorated NETs enhance antigen presentation of dendritic cells to activate CD4⁺ T cells⁹⁴. In a vicious cycle, B-cells and plasma cells are also continuously stimulated to produce more ANCAs by released B cell-activating factor and attracted CD4⁺ T cells⁹⁵.

The exact mechanisms of ANCA induction in patients is not fully understood but a variety of factors seem to be related. Genetically, the major-histocompatibility complexes *HLA-DP* and *HLA-DQ* are associated with PR3- and MPO-ANCA, respectively[%]. The microbiome also appears to influence disease activity in AAV, particularly nasal colonization with Staphylococcus aureus and Corynebacterium are associated with relapses in GPA⁹⁷. Cross-reactivity and molecular mimicry of host and pathogen peptides may hereby lead to the induction of ANCAs^{98,99}. Furthermore, colonization may locally result in low-grade inflammation, attracting macrophages and priming neutrophils¹⁰⁰. Environmental factors like silica dust exposure and drugs also contribute to ANCA induction^{101,102}.

AAV and thrombotic events

The rate of thrombotic events in patients with AAV is high, ranging from 6.3% to 13.7%¹⁰³⁻¹⁰⁵. Clinical features associated with an increased risk for venous thrombotic events are a high Birmingham Vasculitis Activity Score, MPO-ANCA positivity, skin or pulmonary involvement, and impaired kidney function^{105,106}. These risk factors, however, could be more helpful in identifying those patients that might benefit from prophylactic anticoagulation considering that pulmonary and renal involvement are frequent symptoms in AAV. Particularly when severe hemoptysis or end-stage renal disease is present, anticoagulation should be used with caution. Therefore, recommendations for the prevention and management of thrombotic events in AAV are currently limited^{80,107}. Most thrombotic events occur during active disease^{103,108}, pointing towards an important link between inflammation and hypercoagulability. Excessive immune activation, NETs

link between inflammation and hypercoagulability. Excessive immune activation, NETs formation and endothelial cell damage may result in hypercoagulability that is reflected by higher prothrombin fragments and D-dimers in patients with active AAV¹⁰⁹. Damaged endothelial cells express vWF that, in turn, binds and activates platelets. Furthermore, a postmortem biopsy study identified that NETs co-localized with thrombi and glomerular crescents in a patient with MPA¹¹⁰. Neutrophils derived from patients with active AAV express higher levels of TF as well as TF containing NETs and microvesicles, whereas remission-induction therapy diminished these findings¹¹¹. Interestingly, neutrophil dependent TF expression is directly induced after priming neutrophils with C5a and ANCA stimulation¹¹². Subsequently, circulating TF combined with FVIIa activates thrombin generation via the extrinsic pathway of coagulation.

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Patients in remission were also found to have increased endogenous thrombin potential, FVIII and tissue factor pathway inhibitor (TFPI) levels compared to healthy controls¹¹³. This points towards a persistent hypercoagulable state, eventually due to ongoing low-grade inflammation and endothelial cell activation, even in patients that were assumed to have achieved, at least clinically, stable remission.

The presence of autoantibodies that interfere with coagulation may also contribute to an increased incidence of thrombotic events in AAV. Anti-plasminogen antibodies that bind to fibrinogen, thereby reducing the fibrinolysis capacity to dissolve blood clots, were reported in several PR3-ANCA positive patients with thrombotic events¹¹⁴. Furthermore, antiphospholipid antibodies (APL) were detectable in 13% of patients with AAV¹¹⁵. Another study documented in 3.8% of the patients anticardiolipin antibodies¹¹⁶. Therefore, defective fibrinolysis and persistent APL positivity may be independent risk factors of thrombotic events in patients with AAV not only during active disease.

Taken together, the incidence of thrombotic events is high in patients with AAV. An understanding of the driving mechanisms is currently limited but it appears to be related to inflammation, endothelial damage, platelet activation and in some patients to APL and anti-plasminogen antibody positivity. As pointed out in the section *"Immunothrombosis"*, there is a strong link between inflammation, complement activation, NETs formation and coagulation activation that might result in thrombotic events in patients with AAV. Nevertheless, little is known of the delicate balance and activity of the intrinsic and extrinsic coagulation pathways in AAV. It is essential to improve the understanding of thrombotic events in AAV since guided treatment strategies to identify and treat those patients at risk are scarce.

AIMS AND OUTLINE OF THIS THESIS

The thesis aimed to better understand the link between hyperinflammation and hypercoagulability in COVID-19 and to apply the knowledge gained to AAV. Therefore, several aspects of the immune and coagulation system were investigated in two large and well-defined prospective cohorts of patients with COVID-19 and AAV. In this thesis, markers of inflammation, endothelial activation and coagulation were assessed in patients with COVID-19, the effects of C5a inhibition with vilobelimab on clinical outcomes were studied, and the role of AnxA1 in patients with COVID-19 was investigated. Based on the lessons learned from the observations in COVID-19, the underlying mechanisms behind hypercoagulability in AAV were studied.

Since knowledge about the interplay of hyperinflammation and hypercoagulability was scarce at the beginning of the COVID-19 pandemic, we performed a comprehensive analysis on the intrinsic pathway of coagulation in a large and well-defined prospective cohort of 228 consecutive patients with COVID-19 in Chapter 2. We also assessed whether complement activation, neutrophils, and NETs formation are potential drivers of this COVID-19 induced hypercoagulability. We extend our findings in Chapter 3. By assessing free FVIIa and FVIIa:AT, we aimed to decipher the interplay and dynamics between the intrinsic and extrinsic pathways of coagulation longitudinally in the COVID-19 cohort. We also aimed to identify associations between activation markers of coagulation, endothelial activation and disease severity, including ICU admission, thrombosis, and mortality in this study. Based on these findings, we hypothesized that the neutrophil driven inflammation and NETs formation in COVID-19 may be reduced by blocking the C5a-C5a receptor axis. C5a is a potent anaphylatoxin that stimulates neutrophil attraction and activation and is increased in COVID-19. In Chapter 4 we were able to conduct together with colleagues from Amsterdam UMC an explorative phase 2 trial to investigate the safety and preliminary efficacy of C5a inhibition with vilobelimab in patients with severe COVID-19. In Chapter 5 we explored the role of AnxA1 in COVID-19 by longitudinally assessing AnxA1 and by correlating AnxA1 levels with inflammatory markers and clinical outcomes like thrombosis in patients with COVID-19. To better understand the driving mechanisms behind hypercoagulability in AAV in line with lessons learned from COVID-19, we assessed coagulation factors of the intrinsic and extrinsic coagulation pathway in a prospective cohort of 75 patients with active AAV before treatment and after 6 months in Chapter 6. All results from the research on hyperinflammation and hypercoagulability in COVID-19 and AAV presented in this thesis are summarized and reflected upon in Chapter 7, including clinical implications of this research, and suggestions for future research are presented.

Π

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Chapter 2

Neutrophils and contact activation of coagulation as potential drivers of COVID-19

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Adapted from Circulation. 2020;142:1787–1790

ABSTRACT

The spectrum of coronavirus disease 2019 (COVID-19) ranges from mild disease to lifethreatening acute respiratory distress syndrome, with hyperinflammation, vascular damage, and high rates of thrombotic events. The underlying mechanism need to be clarified. We hypothesized that activated neutrophils damage the endothelium and constantly trigger coagulation. We prospectively analyzed biomarkers of inflammation and early coagulation in 228 consecutive patients with COVID-19. At presentation and during follow-up, complement C5a, histone H3, either citrullinated or not, formation of neutrophil extracellular traps (NETs), and activated factors of coagulation in complex with natural inhibitors were measured. Elevated C5a (n/N=153/201, 76%) was common, with the highest levels in patients with moderate and severe COVID-19. Histone H3 was found in 8 (N=65, 12%) and 15 (N=102, 15%) patients with moderate and severe disease, the prevalence of which increased to 5 (N=23, 24%) and 30 (N=52, 58%) during the course of disease (P=0.008). Histone H3 was citrullinated in 38 (73%) out of 52 patients with extracellular histones detected, confirming NETs formation as the source. Serum from patients with severe COVID-19 induced in vitro NETs formation. Elevated von Willebrand factor antigen (n/N=207/217, 95%) and plasma kallikrein (n/N=194/217, 89%), activated factor XI (n/N=206/217, 95%), activated factor IX (n/N=145/217, 67%), and thrombin (n/N=131/217, 60%) in complex with natural inhibitors were common, with highest levels in those with severe COVID-19. We demonstrate that with increasing severity of disease, complement activation, NETs formation, and activation of coagulation, particularly the intrinsic coagulation pathway, becomes more dominant, pointing to potential treatment targets.

INTRODUCTION

Ever since the first case of pneumonia related to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), known as coronavirus disease 2019 (COVID-19)¹, a pandemic has occurred, with a dramatic impact on global society. COVID-19 varies from mild disease with full recovery to life-threatening acute respiratory distress syndrome (ARDS). Mortality rates are high and exceed 25% in patients with ARDS admitted to the intensive care unit². The pathogenesis underlying the rapid deterioration in a subset of patients remains poorly understood and, consequently, targeted treatment is lacking. It is important to distinguish targets for treatment in the early phase of disease progression, not only to clear the virus, but also to prevent irreversible organ damage and permanent disability. Critically ill patients typically show hyperinflammation^{1,3}, vascular damage, and coagulopathy⁴, with high rates of thrombotic events. Unraveling the connection between these features can be the bridge to timely and targeted therapies in COVID-19. We hypothesize that activated neutrophils damage the endothelium, activate platelets, and constantly trigger coagulation in patients with COVID-19, particularly in those patients with severe disease. This is in line with the neutrophil infiltration, capillaritis, and fibrin deposits found in lung tissue specimens from deceased COVID-19 patients^{5,6}. We consider complement 5a (C5a) to be a main neutrophil driving factor, neutrophil extracellular traps (NETs) formation a key process, and the release of cytotoxic histones as downstream effectors for severe COVID-19 to occur. We studied this premise in a welldefined cohort of 228 patients with COVID-19 by measuring circulating biomarkers of inflammation and vascular damage, NETs formation, and early activation of coagulation.

MATERIALS AND METHODS

Patient population and sampling

Consecutive patients with COVID-19 who presented at the Maastricht University Medical Center, Maastricht, the Netherlands, from March 21 through April 29, 2020, were included. COVID-19 was diagnosed in patients with typical radiologic findings on computed tomography, such as diffuse ground glass opacities and/or bilateral consolidations³, and confirmed by reverse transcriptase polymerase chain reaction of nasopharyngeal swab and/or sputum (i.e., SARS-CoV-2 RNA with a cycle threshold value <40)⁷. Disease severity was classified as mild in patients not admitted to the hospital, moderate in patients admitted to the general ward requiring supplemental oxygen via a nasal cannula (up to 5 l/min), severe in patients requiring supplemental oxygen via a face mask, in patients admitted to the intensive care unit for invasive ventilation and/or those who died \leq 7 days of admission.

At the time of presentation, blood samples were obtained as part of standard care and routine practice. Citrated platelet poor plasma was processed as quickly as possible at 4 degrees Celsius. Serum tubes were allowed to coagulate for 30 minutes at room temperature. Plasma and serum samples were aliquoted, and stored at -80 degrees Celsius until testing. Follow-up samples, obtained every 5 (±2) days, were used when available. In addition, sputum samples were collected in selected cases.

This study was approved by the appropriate ethics committee (2020-1315), with a waiver of informed consent.

Soluble C5a

C5a was quantified in plasma by enzyme immunoassay (Quidel, San Diego, CA), according to the manufacturer's instructions; the assay measures the amount of desarginated C5a.

Histone H3 and citrullinated histone H3

Extracellular histone H3, either citrullinated (citH3) or not, was detected in plasma and sputum by Western blot. In short, plasma or sputum samples diluted in test buffer (1:10; 25 mM HEPES, 150 mM NaCl, 5 mg/mL BSA, pH 7.7) were separated via SDS-PAGE and transferred to PVDF membranes (Pall Corporation, Port Whasington, NY) by semi-dry blotting. After blocking, the membranes were incubated overnight at 4 degrees Celsius with rabbit anti-human histone H3 pAb (1:10,000; Abcam) followed by biotinylated donkey anti-rabbit Ab (1:10,000; Abcam) for 30 minutes and streptavidin/biotinylated HRP solution (1:500, Dako) for 30 minutes. To test whether histone H3 was citrullinated or not, membranes were incubated with mouse anti-citrulline mAb (1:5,000; Antibodies-online, Aachen, Germany) followed by HRP labeled goat anti-mouse Ab (1:5,000, Dako).

Neutrophil extracellular trap formation

Neutrophils from healthy donors were prepared from 10 mL whole blood collected in EDTA tubes. Blood was fractionated by density gradient centrifugation using Lymphoprep (Stemcell, Vancouver, Canada) before lysing residual RBCs with 0.84% ammonium chloride. Neutrophils, labeled with PKH26 (Sigma-Aldrich, St. Louis, MA), were resuspended in medium (phenol red free RPMI, 2% inactivated FBS, and 10% penicillin/streptomycin), seeded on glass culture slides, and incubated with serum diluted in medium to undergo NETs formation for 4 hours; pooled normal human serum and medium were used as a negative control. Neutrophils were fixed and blocked with 1% BSA, incubated with SYTOX green (Thermo Fisher, Waltham, MA) for 15 minutes and DAPI for 5 minutes. In additional experiments, neutrophils were incubated with rabbit anti-citH3 pAb (1:200; Abcam, Cambridge, United Kingdom), rabbit anti-neutrophil

elastase pAb (1:100; Abcam), or rabbit anti-myeloperoxidase pAb (Dako, Glostrup, Denmark) for 2 hours, Alexa 488 labeled anti-rabbit Ab (1:100; Life Technologies, Carlsbad, CA) for 30 minutes, and DAPI for 5 minutes.

Coagulation activation markers

PKa in complex with CINH (PKa:C1INH), FXIa in complex with antithrombin (FXIa:AT) and α 1-antitrypsin (FXIa: α 1AT), FIXa in complex with AT (FIXa:AT), thrombin in complex with AT (T:AT), and vWF:Ag were quantified by in-house developed and validated (ISO 9001) enzyme-linked immunosorbent assays (**Supplementary 2.1**).

Statistical analysis

Continuous variables were presented as mean (±SD) or median (interquartile range [IQR]) as appropriate; between-group differences were analyzed by unpaired sample t test, Mann Whitney U test, one-way ANOVA, or Kruskal Wallis. Differences in categorical variables were analyzed by Fisher's exact test. P<0.05 was considered significant.

RESULTS

Patient population

The baseline characteristics of 228 consecutive patients with COVID-19 are listed in **Table 2.1.** In total, 149 (65%) out of 228 patients were male; the mean age was 67 (±14) years. Patients presented with dyspnea and typical biochemical features of COVID-19, that is, elevated C reactive protein (n=205, 90%), lactate dehydrogenase (n=169, 74%), aspartate transaminase (n=158, 69%), and lymphopenia (n=131, 63%). Also, fever (n=70, 31%) was common. The median duration from onset of symptoms to presentation at our hospital was seven (IQR, 5-12) days. Mild, moderate, and severe COVID-19 was classified in 54, 68, and 106 patients, respectively. Of note, comorbidities did not differ between groups. The moderate and severe COVID-19 patients (n=174, 76%) were admitted to the hospital, 59 (34%) of whom were admitted to the intensive care unit; these patients had elevated D-dimer (n/N=51/53, 96%) and fibrinogen (n/N=49/54, 91%), with median levels of 5,498 (IQR, 1,623-10,000) µg/L and 6.9 (IQR, 5.8-8.0) g/L, respectively. Prolongation of the activated partial thromboplastin time (aPTT) and low platelet counts (i.e., <100×109/L) were found in 13 (N=55, 24%) patients and one (N=58, 2%) case, respectively. None of the patients showed disseminated intravascular coagulation⁸.
Table 2.1 Baseline characteristics of 228 patients with COVID-19.

	Normal	Mild	Moderate	Severe	Overall
	Range	(<i>n</i> =54)	(<i>n</i> =68)	(<i>n</i> =106)	Р
M/F		29/25	42/26	78/28+	0.03
Age, yr.		62 (±16)	69 (±13)‡	69 (±12) ⁺	0.003
Days from illness onset		7 (5-11)	7 (5-14)	7 (5-14)	0.88
SBP, mmHg		130 (±18)	138 (±22)	138 (±25)	0.09
DBP, mmHg		80 (70-89)	83 (75-87)	80 (70-88)	0.65
Heart rate, bpm		90 (±21)	89 (±18)	96 (±20)	0.08
Body temperature, °C		37.6 (±0.8)	38.1 (±1.0)‡	38.1 (±1.0) ⁺	0.009
Fever, %		10, 19	24, 36	36, 40+	0.03
SARS-CoV-2, Ct		31 (±4)	29 (±5)	29 (±5)	0.15
Medical history		. ,			
Hypertension, %		16,30	27,40	35, 33	0.48
Diabetes, %		12, 22	10, 15	25, 24	0.37
CVA, %		7,13	8, 12	15, 14	0.94
Cardiac disease, %		15, 28	23, 34	32, 30	0.76
COPD/asthma, %		9,17	16, 24	13, 12	0.08
None, %		14, 26	16, 24	28, 26	0.96
Platelets, ×10 ⁹ /L	130-350	225 (±97)	213 (±88)	209 (±66)	0.55
Leukocytes, ×10 ⁹ /L	3.5-11.0	5.9 (5.1-8.5)	6.6 (4.7-9.0)	7.5 (5.8-10.1)+	0.006
Neutrophils, ×10 ⁹ /L	1.4-7.7	4.5 (3.5-6.3)	5.0 (3.4-7.4)	$5.9(4.6-8.1)^{\dagger}$	0.02
Lymphocytes, ×109/L	1.1-4.0	1.2 (0.7-1.5)	0.8 (0.6-1.2)‡	0.7 (0.5-1.1) ⁺	< 0.001
AST, U/L	<35	38 (27-55)	49 (36-64) [‡]	54 (39-79) ⁺	< 0.001
LDH, U/L	<250	273 (±91)	362 (±142)‡	480 (±191) ^{*, †}	< 0.001
Serum creatinine, µmol/L	60-115	83 (61-110)	86 (71-112)	92 (71-121)	0.26
Albumin, g/L	32.0-47.0	34 (31-38)	34 (30-36)	29 (26-32)* ⁺	< 0.001
CRP, mg/L	<10	57 (17-96)	66 (39-123)	101 (56-179)*+	< 0.001
Coagulation factors		n/N=48/54	n/N=66/68	n/N=103/106	
PKa:C1INH, nM	≤0.3	2.0 (0.8-3.9)	2.2 (0.9-4.8)	1.9 (1.0-3.5)	0.68
High PKa:C1INH		43, 89%	59,89%	93, 90%	1.00
FXIa:AT, pM	≤42	24.4 (17.4-36.2)	25.3 (19.8-36.4)	30.0 (22.2-65.2)*+	0.002
High FXIa:AT		7,15%	13, 20%	40, 39%*+	0.002
FXIa:α1AT, pM	≤248	391 (309-625)	513 (395-751)	547 (403-809)+	0.007
High FXIa:α1AT		41,85%	63,96%	102, 99%+	0.002
FIXa:AT, pM	≤56	53.8 (33.0-80.9)	63.7 (43.4-91.4)	85.8 (61.7-126.7)*+	< 0.001
High FIXa:AT		20, 42%	42, 64%‡	83, 81%*+	< 0.001
T:AT, ng/mL	≤5	4.2 (3.0-5.8)	5.5 (3.7-10.2)	8.7 (4.9-21.6)*+	< 0.001
High T:AT		19,40%	35, 53%	77, 75%*+ [´]	< 0.001
Vascular damage		,	,		
vWF:Ag, %	≤160	337 (±153)	383 (±148)	462 (±161)*+	< 0.001
High vWF:Ag		43,90%	64,97%	100, 97%	0.11
Neutrophils		·	·		
C5a, ng/mL	≤21.1	15.4 (9.0-25.4)	21.9 (16.5-29.7)‡	22.1 (10.8-31.6)+	0.02
High C5a		29,63%	50, 89%‡	71, 74%+	0.007
Histone H3, <i>n</i> /N		0/44	8/65‡	15/102+	0.02
In vitro NETs formation, n/N		0/5	0/5	9/9*, +	< 0.001

Differences between groups were analyzed by the unpaired sample *t* test, Mann Whitney U test, one-way ANOVA, or Kruskal Wallis. Differences in categorical variables were analyzed by chi square test or Fisher's exact test when appropriate; significant differences between patients groups: severe versus *moderate or †mild disease; moderate versus ‡mild disease. The detection limit of extracellular histone H3 was 0.005 μ g/mL. *Abbreviations:* α 1AT, α 1-antitrypsin. AST, aspartate transaminase. AT, antithrombin. C1INH, C1 esterase inhibitor. COPD, chronic obstructive pulmonary disease. CRP, C-reactive protein. CVA, cerebrovascular accident. DBP, diastolic blood pressure. FIXa, activated factor IX. FXIa, activated factor XI. LDH, lactate dehydrogenase. PKa, plasma kallikrein. SBP, systolic blood pressure. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.

Disease course of COVID-19

In most of the 174 admitted patients treatment consisted of antibiotics (n=161, 93%), chloroquine (n=134, 77%), and anticoagulation (n=155, 89%; therapeutic dose, n=29), in addition to oxygen support. The median follow-up of these patients was 8 (IQR, 4-18) days; the median time of hospitalization was 6 (IQR, 4-9) days in patients with moderate COVID-19 and 9 (IQR, 4-19) days in patients with severe disease. The median time at the intensive care unit was 15 (IQR, 7-21) days and the duration of invasive ventilation was 14 (IQR, 5-20) days.

The cumulative incidence of thrombotic events was highest in patients with severe COVID-19 (n/N=23/106, 22%; pulmonary embolism, n=19) as compared to patients with moderate disease (n/N=3/68, 4%; no pulmonary embolism; P=0.0018) and those with mild disease (n/N=2/54, 4%; pulmonary embolism, n=2; P=0.0024). Fifty-seven (33%) out of 174 admitted patients died after a median of six (IQR, 3-14) days.

Complement C5a, activation of neutrophils, and NETs formation

The anaphylatoxin C5a was measured as indicator of complement activation. At presentation, elevated C5a was found in 153 (76%) out of 201 patients with COVID-19 (Table 2.1); highest levels were found in patients admitted to the hospital as compared to those with mild COVID-19. Neutrophils, recruited and activated by C5a, have been shown to infiltrate the lungs in COVID-19. Neutrophils can undergo NETs formation, a process accompanied by the release of histones. At presentation, histone H3 was found in plasma of eight patients with moderate COVID-19 (n/N=8/65, 12%) and 15 patients with severe disease (n/N=15/102, 15%). Extracellular histore H3 was not found in patients with mild COVID-19. During follow-up, histone H3 was detectable in 33 (58%) out of 57 patients with severe COVID-19, including 28 patients admitted to the intensive care unit and 2 patients with palliative care; compared to 6 (24%) out of 25 patients with moderate COVID-19 (P=0.006). Histone H3 was citrullinated in 38 (73%) out of 52 patients with extracellular H3 detected, indicating NETs formation as the origin of histone H3 in the circulation. In addition, we found histone H3 in sputum samples from nine randomly selected patients admitted to the intensive care unit; all samples contained citrullinated histone H3 (citH3), indicating that NETs formation occurred in the lungs.

Most patients also presented with elevated vWF:Ag (n/N=207/217, 95%). Again, vWF:Ag was significantly higher in patients with severe COVID-19 as compared to patients with moderate and mild disease (**Table 2.1**). Persistent elevated levels of vWF:Ag were found in admitted patients, indicating ongoing vascular damage during the course of disease (**Table 2.2**).

Chapter 2

		Moderate		Severe	P value
	п	median (IQR)	п	median (IQR)	
PKa:C1Inh, nM					
Baseline	66	2.2 (0.9-4.8)	103	1.9 (1.0-3.5)	0.82
≤7 days	25	1.9 (1.0-5.0)	53	2.3 (1.0-12.5)	0.39
8–14 days	8	2.0 (1.7-5.6)	40	2.7 (1.1-9.4)	0.79
FXIa:AT, pM					
Baseline	66	25.3 (19.8-36.4)	103	30.0 (22.2-65.2)	0.004
≤7 days	25	21.4 (19.4-31.2)	53	39.7 (25.0-75.9)	< 0.001
8–14 days	8	20.6 (14.3-28.8)	40	33.2 (22.4-62.0)	0.017
FXIa:α1AT, pM					
Baseline	66	513 (395-751)	103	545 (402-806)	0.49
≤7 days	25	399 (340-569)	53	603 (442-905)	< 0.001
8–14 days	8	385 (294-614)	40	589 (491-860)	0.014
FIXa:AT, pM					
Baseline	66	63.7 (43.4-91.4)	103	85.8 (61.7-126.7)	< 0.001
≤7 days	25	66.4 (49.9-89.7)	53	92.0 (73.7-127.2)	0.003
8–14 days	8	61.7 (39.1-78.4)	40	75.4 (58.8-107.4)	0.091
T:AT, ng/mL					
Baseline	66	5.5 (3.7-10.2)	103	8.7 (4.9-21.6)	< 0.001
≤7 days	25	4.3 (2.8-9.3)	53	7.6 (5.3-22.2)	< 0.001
8–14 days	8	4.6 (3.8-5.2)	40	6.7 (4.8-10.9)	0.026
vWF:Ag, %					
Baseline	66	383 (±148)	103	462 (±161)	0.015
≤7 days	25	391 (±143)	53	497 (±158)	0.005
8–14 days	8	342 (±132)	40	520 (±148)	0.003

Table 2.2 Markers of coagulation and vascular damage in admitted patients with COVID-19 during the course of disease. None of the markers did significantly change over time.

Differences between groups were tested by the Mann Whitney U test. *Abbreviations*: PKa, plasma kallikrein. C1INH, C1 esterase inhibitor. FXIa, activated factor XI. AT, antithrombin. FIXa, activated factor IX. α 1AT, α 1-antitrypsin. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.

Serum-induced in vitro NETs formation

Serum from patients with severe COVID-19 (n/N=9/9) induced abundant in vitro formation of NETs as indicated by extracellular fibers that contain DNA with citH3, neutrophil elastase, and myeloperoxidase (**Figure 2.1**). In vitro NETs formation was observed when using serum with extracellular H3 (n=5) or not (n=4). Extracellular histone H3, however, became detectable in 3 out of 4 latter patients during the course of disease.

Serum from patients with moderate COVID-19 (n/N=2/5) showed scant NETs formation, while NETs did not form upon incubation with serum from patients with mild COVID-19 (n/N=0/5), pooled normal human serum, or medium alone. The presence of citH3 indicates that sera of patients with severe COVID-19 induce peptidylarginine deiminase 4 (PAD4)-dependent NETs formation.



Figure 2.1 Serum from patients with severe COVID-19 (n=3) induced excessive in vitro formation of neutrophil extracellular traps. The presence of citrullinated histone H3 indicates peptidylarginine deiminase 4 -dependent chromatin decondensation; original magnification, 400×. *Abbreviations*: NHS, normal human serum. citH3, citrullinated histone H3. MPO, myeloperoxidase. NE, neutrophil elastase.

Hypercoagulability

Next, we dissected the coagulation cascade. We measured PKa, which reflects activation of FXII, and the activated factors FXIa, FIXa, and thrombin, in complex with their natural inhibitors, that is, C1 esterase inhibitor, antithrombin, and/or α 1 antitrypsin. At baseline, most patients presented with elevated levels of these factors (**Table 2.1**).

Elevated PKa:C1INH indicates activation via the contact pathway of coagulation. Also, FXIa: α 1AT, FIXa:AT, and T:AT were elevated, with highest levels in patients with severe COVID-19. During follow-up, these markers remained elevated with highest levels in patients with severe COVID-19 (**Table 2.2**), indicating that with increasing severity of disease the contact pathway becomes more activated.

Ninety out of 106 patients with severe COVID-19 were treated with low molecular weight heparin (LMWH); therapeutic dose, n/N=7/106 (7%), intermediate dose, n/N=83/106 (78%). Twelve patients not treated with LMWH continued vitamin K

antagonists (VKA) or non-VKA oral anticoagulants. Neither FXIa: α 1AT nor FXIa:AT decreased in patients treated with LMWH (**Supplementary 2.2**). FXI can be activated via contact activation and the extrinsic pathway through the positive feedback loop of thrombin. Because LMWH accelerates inactivation of FXa and thrombin our findings indicate that coagulation is initiated, at least partly, through the contact pathway.

DISCUSSION

Here, we provide evidence for the pathogenic role of the neutrophil-complementcoagulation axis in COVID-19. We demonstrate that with increasing disease severity neutrophils undergo NETs formation, the anaphylatoxin C5a is generated, and the contact pathway is activated.

The clinical spectrum of COVID-19 ranges from mild manifestations to severe disease, often characterized by a rapid deterioration. Neutrophilia is common and may predict worse outcomes9. We demonstrate elevated C5a, a potent anaphylatoxin, in most patients with COVID-19, with the highest levels in patients with moderate and severe disease. Murine data have shown that complement activation contributes to severe disease caused by viruses that share genetic homology with SARS-CoV-2, that is, SARS-CoV-1 and Middle East respiratory syndrome-CoV^{10,11}. C5a primes and activates neutrophils, causing more generation of C5a via the alternative complement pathway and thus, a feedback amplification of neutrophil activation¹². C5a can also stimulate NETs formation in case neutrophils are primed with interferon α^{13} . NETs formation is accompanied by the release of DNA fragments and histones. NETs can cause endothelial damage and the release of vWF:Ag¹⁴. Indeed, vWF:Ag was elevated in our patients with COVID-19. Moreover, we observed a high cumulative incidence of histone H3 and, in particular, citH3, in plasma and sputum from patients with severe COVID-19, indicating that the extent of NETs formation is associated with disease severity. This was confirmed by the in vitro experiments showing that serum samples from patients with severe COVID-19 induce NETs formation. In contrast, samples from patients with mild and moderate disease did not. Chromatin decondensation but not NETs formation, however, was found in patients with moderate COVID-19. Thus, complement activation and neutrophils play a pathogenic role in disease progression. We confirmed the findings of Zuo et al.¹⁵ and the observation that NETs are linked to disease severity in ARDS¹⁶.

Extracellular histones, released during NETs formation, exhibit cytotoxic and activating effects on the endothelium and platelets^{17,18}. Histones injected into mice cause death, with neutrophil margination to the lungs, alveolar fibrin deposition, and microvascular thrombosis¹⁹; identical lesions have been found on lung tissue specimens from deceased COVID-19 patients^{5,20}. Histones, although often below the detection limit at presentation, were most prevalent during the course of disease in patients with severe COVID-19. In a subset of these patients no extracellular histone H3 was detected, although in vitro NETs formation was observed, suggesting that NETs formation and histone-induced effects on the endothelium and platelets occurred.

The most striking and poorly understood feature of COVID-19 is the high cumulative incidence of thrombotic manifestations, particularly pulmonary embolism, that was found in ~40% of patients admitted to the intensive care unit^{4,21}. Thrombosis was proven in 22% of our patients with severe COVID-19 despite using LMWH. The lower incidence in our cohort may be an underestimation as CT angiography was not routinely performed in suspected cases.

The high incidence of thrombotic events may be linked to NETs formation. It has been shown that DNA and histones can activate FXII either directly or through the activation of platelets^{17,22}. We thoroughly assessed the coagulation cascade and clearly showed the important contribution of the contact pathway in COVID-19. At presentation, elevated PKa:C1INH was found in almost all patients, indicating activation of the contact pathway. This pathway becomes more dominant with increasing disease severity, as reflected by elevated levels of FXIa: α 1AT, FXIa:AT, and FIXa:AT that persist during the disease. FXI can be activated both via the contact and extrinsic/common pathway. LMWHs are known to have only mild effects on PKa and FXIIa²², which are key for contact activation. LMWHs did not affect FXIa:AT and FXIa: α 1AT, confirming that activation of FXI occurred through PKa and/or FXIIa. NETs contained citH3, indicating PAD4-induced chromatin decondensation. Mice deficient for PAD4 are protected from thrombosis to occur²⁴. Thus, we postulate that NETs formation is critical for hypercoagulability in COVID-19.

Previous studies on COVID-19 associated coagulopathy documented elevated D-dimer and, although less common, prolonged aPTT^{7,25}. D-dimer was elevated in patients admitted to the intensive care unit, often accompanied by fibrinogen concentrations above the upper limit of normal. Of note, D-dimer reflects plasmin activity, which can further enhance thrombo-inflammation²⁶. The prolonged aPTT did not reflect disseminated intravascular coagulation in our cohort and has recently been linked to lupus anticoagulant, either with FXII deficiency or not²⁷. Lupus anticoagulant, however, was not linked to thrombosis and its role in COVID-19 associated coagulopathy, if any, remains to be determined.

The hyperinflammation in severe COVID-19, characterized by high levels of ferritin and interleukin-6 among other cytokines²¹, can also be triggered by NETs formation. DNA can promote the localization of Toll-like receptor 4 to endosomes containing histones in human monocytes, inducing the release of pro-inflammatory cytokines²⁸. Bronchoalveolar lavage fluid from patients with severe COVID-19, indeed, showed a high proportion of pro-inflammatory monocyte-derived macrophages and neutrophils²⁹.

In conclusion, we analyzed complement, neutrophils, and coagulation in a large and well-defined cohort of patients with COVID-19, providing a deeper understanding of the intricate link between these factors. The feedforward and feedback loops intricately connecting innate immunity and hemostasis can amplify hypercoagulability. These findings provide a rationale for novel therapies for treating COVID-19, such as inhibition of complement, attenuating NETs formation, and neutralizing extracellular histones. Early intervention in those patients with excessive activation of the neutrophil-complement-coagulation axis may be lifesaving.

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SUPPLEMENTALS

Supplementary 2.1

Supplemental methods for the assessment of coagulation activation markers.

Von Willebrand factor antigen (vWF:Ag) was measured in plasma by using an in-house developed enzyme-linked immunosorbent assay (ELISA). Microplates were coated with rabbit anti-human vWF pAb (2 µg/mL; Dako, Glostrup, Denmark). HRP-labeled rabbit anti-human vWF pAb (2 µg/mL; Dako) was used to detect vWF:Ag; quantification was determined using a standard concentration, i.e., 14-187.04%, Plasma kallikrein (PKa), factor XIa (FXIa), FIXa, and thrombin were measured in complex with their natural inhibitors antithrombin (AT), α 1-antitrypsin (α 1AT), or C1 esterase inhibitor (C1INH) in plasma by using ELISAs. Briefly, purified K15 mAb (1.25 µg/mL; in-house), goat antihuman FXI pAb (2 µg/mL; R&D Systems, Minneapolis, MN), sheep anti-human FIX pAb (2 µg/mL; Affinity Biologicals, Ancaster, Canada), and mouse anti-human thrombinantithrombin (T:AT) pAb (3 µg/mL; ITK Diagnostics, Uithoorn, the Netherlands) were used to capture complexes. For detection, sheep anti-human AT pAb (1 µg/mL; Affinity Biologicals), sheep anti-humana1AT pAb (2.5 µg/mL; Abcam, Cambridge, United Kingdom), and purified R11 mAb ($2.5 \mu g/mL$; in-house) were applied. The quantification of complexes was determined using standard concentrations, i.e., PKa:C1INH (0.019-1.25 nM), FXIa:AT (0.62-15 pM), FXIa:α1AT (2.5-150 pM), FIXa:AT (0.34-16.2 pM), and T:AT (2.4-46.2 ng/mL). Precision and accuracy for all ELISAs was evaluated through repeated analysis of normal pooled plasma (in house collected from ≥ 80 healthy controls) and 2 controls with spiked complexes; overall within and between run variation was <15%.

Supplementary 2.2

Treatment with low molecular weight heparin did not affect levels of FXIa: α 1AT and FXIa:AT, indicating that FXI was mainly activated via the intrinsic pathway. Eighty-seven, 45, and 36 patients were tested at to (baseline), ti (\leq 7 days), and t₂ (8-14 days).



Abbreviations: FXIa, activated factor XI. α1AT, α1-antitrypsin. AT, antithrombin.

2



Chapter 3

Thrombin formation via the intrinsic coagulation pathway and von Willebrand factor reflect disease severity in COVID-19

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Adapted from Haematologica 2023;108:1417–1422

ABSTRACT

Both the intrinsic and extrinsic coagulation pathways are activated in coronavirus disease 2019 (COVID-19) and associated with hypercoagulability. However, data on the interplay between these pathways and their implications on clinical outcomes are limited. We longitudinally investigated the dynamics of the intrinsic and extrinsic pathway and von Willebrand factor: antigen (vWF:Ag) in relation to disease severity, thrombosis, and mortality in a prospective observational cohort of 220 patients with COVID-19. At presentation and during follow-up, activation markers of coagulation and vWF:Ag were assessed, FXIa:antithrombin (AT), FIXa:AT, thrombin:antithrombin (T:AT), and vWF:Ag but not FVIIa:AT or free FVIIa increased with disease severity and remained stable over time. FXIa:AT (r=0.64) and FIXa:AT (r=0.74) but not FVIIa:AT (r=0.14) or free FVIIa (r=0.16) correlated with T:AT, pointing to activation of the intrinsic pathway. FXIa:AT, FIXa:AT, and T:AT were higher in patients admitted to the ICU, with thrombosis, and/or non-survivors. Multivariable logistic regression indicated T:AT as a predictor for ICU admission (OR 1.449 [95% confidence interval [CI] 1.092-1.922]; P=0.01) and thrombotic events (OR 1.336 [95% CI 1.025-1.740]; P=0.032). Linear mixed models predicted that vWF:Ag increased over time in patients admitted to the ICU (+58 [95% CI 1-116] %; P < 0.001) and non-survivors (+77 [95% CI 15-137] %; P = 0.023). We conclude that thrombin formation is driven via the intrinsic pathway in COVID-19. T:AT and vWF:Ag are important markers of disease severity, thrombosis, and mortality.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) varies from mild disease to life-threatening acute respiratory distress syndrome (ARDS). Severe COVID-19 has been characterized by hyperinflammation, vascular damage, and thrombosis^{1,2}. COVID-19 induced hypercoagulability has distinctive features and differs from thrombotic events associated with disseminated intravascular coagulation³. In COVID-19, excessive proinflammatory reactions activate and damage the pulmonary vasculature. Indeed, expression of endothelial activation markers such as von Willebrand factor (vWF) are associated with severe COVID-19^{4,5}. Vascular damage can trigger the extrinsic coagulation cascade by exposure of tissue factor (TF) to blood and to factor VII, thereby generating activated FVII (FVIIa) that subsequently activates a prothrombotic pathway including the FXa driven generation of thrombin and fibrin, resulting in clot formation. Moreover, hypercoagulability has been linked to activation of the intrinsic pathway due to neutrophil extracellular traps (NETs) formation and/or complement activation in general and which was recently also confirmed in COVID-19⁶⁻⁹.

We previously demonstrated that increased intrinsic activation shown by elevated levels of plasma kallikrein:C1 esterase inhibitor (PKa:C1Inh), FXIa:antithrombin (AT), and FIXa:AT complexes were associated with disease severity in COVID-19¹. Moreover, low molecular weight heparin (LMWH) did not affect these complexes during the course of disease, suggesting activation via FXIIa. Yet, the extrinsic pathway has not been studied in relation to the intrinsic pathway in COVID-19 hypercoagulability and thus, firm conclusions cannot be drawn.

Here, we assessed the intrinsic and extrinsic pathway of coagulation in a large and welldefined cohort of patients with COVID-19. The dynamics of both pathways were studied longitudinally in the first wave of the pandemic. Also, associations between activation markers of coagulation, endothelial activation and disease severity, including intensive care unit (ICU) admission, thrombosis, and mortality, were studied.

METHODS

Patient population and sampling

Consecutive patients with COVID-19 who presented at the Maastricht University Medical Center, Maastricht, the Netherlands, from March 21, 2020, through April 28, 2020, were included in our prospective cohort. COVID-19 was diagnosed in patients with typical radiologic findings on computed tomography and confirmed by reverse transcriptase polymerase chain reaction of nasopharyngeal swab and/or sputum. Disease severity was classified as mild in patients not admitted to the hospital, moderate in

patients admitted to the general ward requiring supplemental oxygen via nasal cannula (up to 5 L/min), and severe in patients requiring supplemental oxygen via a face mask, admitted to the ICU for mechanical ventilation, and/or those who died due to COVID-19. At presentation and fixed time points during follow-up (i.e., every 5 [±2] days), blood samples were obtained using vacutainer tubes containing 3.2% trisodium citrate and serum tubes; citrated blood was processed immediately and centrifuged at 2000 g for 10 minutes at room temperature (RT), while serum tubes were allowed to clot for 30 minutes and centrifuged at 1885 g for 10 minutes at RT. Plasma and serum samples were aliquoted and stored at –80 degrees Celsius until testing. Follow-up samples were used when available. This study was approved by the appropriate ethics committee (2020-1315), with a waiver of informed consent.

Data collection

Demographics and clinical findings as well as outcomes (i.e., ICU admission, thrombotic events, 28 days in-hospital mortality) were obtained from electronic patient records.

Measurement of coagulation factors, complement 5a and vWF:Ag

Activated coagulation factors in complex with their natural inhibitors (i.e., activated FVII:antithrombin [FVIIa:AT], plasma kallikrein:C1 esterase inhibitor [PKa:C1Inh], FXIa:AT, FXIa:alpha1-antitrypsin [α 1AT], FIXa:AT, and thrombin:antithrombin [T:AT]), desarginated complement 5a (C5a), and vWF:antigen (vWF:Ag), were quantified as described^{1,10}. Free FVIIa was measured using the Staclot® VIIa-rTF kit (Diagnostica Stago, Asniéres-sur-Seine, France) according to the manufacturer's instructions.

Statistical analysis

Continuous variables were presented as mean (±standard deviation [SD]) or median (interquartile range [IQR]) as appropriate; between-group differences were analyzed after exploration for normality and equal variances by the independent-samples t-test, Mann Whitney U test, one-way ANOVA, or Kruskal Wallis. Differences in categorical variables were analyzed by Fisher's exact test. Correlations between coagulation factors, vWF:Ag and inflammatory markers were assessed with Spearman's rank correlation coefficient. Univariable and multivariable logistic regression was used to assess associations between activated coagulation factors and vWF:Ag levels (per ten units) with ICU admission, thrombotic events, and 28 days mortality, respectively. Results were expressed as odds ratio (OR) including 95% confidence interval (CI). To assess the discriminative ability of each factor (univariable) and each factor in combination with other variables (multivariable), we computed the area under the receiver operating characteristic (ROC) curve (AUC). Linear mixed-effects models were used to model the effects of the

longitudinal coagulation factor and vWF:Ag data on clinical outcome measures (i.e., ICU admission, thrombotic events, and 28 days in-hospital mortality). The statistical analyses were performed with IBM SPSS Statistics version 28, RStudio version 4.0.4 and GraphPad Prism version 9. *P*<0.05 was considered significant.

RESULTS

Patient characteristics

The coagulation factors were assessed in 220 out of the 228 (96%) patients with COVID-19¹. Eight patients were excluded due to an insufficient amount of blood samples for the analysis. Baseline characteristics of the 220 included patients are depicted in **Table 3.1**.

Patients presented after a median of 7 (IQR, 5-14) days after the onset of symptoms. 146 (66%) patients were male and median age at presentation was 71 (IQR, 59-78) years. 46 (21%) patients had mild, 68 (31%) had moderate and 106 (48%) had severe COVID-19, respectively. Patients were admitted to the hospital for a median of 8 (IQR, 5-18) days; 6 (IQR, 4-10) days and 13 (IQR, 5-23) days for patients with moderate and severe COVID-19, respectively.

Most admitted patients were treated with antibiotics (n/N=157/174, 90%), chloroquine (n/N=131/174, 75%), and anticoagulation (n/N=154/174, 88%; prophylactic dose of LMWH [n=126], therapeutic dose of LMWH [n=6], and continuation of direct oral anticoagulant or vitamin K antagonists [n=22]), in addition to oxygen support. Of note, steroids were not routinely prescribed at that time (n/N=6/174, 3%).

Routine coagulation tests were not consistently measured, particularly not in patients with mild or moderate COVID-19. Patients with severe COVID-19 had elevated D-dimer (n/N=62/64, 97%) and fibrinogen (n/N=55/62, 89%), with a median level of 2774 (IQR, 1167-10000) µg/L and 6.6 (IQR, 5.3-8.0) g/L, respectively. The activated partial thromboplastin time (aPTT) and prothrombin time (PT) were prolonged in 19 (18%) and 14 (13%) patients.

	Normal	Mild	Moderate	Severe	Overall P
	Range	(<i>n</i> =46)	(<i>n</i> =68)	(<i>n</i> =106)	
M/F		26/20	42/26	78/28	0.077
Age, yr.		64 (52-74)	73 (60-79)‡	73 (60-77)+	0.014
Days from illness onset		7 (5-11)	7 (5-14)	7 (5-14)	0.975
SBP, mmHg		129 (±18)	138 (±22)	138 (±25)	0.063
DBP, mmHg		80 (70-86)	83 (74-87)	80 (70-88)	0.714
Heart rate, bpm		88 (75-100)	90 (80-100)	95 (80-110)*, †	0.013
Body temperature, °C		37.6 (±0.9)	38.1 (±1.0)‡	38.1 (±1.0) ⁺	0.006
Fever, (%)	>37.9	12 (27)	39 (58)‡	55 (62)+	< 0.001

Table 3.1 Baseline characteristics of 220 patients with COVID-19.

Table 3.1 (continued)

	Normal	Mild	Moderate	Severe	Overall P
	Range	(<i>n</i> =46)	(<i>n</i> =68)	(<i>n</i> =106)	
Medical history			\$ 7	· · · · ·	
Hypertension, (%)		13 (28)	27 (40)	35 (33)	0.452
Diabetes, (%)		9 (20)	11 (16)	24 (23)	0.603
CVA, (%)		5 (11)	9 (13)	14 (13)	0.931
Cardiac disease, (%)		11 (24)	23 (34)	32 (30)	0.560
COPD/asthma, (%)		6 (13)	15 (22)	11 (10)	0.110
None, (%)		12 (26)	16 (24)	28 (26)	0.918
Platelets, ×10 ⁹ /L	130-350	187 (154-292)	214 (147-260)	211 (168-247)	0.985
Leukocytes, ×109/L	3.5-11.0	6.0 (4.8-6.5)	6.6 (4.7-9.0)	7.4 (5.8-10)*, ⁺	0.016
Neutrophils, ×10 ⁹ /L	1.4-7.7	4.8 (3.4-6.5)	5.0 (3.4-7.4)	5.9 (4.7-8.1)+	0.023
Lymphocytes, ×10 ⁹ /L	1.1-4.0	1.1 (0.7-1.5)	0.8 (0.6-1.2)	$0.7(0.5-1.1)^{+}$	0.013
NLR		4.7 (2.9-6.9)	6.0 (4.1-9.0) [‡]	8.6 (5.2-12.5) ^{*, +}	< 0.001
AST, U/L	<35	36 (26-58)	49 (37-64)‡	55 (40-80)*	< 0.001
LDH, U/L	<250	253 (202-344)	328 (266-451)‡	451 (358-595)*, +	< 0.001
Serum creatinine, µmol/L	60-115	83 (62-113)	88 (71-119)	91 (71-120)	0.369
Albumin, g/L	32.0-47.0	34 (31-38)	33 (30-36)	29 (26-32)*,+	< 0.001
CRP, mg/L	<10	57 (17-95)	69 (39-130)‡	103 (56-178)*, +	< 0.001
C5a, ng/mL	≤21.1	15.4 (9.0-25.4)	21.8 (16.8-28.7)‡	22.1 (11.0-31.5)*	0.024
High C5a, (%)		27 (61%)	51 (90%)‡	73 (75%)*	0.004
D-dimer, μg/L	<500	-	-	2774 (1167-10000)	-
Fibrinogen, g/L	1.7-4.0	-	-	6.6 (5.3-8.0)	-
aPPT, sec	23-32	-	-	29 (27-33)	-
PT, sec	9.9-12.4	-	-	11.8 (11.0-12.0)	-
FVIIa, mIU/mL	-	33.1 (21.0-50.8)	31.7 (20.0-47.6)	39.8 (20.5-55.3)	0.360
High FVIIa, (%)		-	-	-	-
FVIIa:AT, pM	≤910	599 (485-719)	556 (476-741)	607 (458-784)	0.790
High FVIIa:AT, (%)		3 (7)	3 (4)	15 (14)	0.282
PKa:C1Inh, nM	≤0.3	1.6 (0.7-4.1)	2.5 (0.9-4.8)	1.9 (1.0-3.5)	0.733
High PKa:C1Inh, (%)		40 (89)	60 (90)	94 (90)	0.957
FXIa:AT, pM	≤42	23.4 (17.3-35.9)	25.7 (19.8-38.0)	30.0 (22.3-65.2)*, *	0.001
High FXIa:AT, (%)		7 (16)	13 (19)	40 (39)*, +	0.003
FXIa:α1AT, pM	≤248	377 (308-597)	515 (396-751)‡	545 (402-806)†	0.003
High FXIa:α1AT, (%)		38 (84)	64 (96)‡	103 (99)+	0.002
FIXa:AT, pM	≤56	53.7 (33.0-71.8)	64.2 (43.4-91.0)	86.8 (62.0-125.6)*, *	< 0.001
High FIXa:AT, (%)		18 (39)	43 (64)‡	84 (81)*, †	< 0.001
T:AT, ng/mL	≤5	4.2 (3.0-5.7)	5.5 (3.7-10.1)‡	8.7 (4.9-21.2) ^{*, +}	< 0.001
High T:AT, (%)		17 (38)	36 (54)	78 (75)*, †	< 0.001
vWF:Ag, %	≤160	323 (214-422)	361 (263-488)	438 (343-557) ^{*, †}	< 0.001
High vWF:Ag, (%)		40 (89)	65 (97)	101 (97)	0.084

Continuous variables were presented as mean (±standard deviation) or median (interquartile range) as appropriate. Differences between groups were analyzed by unpaired sample *t* test, Mann Whitney U test, one-way ANOVA, or Kruskal Wallis. Differences in categorical variables were analyzed by chi square test or Fisher's exact test when appropriate; significant differences between patients groups: severe versus *moderate or †mild disease; moderate versus ‡mild disease. *Abbreviations*: NLR, neutrophil-lymphocyte ratio. AST, aspartate transaminase. COPD, chronic obstructive pulmonary disease. CRP, C-reactive protein. CVA, cerebrovascular accident. DBP, diastolic blood pressure. LDH, lactate dehydrogenase. APTT, activated partial thromboplastin time. PT, prothrombin time. FVIIa, activated FVII. AT, antithrombin. PKa, plasma kallikrein. C1INH, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.

Activated coagulation factors at baseline and during follow-up

We previously demonstrated that T:AT is elevated and linked to disease severity in COVID-19. Neutrophils, NETs formation, and activation of the intrinsic pathway drive T:AT levels and COVID-19's hypercoagulability¹. NETs formation, with release of TF, may also activate the extrinsic pathway¹¹. To better understand the balance between the intrinsic and extrinsic pathways, we assessed markers of the extrinsic pathway. FVIIa:AT, a marker of circulating FVIIa-TF complexes, and free FVIIa did not differ between patients with mild, moderate, or severe COVID-19 (**Table 3.1**) and remained stable over time (**Supplementary 3.1**). Spearman's Q indicated a strong positive correlation between T:AT and FXIa:AT (r=0.64, *P*<0.001) as well as FIXa:AT (r=0.74, *P*<0.001), but not FVIIa:AT (r=0.14, *P*=0.097) or free FVIIa (r=0.16, *P*=0.021; **Figure 3.1**). The role of FVIIa:AT and free FVIIa decreased even more whereas the association of FXI:AT, FIX:AT and T:AT persisted with increasing disease severity.

Correlation between activated coagulation factors and inflammatory markers

The Spearman rank correlation coefficients for the coagulation markers compared with different markers of inflammation (i.e., CRP, neutrophil and lymphocyte counts, neutrophil-lymphocyte ratio [NLR] and soluble C5a) at baseline are presented in **Supplementary 3.2**. A positive correlation was observed between CRP and FXIa: α 1AT (r=0.38, P<0.001), FIX:AT (r=0.41, P<0.001), T:AT (r=0.35, P<0.001), and vWF:Ag (r=0.38, P<0.001). FXI:AT (r=0.22, P=0.002) was weakly correlated with CRP, whereas FVIIa:AT and free FVIIa activity did not correlate with CRP. In contrast to a positive correlation between neutrophil counts and vWF:Ag levels (r=0.31, P<0.001), other inflammatory markers were only weakly or not associated with increased coagulation activity assessed by coagulation complexes or with the vWF:Ag levels. Taken together, CRP appeared to correlate with activation of the intrinsic pathway and endothelial damage.

Activated coagulation factors and clinical outcome

Differences in activated coagulation complexes and vWF:Ag between patients admitted to the ICU or not, patients with thrombotic events or not, and survivors or non-survivors are presented in **Figure 3.2**.

ICU admission

Sixty-four (29%) patients with COVID-19 were admitted to the ICU. The median admission time to the ICU was 16 (IQR, 7-22) days and the duration of invasive ventilation was 15 (IQR, 6-20) days. At baseline, FXIa:AT (P=0.003), FXIa: α IAT (P=0.001), FIXa:AT (P<0.001), T:AT (P<0.001) as well as vWF:Ag (P<0.001) were significantly higher in ICU versus non-ICU admitted patients with COVID-19 (**Figure 3.2a**). FVIIa:AT and

51

3

free FVIIa were similar in both groups. Logistic regression analysis (**Table 3.2**) indicated that baseline coagulation and vWF:Ag (per ten units) were associated with ICU admission: T:AT (OR 1.589 [95% CI 1.253-1.999]; P<0.001), FXIa: α 1AT (OR 1.001 [95% CI 1.000-1.001]; P=0.028), and vWF:Ag (OR 1.026 [95% CI 1.008-1.045]; P=0.005). Multivariable logistic regression, adjusted for age (in years), CRP (mg/L), and a positive medical history for cardiovascular disease (i.e., cardiac disease and/or hypertension) indicated that T:AT (OR 1.449 [95% CI 1.092-1.922]; P=0.010) and FXIa: α 1AT (OR 1.005 [95% CI 1.000-1.010]; P=0.047) but not vWF:Ag (OR 1.020 [95% CI 0.999-1.043]; P=0.068) predicted the risk for ICU admission.



Figure 3.1 Correlation matrix of baseline extrinsic and intrinsic coagulation factors in patients with COVID-19. Spearman rank correlation coefficients were calculated for all patients (A) and stratified by disease severity into mild (B), moderate (C), and severe patients (D). *indicates a P value < 0.05. *Abbreviations*: FVIIa, activated factor VIIa. AT, antithrombin. PKa, plasma kallikrein. C1INH, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. T:AT, thrombin in complex with antithrombin.



Figure 3.2 Differences of activated intrinsic coagulation factors, T:AT and vWF:Ag at baseline stratified by different clinical outcomes. Scatter plots with depicted medians and interquartile ranges show FXIa:AT, FXIa: α 1AT, FIXa:AT, T:AT and vWF:Ag levels for patients admitted to the intensive care unit (ICU) or not (A), with thrombotic events (TE) or not (B), and for survivors and non-survivors (C). *P*-values are calculated with the Mann-Whitney test. *Abbreviations*: FXIa, activated factor XI. AT, antithrombin. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.

Thrombotic events

Thrombotic events were observed in 29 (13%) out of 220 patients with COVID-19. Of these patients, 3 had mild, 3 had moderate, and 23 had severe COVID-19. Pulmonary embolisms, cerebrovascular accidents, peripheral arterial occlusions, and acute coronary syndromes were found in 22 (75%), 3 (11%), 3 (11%), and 1 (3%) patient, respectively. Patients with thrombotic events had significantly higher FXIa:AT (*P*=0.022), FIXa:AT (*P*<0.001), and T:AT (*P*=0.001) when compared to patients without thrombotic events (**Figure 3.2b**). Logistic regression showed that T:AT (OR 1.402 [95% CI 1.078-1.824]; *P*=0.012) and FXIa:AT (OR 1.024 [95% CI 1.000-1.048]; *P*=0.048) were associated with an increased risk of thrombotic events in patients with COVID-19 (**Table 3.2**). The association of T:AT remained significant (OR 1.336 [95% CI 1.025-1.740]; *P*=0.032) in a multivariable model adjusting for sex.

Mortality

Fifty-eight (26%) patients with COVID-19 died in the hospital within 28 days. Two (3%) of these patients had moderate disease and their death was not related to COVID-19 (i.e., in both cases palliation because of fatal subdural hematoma on the background of traumatic brain injury). Both patients were excluded from the following analysis. Non-survivors had significantly higher free FVIIa (*P*=0.045), FIXa:AT (*P*<0.001), T:AT (*P*=0.002), and vWF:Ag (*P*<0.001) at baseline when compared to survivors (**Figure 3.2c**). T:AT (OR 1.403 [95% CI 1.116-1.769]; *P*=0.004) and vWF:Ag (OR 1.034 [95% CI 1.015-1.054]; *P*=0.001) were associated with 28 days in-hospital mortality but after correction in a multivariable models adjusting for age (in years), CRP (mg/L), and diabetes as comorbidity was performed (**Table 3.2**).

Activated coagulation factors over time and clinical outcome

We assessed the prognostic value of activated coagulation factors in complex with their natural inhibitors and vWF:Ag over time in admitted patients using linear mixed models (**Figure 3.3** and **Supplementary 3.3**). The dynamics of vWF:Ag were associated with clinical outcomes, whereas none of the activated coagulation markers (i.e., FVIIa:AT, free FVIIa, PKa:C1Inh, FXIa:AT, FXIa: α 1AT, FIXa:AT and T:AT) was; at presentation, however, FXIa:AT (+85.2 [95% CI 13.5-156.8]; *P*=0.020) and T:AT (+9.6 ([95% CI, 1.4-18], *P*=0.023) were higher in patients with thrombotic events. vWF:Ag increased over time, particularly in patients admitted to the ICU and those who died. The increase in vWF:Ag was steeper in patients who died as compared to those who survived. Of note, elevated levels of PKa:C1Inh, FXIa: α 1AT, FIXa:AT and T:AT remained stable during hospital admission (data not shown).

Univariable	OR (95% CI)	P value	AUC (95% CI)
ICU admission			
FVIIa	0.961 (0.858-1.077)	0.497	0.507 (0.421-0.592)
FVIIa:AT	1.005 (0.999-1.010)	0.097	0.507 (0.404-0.611)
PKa:C1Inh	1.022 (0.990-1.054)	0.178	0.515 (0.431-0.599)
FXIa:AT	1.023 (0.999-1.047)	0.064	0.630 (0.548-0.712)
FXIa:α1AT	1.001 (1.000-1.001)	0.028	0.646 (0.570-0.723)
FIXa:AT	1.007 (0.997-1.018)	0.163	0.682 (0.606-0.759)
T:AT	1.583 (1.253-1.999)	< 0.001	0.680 (0.603-0.757)
vWF:Ag	1.026 (1.008-1.045)	0.005	0.644 (0.566-0.722)
Thrombotic events			
FVIIa	0.929 (0.790-1.093)	0.373	0.521 (0.424-0.618)
FVIIa:AT	1.001 (0.999-1.004)	0.192	0.449 (0.318-0.580)
PKa:C1Inh	1.005 (0.975-1.037)	0.741	0.545 (0.435-0.655)
FXIa:AT	1.024 (1.000-1.048)	0.048	0.632 (0.523-0.741)
FXIa:α1AT	1.001 (0.997-1.005)	0.538	0.605 (0.492-0.718)
FIXa:AT	1.008 (0.997-1.019)	0.141	0.696 (0.591-0.800)
T:AT	1.402 (1.078-1.824)	0.012	0.685 (0.589-0.780)
vWF:Ag	1.014 (0.991-1.038)	0.239	0.561 (0.447-0.674)
28 Days Mortality			
FVIIa	1.112 (0.995-1.243)	0.061	0.593 (0.497-0.689)
FVIIa:AT	1.001 (0.999-1.003)	0.305	0.474 (0.364-0.584)
PKa:C1Inh	0.899 (0.721-1.121)	0.344	0.559 (0.473-0.645)
FXIa:AT	1.003 (0.978-1.027)	0.837	0.588 (0.501-0.674)
FXIa:α1AT	0.998 (0.994-1.003)	0.503	0.512 (0.425-0.598)
FIXa:AT	1.003 (0.993-1.014)	0.520	0.664 (0.582-0.747)
T:AT	1.403 (1.116-1.769)	0.004	0.638 (0.553-0.722)
vWF:Ag	1.034 (1.015-1.054)	0.001	0.660 (0.579-0.740)
Multivariable	OR (95% CI)	P value	AUC (95% CI)
ICU admission*			
FVIIa	0.967 (0.849-1.102)	0.614	0.734 (0.659-0.808)
FVIIa:AT	1.007 (1.000-1.015)	0.059	0.773 (0.692-0.854)
PKa:C1Inh	1.018 (0.983-1.055)	0.306	0.744 (0.673-0.815)
FXIa:AT	1.027 (0.996-1.059)	0.085	0.734 (0.660-0.808)
FXIa:α1AT	1.005 (1.000-1.010)	0.047	0.747 (0.674-0.820)
FIXa:AT	1.008 (0,995-1.020)	0.240	0.724 (0.647-0.801)
T:AT	1.449 (1.092-1.922)	0.010	0.751 (0.677-0.825)
vWF:Ag	1.020 (0.999-1.043)	0.068	0.738 (0.662-0.814)
Thrombotic events ⁺			
FVIIa	0.969 (0.823-1.140)	0.702	0.594 (0.500-0.687)
FVIIa:AT	1.001 (0.999-1.004)	0.226	0.561 (0.444-0.679)
PKa:C1Inh	1.002 (0.971-1.034)	0.913	0.640 (0.542-0.739)
FXIa:AT	1.019 (0.995-1.044)	0.113	0.699 (0.592-0.806)
FXIa:α1AT	1.001 (0.997-1.005)	0.772	0.672 (0.569-0.775)
FIXa:AT	1.006 (0.995-1.017)	0.257	0.740 (0.639-0.841)
T:AT	1.336 (1.025-1.740)	0.032	0.725 (0.630-0.821)
vWF:Ag	1.010 (0.987-1.034)	0.399	0.635 (0.535-0.735)

Table 3.2 Logistic regression was performed to ascertain the effects of the coagulation factors (per ten units) alone (univariable) and together with other predictors (multivariable) on the likelihood of ICU admission, thrombosis and 28 days mortality.

Multivariable	OR (95% CI)	P value	AUC (95% CI)
28 Days Mortality [‡]			
FVIIa	1.101 (0.966-1.256)	0.149	0.803 (0.733-0.872)
FVIIa:AT	1.000 (0.998-1.002)	0.910	0.817 (0.729-0.905)
PKa:C1Inh	0.904 (0.706-1.157)	0.421	0.778 (0.707-0.850)
FXIa:AT	0.992 (0.959-1.027)	0.652	0.774 (0.702-0.847)
FXIa:α1AT	0.997 (0.991-1.004)	0.379	0.777 (0.705-0.849)
FIXa:AT	0.997 (0.982-1.013)	0.725	0.774 (0.701-0.847)
T:AT	1.316 (0.990-1.750)	0.059	0.789 (0.718-0.859)
vWF:Ag	1.007 (0.984-1.031)	0.555	0.773 (0.701-0.845)

Table 3.2 (continued)

*Combined with male sex (OR 2.112 [95% CI 1.090-4.090]; P=0.027), a medical history of hypertension (OR 2.170 [95% CI 1.121-4.201]; P=0.022), cardiac disease (OR 2.139 [95% CI 1.071-4.273]; P=0.031), and CRP in mg/L (OR 1.008 [95% CI 1.004-1.0011]; P<0.001). *combined with male sex (OR 3.616 [95% CI 1.209-10.816]; P=0.022). *combined with age in years (OR 1.073 [95% CI 1.040-1.107]; P<0.001), a medical history of diabetes (OR 3.510 [95% CI 1.729-7.124]; P=0.001), and CRP in mg/L (OR 1.005 [95% CI 1.001-1.009]; P=0.010). *Abbreviations*: FVIIa, activated FVII. AT, antithrombin. PKa, plasma kallikrein. C1INH, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.



Figure 3.3 Predicted estimates of vWF:Ag stratified by different clinical outcomes at baseline and over time in patients with COVID-19. Linear mixed-effects models were used to illustrate the effects of vWF:Ag on ICU admission, thrombosis, and 28 days in-hospital mortality. (A) Estimated vWF:Ag was for ICU admitted patients at baseline +9.0 ([95% CI, -55-73], *P*=0.782) higher than non-ICU admitted patients. Over time, vWF:Ag decreased (-3.0 [95% CI, -27-22], *P*=0.826) overall, but increased significantly in ICU admitted patients (+61 [95% CI, 28-94]; *P*<0.001). (B) Baseline vWF:Ag was comparable between patients with (+7.0 [95% CI, -80-93], *P*=0.879) and without thrombosis. Over time, vWF:Ag increased significantly in both groups (+28 [95% CI, 9.0-48], *P*=0.005) without a statistical significant difference for patients with thrombosis (+34 [95% CI, -7.0-74], *P*=0.103). (C) vWF:Ag tend to be higher in non-survivors (+45 [95% CI, -28-117], *P*=0.225) and increased over time (+28 [95% CI, 8-47], *P*=0.006) in both groups with a statistically significant sharper increase in non-survivor (+49 [95% CI, 7-90], *P*=0.023). *Abbreviations*: vWF:Ag, von Willebrand factor antigen. ICU, intensive care unit.

DISCUSSION

In this prospective observational study, we deciphered the coagulation cascade over time and showed that the intrinsic pathway becomes more dominant in patients with increasing severity of COVID-19. Furthermore, we found that ICU admission, thrombotic events and in-hospital mortality were mainly associated with vWF:Ag and T:AT levels. Finally, for the first time in the literature, we predicted varying courses of coagulation and endothelium activation, i.e. T:AT and vWF:Ag, in relation to different clinical outcomes.

We previously reported that FXIa:AT, FXIa: α 1AT, FIXa:AT, and T:AT levels were elevated in patients with COVID-19 and associated with COVID-19 hypercoagulability¹. which has been confirmed by others^{6,12,13}. However, firm conclusions on the interplay and balance between the intrinsic and extrinsic coagulation pathways in COVID-19 could not be drawn because data on the extrinsic pathway were lacking. Of note, TF-driven coagulation is phospholipid surface dependent and not readily detectable in plasma^{14,15}. We therefore assessed FVIIa:AT, a marker of circulating FVIIa-TF complexes, and free FVIIa, a general marker for activation of the extrinsic pathway. FVIIa:AT and FVIIa, however, did not differ between mild, moderate and severe COVID-19. In contrast, we found a strong positive correlation between the intrinsic pathway (i.e., FXIa:AT and FIXa:AT) and T:AT, which was not found between FVIIa:AT as well as FVIIa and T:AT, pointing to the intrinsic pathway. Moreover, the role of FVIIa:AT and FVIIa decreased even more with increasing disease severity, suggesting that the role of the extrinsic pathway in the amplification of thrombin generation is limited. Thus, amplification of thrombin generation may occur via FXIa^{16,17}. The observation that markers of activated FXI:AT, FIX:AT and thrombin generation, but not kallikrein, were related to disease severity suggests that besides initiation, contact activation (FXIIa mediated PKa formation) is not directly linked to propagation of thrombin generation. Unfortunately, we do not have information about the degree of FXII activation in these patients.

Activation of the intrinsic pathway in COVID-19 may be multifactorial, but an important role of hyperinflammation has been found¹. CRP levels as a general marker of inflammation correlated with FXIa:α1AT, FIXa:AT and T:AT levels in our cohort. Previous studies reported on the role of complement activation and NETs formation in COVID-19 and suggested that the release of RNA and DNA can trigger the intrinsic coagulation via activation of FXII⁷⁻⁹. In fact, NETs formation is associated with microthrombi and platelet deposition in lung biopsies of non-survivors with COVID-19¹⁸. Of importance, FXII was found to co-localize with NETs on lung biopsies and improved clearance of NETs reduced activation of FXII *in vitro*⁶.

Baseline and follow-up vWF:Ag levels were elevated in patients with COVID-19 and associated with disease severity. Patients admitted to the ICU and/or those who died within 28 days upon admission had the highest levels of vWF:Ag, corroborating previous

3

observations^{5,19,20}. Thus, endothelial damage is an important contributor to severe COVID-19.

Next, we investigated the associations between poor outcomes and coagulation complexes as well as vWF:Ag in COVID-19. FIXa:AT and T:AT levels at baseline were associated with ICU admission, thrombotic events, and 28 days in-hospital mortality. ICU admission, in particular, was associated with the activation of the intrinsic pathway. In a small cohort, FXIa:AT and FIXa:AT levels were associated with the length of hospital and ICU admission and radiologic progression of lung disease on computed tomography¹². Here, we found that $FXIa:\alpha 1AT$ and T:AT predicted the risk of ICU admission after correction for sex, a medical history of hypertension and cardiac disease and CRP. T:AT also predicted the risk of thrombotic events after adjusting for sex. In line with previous studies^{5,21,22}, neither coagulation complexes nor vWF:Ag at baseline predicted the 28 days in-hospital mortality as based on the multivariable model. Most of the studies, however, were small, retrospective or not longitudinally designed. We therefore assessed coagulation activity and vWF:Ag also longitudinally and estimated with linear mixed models the changes of these laboratory markers over time in relation to clinical outcomes, Remarkably, vWF:Ag levels increased over time, particularly in patients admitted to the ICU and those who died. Interestingly, vWF:Ag levels were comparable at baseline between survivors and non-survivors and vWF:Ag rose in both groups over time albeit significantly steeper in non-survivors. T:AT levels, on the other hand, were significantly higher in ICU admitted compared to non-ICU admitted patients as well as in patients with thrombotic events compared to patients without thrombotic events at baseline and remained stable over time. These observations suggest that endothelial damage is associated with poor outcomes, whereas activation of coagulation reflects COVID-19 hypercoagulability and relates to thrombotic events. Willems et al. showed that activation markers of the intrinsic pathway, T:AT and vWF:Ag remain elevated in a subset of patients 3 months after the onset of COVID-1910. Hypercoagulability was also noted in some patients with impaired exercise capacity suffering from post COVID-19 syndrome²³. However, future studies are needed to elucidate to what degree ongoing coagulation and/or endothelial damage contribute to the post COVID-19 syndrome.

Our study has several limitations. First, this cohort was collected at the beginning of the pandemic when thrombotic complications were not routinely screened; therefore, the rate of actual thrombotic complications may be higher than it is currently presented. A second limitation may be the limited sample size of the follow-up samples as compared to the baseline due to the study design with only a limited sample availability. On the other hand, this large and well-defined prospective cohort of patients with COVID-19 with follow-up samples is unique to our analysis.

In conclusion, we showed that thrombin formation, mainly via the intrinsic pathway, is critical for COVID-19's hypercoagulability to occur. Thrombin formation and vascular

damage are important markers of disease severity, thrombosis, and mortality. The intrinsic pathway may therefore be a potential target for the treatment of this devastating disease. Future studies should address whether our findings can be extrapolated to other (viral) respiratory conditions or not.

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SUPPLEMENTALS

		Moderate		Severe	P value
	п	median (IQR)	п	median (IQR)	
FVIIa, mIU/mL					
Baseline	62	31.7 (20.0-47.6)	98	39.8 (20.4-55.3)	0.157
≤7 days	24	37.5 (19.3-48.9	45	32.9 (21.0-48.1)	0.753
8–14 days	8	32.8 (22.1-43.1)	43	33.2 (21.9-45.7)	0.929
FVIIa:AT, pM					
Baseline	31	556 (476-741)	77	607 (458-784)	0.514
≤7 days	11	567 (524-844)	43	642 (540-1110)	0.335
8–14 days	4	704 (512-807)	27	710 (567-711)	0.842
PKa:C1Inh, nM					
Baseline	67	2.5 (0.9-4.8)	104	1.9 (1.0-3.5)	0.750
≤7 days	25	1.9 (1.0-4.7)	51	2.2 (1.0-14.4)	0.367
8–14 days	10	2.5 (1.8-5.7)	35	2.5 (1.1-10.4)	0.925
FXIa:AT, pM					
Baseline	67	25.7 (19.8-38.0)	104	30.0 (22.2-65.2)	0.004
≤7 days	25	21.3 (19.4-31.2)	51	37.8 (24.9-74.0)	0.001
8–14 days	10	20.6 (14.4-31.2)	35	31.7 (22.2-60.9)	0.024
FXIa:α1AT, pM					
Baseline	67	515 (396-751)	104	545 (402-806)	0.418
≤7 days	25	399 (340-569)	51	603 (442-905)	0.003
8–14 days	10	387 (312-661)	35	589 (491-860)	0.014
FIXa:AT, pM					
Baseline	67	64.2 (43.4-91.0)	104	86.8 (62.0-125.6)	<0.001
≤7 days	25	68.2 (49.9-94.3)	51	92.0 (74.8-128.1)	0.007
8–14 days	10	66.2 (51.1-74.6)	35	75.0 (60.3-108.0)	0.073
T:AT, ng/mL					
Baseline	67	5.5 (3.7-10.1)	104	8.7 (4.9-21.2)	< 0.001
≤7 days	25	4.3 (2.8-9.3)	51	7.6 (5.2-21.5)	0.003
8–14 days	10	4.6 (3.8-5.1)	35	6.9 (4.9-12.2)	0.006
vWF:Ag, %					
Baseline	67	361 (263-488)	104	438 (342-557)	0.001
≤7 days	25	374 (295-498)	51	520 (397-633)	0.002
8–14 days	10	324 (245-420)	35	557 (461-594)	< 0.001

Supplementary 3.1 Markers of coagulation and vascular damage in admitted patients with COVID-19 during the course of disease.

Differences between groups were tested with the Mann Whitney U test. *Abbreviations*: FVIIa, activated factor VIIa. AT, antithrombin. PKa, plasma kallikrein. C1INH, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.

Supplementary 3.2 Correlation matrix between CRP, neutrophil and lymphocyte counts, NLR as well as soluble C5a and coagulation factors as well as vWF:Ag at baseline in patients with COVID-19.

	CRP	(mg/L)	Neutroph	nils ($x10^{9}/L$)	Lymphocy	vtes (x10 ⁹ /L)	N	LR	C5a (ng/mL)
	r	P value	r	P value	r	P value	r	P value	r	P value
FVIIa	0.07	0.329	0.05	0.510	0.06	0.459	-0.05	0.507	-0.14	0.052
FVIIa:AT	0.06	0.515	-0.05	0.605	-0.09	0.333	-0.01	0.944	-0.12	0.153
PKa:C1Inh	0.00	0.949	-0.04	0.562	-0.04	0.579	-0.01	0.922	-0.03	0.725
FXI:AT	0.22	0.002	0.11	0.135	-0.16	0.028	0.19	0.009	0.08	0.285
FXIa:α1AT	0.38	<0.001	0.14	0.055	-0.16	0.032	0.22	0.003	0.24	< 0.001
FIX:AT	0.41	< 0.001	0.21	0.005	-0.11	0.114	0.19	0.010	0.13	0.059
T:AT	0.35	<0.001	0.23	0.002	-0.11	0.141	0.20	0.006	0.18	0.011
vWF:Ag	0.38	< 0.001	0.31	< 0.001	-0.15	0.040	0.27	< 0.001	0.16	0.021

Spearman rank correlation coefficients were used. *Abbreviations*: CRP, C-reactive protein. NLR, neutrophil-lymphocyte ratio. FVIIa, activated factor VIIa. AT, antithrombin. PKa, plasma kallikrein. C1INH, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.

Supplementary 3.3 Mean predicted T:AT and vWF:Ag levels at baseline and over time for ICU admission, thrombotic events, and 28 days in-hospital mortality with linear mixed models in patients with COVID-19, respectively.

	T:AT		vWF:Ag	
ICU admission	Estimate (95% CI)	Р	Estimate (95% CI)	Р
ICU admitted	+9.0(2.7-15.4)	0.006	+9(-55-73)	0.782
Over time	- 0.4 (-3.1-2.3)	0.786	- 3 (-27-22)	0.826
ICU admitted*Over time	- 1.7 (-5.4-2.0)	0.370	+61(28-94)	< 0.001
Thrombotic events	Estimate (95% CI)	Р	Estimate (95% CI)	Р
Thrombosis	+9.6(1.4-17.9)	0.023	+7(-80-93)	0.879
Over time	+0.3(-1.8-2.3)	0.817	+28(9-48)	0.005
Thrombosis*Over time	- 3.0 (-7.5-1.5)	0.187	+ 34 (-7-74)	0.103
28 Days mortality	Estimate (95% CI)	Р	Estimate (95% CI)	Р
Deceased	+6.8(-0.4-14.0)	0.062	+45(-28-117)	0.225
Over time	+0.3(-1.8-2.3)	0.808	+28(8-47)	0.006
Deceased*Over time	- 1.1 (-5.6-3.3)	0.614	+49(7-90)	0.023

Abbreviations: T:AT, thrombin in complex with antithrombin. vWF:Ag, von Willebrand factor antigen.



Chapter 4

Anti-C5a antibody IFX-1 (Vilobelimab) treatment versus best supportive care for patients with severe COVID-19 (Panamo): an exploratory, open-label, phase 2 randomised controlled trial

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Lancet Rheumatol 2020;2:e764-e773

ABSTRACT

Severe coronavirus disease 2019 (COVID-19) is characterized by inflammation and coagulation in the presence of complement activation. We conducted an explorative phase 2 randomized, open label trial of intravenous IFX-1, a monoclonal antibody selectively blocking the anaphylatoxin C5a, in adults with severe COVID-19. Patients were randomly assigned to receive IFX-1 plus best supportive care or best supportive care only. The primary objective was to explore the impact of C5a inhibition in patients with severe COVID-19. 30 patients underwent randomization: 15 assigned to IFX-1 and 15 patients to best supportive care. The PaO2/FiO2 ratio improvement on day 5, chosen as primary outcome parameter, did not show significant differences between groups. However, IFX-1 treatment was associated with consistent trends of disease improvement as evidenced by prevention of decrease in estimated glomerular filtration rates, normalization of lymphocyte counts, and lowering of plasma lactate dehydrogenase concentrations. Kaplan-Meier estimates of mortality by 28 days were 13 percent for IFX-1 and 27 percent for controls (hazard ratio for death, 0.58; 95% confidence interval, 0.09 to 3.65). Serious adverse events rates were comparable between groups but the rate of severe grade pulmonary embolism was three-fold lower in the IFX-1 treatment group (13%) as compared to the best supportive care group (40%). In this exploratory part of the PANAMO study, C5a inhibition with IFX-1 was shown to be safe in severe COVID-19. PaO2/FiO2 ratio at day five was comparable between groups, but a consistent beneficial signal in IFX-1 treatment-group including reduction in renal impairment, normalization of lymphocytopenia, decrease of LDH, and a survival benefit, warrant investigating of this drug within a phase 3 trial.

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes severe respiratory illness and is characterized by viral lung inflammation with lymphocyte infiltration and activation of the coagulation system^{1,2}. Many patients with coronavirus disease 2019 (COVID-19) require intensive care. However, despite optimal care case fatality rates are high due to multi organ failure³, which has been explained by secondary damage due to hyperinflammation⁴.

Autopsies of patients with severe COVID-19 showed widespread complement activation in lung and kidney^{5,6}. Experimental studies showed binding of the SARS-CoV-2 virus Nprotein by complement factor MASP2, activating the final common complement pathway and liberation of complement 5a (C5a)⁶. High levels of C5a and C5b-9 have been reported in patients with severe COVID-19⁷. The anaphylatoxin C5a attracts neutrophils and monocytes to the infection site and has various crosslinks with the coagulation system⁸, inducing release of tissue factor from endothelial cells and activated neutrophils⁹⁻¹¹, and leading to acute respiratory distress syndrome and thrombotic microangiopathy.

IFX-1 is a chimeric immunoglobulin G4 monoclonal anti-human C5a antibody that specifically binds to soluble C5a. IFX-1 showed some beneficial in a monkey model of avian flu virus (H7/N9) induced lung injury¹². It markedly reduced the lung histopathological injury and decreased lung infiltration by macrophages and neutrophils. Furthermore, treatment decreased cytokine levels and virus titers in the infected lungs. Treatment of two Chinese patients with severe COVID-19 with anti-C5a antibody BDB-1 (a drug similar to IFX-1 and produced from the IFX-1 cell line) was reported to result in clinical improvement⁶.

At the beginning of the COVID-19 pandemic, uncertainty about disease course and outcomes prompted us to plan an exploratory phase 2 trial, as part of a phase 2/3 trial, primarily to establish safety and explore preliminary efficacy of IFX-1 in severe COVID-19, with an aim to assess the typical clinical course and adverse outcomes of COVID-19. We designed a pragmatic, adaptive, open-label, randomized phase 2/3 multicenter study of IFX-1 in adults with severe COVID-19 (PANAMO). The phase 2 part of the trial was planned to inform the choice of endpoints and study population specifications for a potential phase 3 study. Here, we describe the preliminary results of the phase 2 part of the trial aiming to explore the potential benefit and safety of IFX-1 in patients with severe COVID-19.

METHODS

Design

PANAMO is a pragmatic adaptive, open-label, randomized phase 2/3 multicenter trial assessing IFX-1 in patients with severe COVID-19. The exploratory phase 2 part of this trial was done at three academic hospitals in the Netherlands (Amsterdam UMC location AMC [Amsterdam]; Amsterdam UMC location VUmc [Amsterdam]; and Maastricht UMC [Maastricht]). The study protocol was approved by the institutional review board of the Academic Medical Center, part of Amsterdam UMC (Amsterdam, Netherlands; IRB 2020_067#B2020179).

Patients

Patient eligibility criteria for the study were as follows: age 18 years or older; severe pneumonia with pulmonary infiltrates consistent with pneumonia, a clinical history of severe shortness of breath within the past 14 days, or a need for non-invasive or invasive ventilation; severe disease defined as a ratio of partial pressure of arterial oxygen to fractional concentration of oxygen in inspired air (PaO2/FiO2) between 100 mm Hg and 250 mm Hg in the supine position; and SARS-CoV-2 infection confirmed by RT-PCR.

Exclusion criteria were as follows: invasive mechanical ventilation for more than 48 h; improvement in PaO2/FiO2 of more than 30% in the past 24 h; known history of progressed chronic obstructive pulmonary disease (COPD; Global Initiative for Chronic Obstructive Lung Disease [GOLD] group C or D); severe congestive heart failure (New York Heart Association class III or IV); known pregnancy; chronic dialysis, cancer, or other life-limiting disease with life expectancy less than 6 months; renal replacement therapy; cardiac resuscitation in the past 14 days; organ or bone marrow transplantation in the past 3 months; anticancer therapy for oncological disease in the past 4 weeks; corticosteroid treatment equivalent to 10 mg prednisone or more per day; treatment with other biological therapy for COVID-19 in the past 14 days; or use of viral replication inhibitor in the past 3 days. Patients who were near death or expected to die within 12 h or with hypersensitivity to IFX-1 were also excluded.

All patients or their legally authorized representatives gave written informed consent for the study. If direct informed consent of patients was not feasible, patients could be included with a deferred consent procedure.

Randomization

Patients were randomly assigned in a 1:1 ratio to IFX-1 plus best supportive care (the IFX-1 group) or to best supportive care only (the control group). Randomization was done by investigators centrally with an online tool within the electronic case report form and was

stratified by study site. The tool used a randomized variable block length of either 2 or 4. The randomization list was only available to contract research organization (Metronomia) staff involved in the production of the randomization list and set-up of the online randomization tool. Treatment allocation was open label.

Procedures

Patients in the IFX-1 group received a maximum of seven doses of IFX-1 800 mg intravenously plus best supportive care, and those in the control group received best supportive care only. Five doses of IFX-1 (days 1, 2, 4, 8, and 15) were administered to all patients assigned to the IFX-1 group who were admitted to hospital alive. A dose at day 22 was administered to patients who were still intubated on day 22. One additional dose of IFX-1 could be given between days 11 and 13 at the discretion of the investigator if signs of weakening of any clinical improvement were detected. Treatment with IFX-1 was discontinued if patients were discharged from hospital. IFX-1 (vilobelimab) was provided by *InflaRx*.

Best supportive care in the participating centres consisted of intensive care therapy according to current guidelines, evidence, and best practice, including but not limited to lung protective ventilation, thrombosis prophylaxis, renal replacement therapy when indicated, and access to advanced therapies including extracorporeal membrane oxygenation. Hydroxychloroquine was allowed during the study; however, active concomitant treatment with antiviral or other immunomodulatory drugs was not allowed. Best supportive care varied in some aspects per site regarding admission criteria for the intensive care unit (ICU) — i.e., one site only admitted patients with COVID-19 when they needed intubation whereas the other two sites also admitted patients when they needed oxygen supply with a non-rebreathing mask. Safety was assessed throughout the study.

Data was collected from the hospital patient files. Estimated glomerular filtration rate (eGFR) was calculated based on the Chronic Kidney Disease Epidemiology Collaboration formula, which adjusts for race. Kidney Disease: Improving Global Outcomes cutoffs were applied. Multiorgan failure was defined as 2 or more failing organs.

Endpoints

The primary outcome was the percentage change in PaO2/FiO2 in the supine position from baseline (day 1, before study drug administration and within 1 h before or after randomization) to day 5. Secondary endpoints were number of patients with an early response (defined as patient alive and extubated or oxygenation index of \geq 300 or improvement of \geq 30% from baseline, temperature <38°C in the absence of fever-decreasing medication for \geq 4 h, and white blood cell count within normal limit of local laboratory quantifications); number of patients with a late response (defined as discharge
from hospital up to day 28 or alive and extubated, discharged from ICU, free of shortness of breath [respiratory rate <20] in absence of oxygen supply, and free of fever [<37.6°C]); percentage change in PaO2/FiO2 in the supine position from baseline to days 3, 7, 9, and 11; 28-day mortality; and treatment-emergent and serious adverse events.

All adverse events, serious and non-serious, were reported. Immediately reportable serious adverse events included adverse events that resulted in death and new life-threatening events.

Various other outcomes (change from baseline in alanine aminotransferase, troponin I adjusted to glomerular filtration rate, creatinine, lymphocyte counts, neutrophil counts, D-dimers, Glasgow outcome scale, time to reach ICU discharge criteria, and assessment of complement activation parameters and plasma concentrations of IFX-1) were predefined in line with the exploratory nature of the initial phase of the clinical study. These outcomes and pharmacokinetic and pharmacodynamic analyses will be reported elsewhere. Subgroup and sensitivity analyses were done for patients intubated at randomization or within 6 h after randomization.

Statistical analysis

For the phase 2 part of the trial, 30 patients was deemed sufficient to learn enough about the uncertainties around the design parameters relevant for the phase 3 part. This initial part of the PANAMO trial was not powered to show statistically significant differences in clinical endpoints.

Oxygenation (PaO2/FiO2) and efficacy-related laboratory parameters (lactate dehydrogenase, lymphocytes, eGFR, and D-dimers) were analyzed at predefined timepoints (days 3, 5, 7, 9, 11, 13, 15, 22, and 29 for PaO2/FiO2 and before IFX-1 infusion and day 29 for other parameters). If a measurement was not available at the exact protocolized timepoint after randomization, values were derived on the basis of linear interpolation between the last available measurement before and the first available measurement after that time. If a patient died, the PaO2/FiO2 was set to 0 mm Hg at the time of death. For patients who recovered and were discharged from the ICU before day 15, the last measured value was carried forward for analysis. The analysis of relative change of oxygenation and laboratory values was based on a linear repeated measures model with the following explanatory variables: baseline value and age and factors for the treatment group; sex; time; interaction between baseline value and time; and interaction between treatment group and time. The model was specified with an unstructured covariance matrix. For the analysis of relative change of oxygenation, intubation status at baseline was also added as an explanatory variable to the model. Based on the model, least squares means and their 95% CIs and p values were derived for the comparison between the two treatment groups at each timepoint.

All-cause mortality was analyzed as a censored time-to-event variable with Kaplan-Meier methods. The proportion of patients still alive at 28 days was derived from the product limits estimator in each treatment group. Adjustment for relevant baseline covariates (age, sex, and PaO2/FiO2) was done using a Cox proportional hazards model. The primary endpoint was assessed in the intention-to-treat population. Safety was assessed in all patients according to treatment received.

All analyses were performed in SAS 9.4 and figures were generated using R, version 4.0.0. An external data safety monitoring committee oversaw the trial and assessed the safety within prespecified interim analyses. Safety was assessed by an independent safety monitoring board. An expert committee consisting of trial investigators, non-trial related experts, and company representatives reviewed data on a weekly basis and was installed to provide recommendations with regards to stopping or moving into a potential phase 3 part of the study based on signals detected and adaption of the choice of endpoints and potential changes in the study population for the phase 3 part. Company representatives acted as non-voting members. This trial was registered at ClinicalTrials.gov (NCT04333420).

RESULTS

Between March 31 and April 24, 2020, we screened 172 patients, of whom 142 were not eligible or declined participation. We enrolled 30 patients and randomly assigned 15 to the IFX-1 group and 15 to the control group (**Figure 4.1**). One patient in the IFX-1 group had a history of COPD Global Initiative for Chronic Obstructive Lung Disease group C (an exclusion criterion) that was unknown at the time of randomization. All 30 patients were included in the intention-to-treat analysis. Of those assigned to the IFX-1 group, all patients received the treatment as assigned. None of the IFX-1 group patients discontinued treatment because of an adverse event or a serious adverse event other than death. As of May 22, all 30 patients had completed the trial up to day 28, recovered, or died. As of July 2, all recovered patients had at least one telephone follow-up and were alive.

Baseline characteristics of the patients

The median age was 60 years (SD, 9) and 22 (73%) were male (**Table 4.1**). Most patients had either one (43%) or two or more (20%) of the prespecified co-existing risk-associated conditions at enrollment, most commonly hypertension (33%) and diabetes mellitus (27%). All patients had symptoms and signs consistent with COVID-19 pneumonia, most commonly dyspnea (90%), cough (67%), and fever (30%). The median number of days between symptom onset and randomization was 11 days (interquartile range, 8 to

13 days). At randomization, 18 patients were intubated (60%), and eight had an oxygen mask, while four patients had lower levels of oxygen delivery through nasal cannulas. Patients were either randomized at the intensive care unit (18 patients), intermediate care unit (7 patients), or ward (5 patients). Within six hours after randomization 20 of 30 patients were intubated (67%). Baseline characteristics were well balanced between treatment groups, although the IFX-1 group tended to have more patients with two or more risk-associated co-morbidities (5 of 15 [33%] in the IFX-1 group versus 1 of 15 in the best supportive care group [7%]).





Efficacy

During the study it became clear that several patients in the prone position could not be assessed regularly in the supine position because of severe hypoxemia. It was therefore decided to focus on all PaO2/FiO2 assessments (irrespective of position) and perform an analysis of supine position values for sensitivity. At day 5 after randomization, mean PaO2/FiO2 (irrespective of position) was 158 mmHg (SD 63; range 84–265) in the IFX-1

group and 189 mmHg (SD 89; range 71–329) in the control group. Linear repeated measures modelling for relative change in PaO2/FiO2 with adjustment for the covariates baseline PaO2/FiO2, timepoint, sex, and age showed no differences between treatment groups. Analyses of the least squares mean relative change in PaO2/FiO2 at day 5 (the primary outcome) showed no differences between treatment groups (17% change in the IFX-1 group vs 41% in the control group; difference –24% [95% CI –58-9], p=0·15; **Figure 4.2**). Sensitivity analysis for PaO2/FiO2 measured in the supine position according to the protocol showed that at day 5, mean values were 148 mmHg (range 0–263) in the IFX-1 group vs 32% in the control group; difference –16% [95% CI –53-20]). Subgroup analyses of patients intubated at baseline or within 6 h after randomization showed similar results.

	IFX1	Control
Age, yr.	58 (±9)	63 (±8)
M/F	11/4	11/4
Days from symptom onset to randomization	11 (4-18)	12 (5-33)
Days from COVID-19 diagnosis to randomization	2 (0-6)	2 (0-6)
No. of relevant coexisting conditions		
None, n (%)	5 (33)	6 (40)
One, n (%)	5 (33)	8 (53)
Two or more, n (%)	5 (33)	1(7)
Selected coexisting conditions		
Hypertension, n (%)	7 (43)	6 (40)
Diabetes, n (%)	8 (57)	6 (40)
Obesity, n (%)	0	2 (13)
Oxygen support		
Intubated at randomization, n (%)	8 (53)	10 (67)
Oxygen mask, n (%)	6 (40)	2 (13)
Nasal cannula, n (%)	1 (7)	3 (20)
Admission department at randomization		
Intensive care unit, n (%)	8 (53)	10 (67)
Intermediate care unit, n (%)	5 (33)	2 (13)
COVID ward, n (%)	2 (13)	3 (20)

Table 4.1 Baseline demographic and clinical characteristics.

Continuous variables were presented as mean (±standard deviation) or median (interquartile range) as appropriate.

eGFR tended to be higher and unchanged in mean for IFX1-treated patients while a trend to worsening could be detected in the best supportive care group (**Figure 4.2**). At day 15, mean eGFR showed a 4% change to baseline in the IFX-1 group versus -12% in the best supportive care group; difference -16%; 95% CI, -2-46; P=0.07). Shift plots demonstrate that eGFR in IFX-1 treated patients remained largely normal or were mildly decreased only with 1 patient developed a kidney injury (7%), while four patients in the best supportive care group (27%) developed moderately to severely decreased values. Two patients received renal replacement therapy. One IFX-1 treated patient developed

vancomycin-induced renal toxicity that quickly recovered after discontinuation of vancomycin and renal replacement therapy. One patient in the best supportive care group developed multi-organ failure prompting renal replacement therapy.



Figure 4.2 Shift plots for eGFR and lymphocyte concentrations and least squares mean plots for relative changes in selected outcome parameters. Relative change in mean PaO2/FiO2 (A) and eGFR (B). eGFR in the IFX-1 group (C) and control group (D); lymphocyte counts in the IFX-1 group (E) and control group (F). Relative change in mean lactate dehydrogenase (G) and D-dimers (H). Error bars show 95% CI. Units for eGFR are mL/min per 1.73 m2. *Abbreviations*: eGFR, estimated glomerular filtration rate. PaO2/FiO2, ratio of partial pressure of arterial oxygen to fractional concentration of oxygen in inspired air.

Lymphocytopenia was present in almost all patients at inclusion (27 of 30 [90%]; **Figure 4.2**). At day 15, lymphocytes counts were normalized in 13 of 15 patients in IFX-1 group (87%) and 8 of 15 in the best supportive care group (53%; Fisher exact, P=0.11). LDH concentrations were elevated at baseline in both treatment groups (median of 443 U/L in the IFX-1 group versus 450 U/L in controls). There was trend for a faster reduction of lactate dehydrogenase in the IFX-1 group when compared to the best supportive care group (**Figure 4.2**); linear repeated measures model for relative change between treatment groups, P=0.08). D-dimer concentrations in IFX-1 patients showed a significant relative increase as compared to the best supportive care group, but only on day two and four (**Figure 4.2**).

Kaplan-Meier estimates of mortality by 28 days were 13% (95% CI, 0-31) for IFX-1 and 27% (95% CI 4-49) for controls (**Figure 4.3**; hazard ratio for death, 0.65; 95% CI, 0.10-4.14). For those intubated within 6 h after randomization, estimates of mortality by 28 days were 20% (95% CI, 0-45) for IFX-1 and 40% (95% CI, 10-70) for the best supportive care group (hazard ratio for death, 0.48 [95% CI, 0.07-3.35]).



Figure 4.3 Kaplan-Meier Curves for 28-day All-cause Mortality. (A) Kaplan-Meier estimates of mortality by 28 days were 13% (95% CI, 0- 31) for IFX-1 and 27% (95% CI, 4- 49) for controls (adjusted hazard ratio for death, 0.65; 95% CI, 0.10-4.14). (B) For those intubated within six hours after randomization, estimates of mortality by 28 days were 20% (95% CI, 0- 45) for IFX-1 and 40% (95% CI, 10-70) for the best supportive care group (hazard ratio for death, 0.48; 95% CI, 0.07 to 3.35). *Abbreviation*: BSC, best supportive care.

Adverse events

An independent safety monitoring board met three times during and after enrolment of the first 30 patients and recommended continuation of the study. Numbers of serious adverse events were similar between groups and reported for nine (60%) patients in the IFX-1 group versus seven (47%) patients in the control group (**Table 4.2**). No deaths were considered related to treatment assignment, as judged by the site investigators. Pulmonary embolism reported as serious adverse events occurred in two (13%) patients in the IFX-1 group and six (40%) patients in the control group. Infections (including positive Staphylococcus test) classified as serious adverse events were reported in three (20%) patients in the IFX-1 group versus five (33%) patients in the control group.

The most commonly reported adverse events were as follows: pulmonary embolism (six [40%] in the IFX-1 group vs seven [47%] in the control group), impaired gastric emptying (four [27%] vs. seven [47%]), hypokalemia (five [33%] vs four [27%]), delirium (five [33%] vs three [20%]), respiratory failure (three [20%] vs three [20%]), deep vein thrombosis (three [20%] vs. three [20%]), decubitus ulcer (two [13%] vs. five [33%]), hypernatremia (three [20%] vs. five [33%]), hypophosphatemia (two [13%] vs. five [33%]), hyperglycemia (three [20%] vs. three [20%]), and acute kidney injury (two [13%] vs. four [27%]).

	IFX1 (n=15)		Control (n=15)	
	Patients	Events	Patients	Events
Total	9 (60)	23	7 (47)	19
Respiratory manifestations	5 (33)	7	6 (40)	8
Pulmonary embolism, n (%)	2 (13)	2	6 (40)	6
Respiratory failure, n (%)	2 (13)	2	1 (7)	2
Hypoxia, n (%)	2 (13)	2	0	0
Dyspnea, n (%)	1 (7)	1	0	0
Infections	3 (20)	6	4 (27)	4
Pneumonia, n (%)	1 (7)	1	3 (20)	3
Device-related sepsis, n (%)	1 (7)	1	0	0
Pseudomonas infection, n (%)	1 (7)	1	0	0
Sepsis, n (%)	0	0	1 (7)	1
Staphylococcal infection, n (%)	1 (7)	1	0	0
Urinary tract infection, n (%)	1 (7)	1	0	0
Vascular device infection, n (%)	1 (7)	1	0	0
Multiple-organ failure, n (%)	0	0	4 (27)	4

 Table 4.2 Serious adverse events.

Data are n (%) or n.

Six (20%) of 30 patients died by day 28. Of patients in the IFX-1 group, one died after a tube failure (leakage) with resulting severe hypoxia, and one patient with a history of severe COPD died of persistent hypoxic failure resulting in withdrawal of care. In the

control group, all four patients died of COVID-19-induced multiorgan failure and three of them had pulmonary embolisms reported as serious adverse events.

DISCUSSION

Results of our explorative trial show that C5a inhibition with IFX-1 was safe in adults with severe COVID-19. Changes in the oxygenation were comparable between groups, but we observed a consistent beneficial effect of IFX-1 on reversal of blood lymphocytopenia and reduction in LDH concentrations, that been reported as biomarkers for COVID-19 disease severity¹³. There was a reduction in COVID-19 induced renal failure, a three-fold decrease in reported severe grade pulmonary embolisms, and a lower death rate. This initial part of the PANAMO trial was exploratory in nature and not powered to show statistically significant differences in clinical endpoints. Nevertheless, results of our study in combination with previous studies on complement in COVID-19, suggest that inhibition of the complement split product C5a may be a promising treatment approach in patients with severe COVID-19.

There was no difference in benefit of IFX-1 on relative change in oxygenation when compared to the standard of care group. This finding seems to be counterintuitive regarding the higher rate of severe grade pulmonary embolism reported in the standard of care patients. There is increasing evidence that worsening of the disease may be related to a primary damaging mechanism of SARS-Cov-2 to the endothelial cells that is pronounced in the lung but can affect all other organs similar to vasculitis. Oxygenation may primarily be impacted by a decreased perfusion through thromboembolic events in the lung vasculature, which might be pronounced in those patients with a strong worsening towards an acute respiratory distress syndrome. This is supported by the finding that patients may demonstrate a strongly compromised oxygenation with a relatively normal lung function especially during the initial phase of disease worsening¹⁴.

Our results point towards a reduction in coagulation activation through inhibition of C5a in COVID-19 as one potential key mechanism of IFX1. Patients with severe COVID-19 are at risk for developing thrombotic complications, with reported rates up to 43 percent¹⁵. Autopsy studies in COVID-19 describe widespread micro-thrombi in several organs including the lungs and kidney in combination with high levels of complement deposition⁸. Coagulation activation in COVID-19 may be initiated by direct virus induced endothelial injury resulting in up-regulation of tissue factor or cytokine and chemokine secretion which results in suppressed fibrinolysis and production of procoagulant proteins¹⁶. C5a activation may also directly induce endothelial tissue factor up-regulation¹⁷, neutrophil mediated coagulation activation and switch inflammatory cells from pro-fibrinolytic (t-PA release) to a pro-thrombotic phenotype (PAI-1 release)¹⁸. Fibrinolysis in the IFX-1 arm was increased with a peak between days two and four after

initiation of treatment, marked by a temporarily significant increase in D-dimer. The timing pattern of this peak was consistent in all patients who showed an increase upon treatment start with IFX-1. We therefore hypothesize that inhibition of C5a by IFX-1 may lead to a decrease of this mechanism and directly or indirectly foster thrombolysis.

Our study was an exploratory randomized open-label study with several limitations. First, the open-label design might have resulted in bias in outcome and safety assessments. Second, the PaO2/FiO2 showed overall very large variability and dependency on patient positioning and intubation status. Signals might not be detected with such large variation and low patient numbers. Third, the study design allowed enrolment of critically ill intubated patients, but also non-intubated patients on the basis of a predefined low PaO2/FiO2. This ratio has several discussed limitations and might result in enrolment of less critically ill patients when not being intubated, thus increasing patient heterogeneity, leading to larger variability in some endpoints. Fourth, although this was a multicenter randomized study, most intubated patients were included at one site. Finally, only 17% of the screened patients were considered eligible to participate. Although the number appears low, this is common for ICU trials and was mainly due to referral of intubated patients with COVID-19 from other centers after 48 h, participation in other trials, or not fulfilling the PaO2/FiO2criteria.

The safety and tolerability analysis of this phase 2 part of the study did not result in any signals of concern. We believe that the totality of observed safety and preliminary efficacy signals support continuation to the phase 3 part. Ultimately, the phase 2 part of the PANAMO trial was exploratory in nature and efficacy of IFX-1 in patients with COVID-19 must be confirmed in an adequately and separately powered controlled phase 3 part of the PANAMO study.

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Chapter 5

Annexin A1 is associated with adverse clinical outcomes in patients with COVID-19

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J Clin Med 2022;142:1787–1790

ABSTRACT

Severe coronavirus disease 2019 (COVID-19) is characterized by hyperinflammation. vascular damage, and hypercoagulability. Insufficient responses of Annexin A1 (AnxA1), a pro-resolving inhibitor of neutrophil infiltration and activation, might contribute to a severe course of disease. We longitudinally evaluated AnxA1's role in terms of inflammation, vascular damage, and clinical outcomes in a large prospective cohort of patients with COVID-19. AnxA1 was measured at presentation and during follow-up in the sera of 220 consecutive patients who presented at our hospital during the first wave. AnxA1 was significantly higher in the moderate and severe cases of COVID-19 compared to the healthy controls. Elevated AnxA1 was associated with markers of inflammation and endothelial damage. AnxA1 was significantly higher in patients with thrombotic events and ICU admission. Multivariable logistic regression indicated baseline AnxA1 (per ten units) as a predictor of thrombotic events. Linear mixed models predicted that AnxA1 tended to increase more steeply over time in patients without adverse events, with a statistically significant rise in patients without thrombotic events. These findings might reflect an insufficient increase in AnxA1 as a response to the excessive hyperinflammation in COVID-19. Future studies should evaluate whether hyperinflammation could be reduced by the administration of human recombinant AnxA1 or Ac2-26 peptide.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), leading to a wide spectrum of clinical manifestations. Most patients remain asymptomatic, although life-threatening acute respiratory distress syndrome may occur against a background of hyperinflammation, vascular damage, and coagulopathy¹. The innate immune system and, more specifically, neutrophils and neutrophil extracellular trap (NET) formation, are key factors in the development of severe COVID-19².

Annexin A1 (AnxA1) is a member of the annexin superfamily and exerts its antiinflammatory effects among a variety of cellular functions in humans through signaling via formyl peptide receptor 2 (FPR2)³. The active form of full-length AnxA1 (37-kDa) enhances neutrophil apoptosis and inhibits neutrophil infiltration and activation⁴⁻⁷. One may assume that a deregulated homeostasis of AnxA1 can therefore play a role in the pathogenesis of severe COVID-19. Dexamethasone, an exogenous glucocorticoid upstream of AnxA1⁸, has in fact been shown to alter type I interferon active neutrophils in COVID-19 and improve outcomes for patients with severe COVID-19^{9,10}. Preliminary observations by *Canacik* et al. showed low levels of AnxA1 in patients with severe COVID-19 compared to those with moderate disease¹¹. However, the sample size of the included patients was rather small. Moreover, longitudinal data on the changes in AnxA1 levels during the course of COVID-19 are lacking.

We studied the serum levels of AnxA1 at presentation and over time in a large and welldefined cohort of patients with COVID-19 to delineate AnxA1's role in terms of inflammation, vascular damage, and clinical outcomes.

METHODS

Patient population and sampling

Consecutive patients with COVID-19 who presented during the first wave (21st March to 28th April 2020) at the emergency department of the Maastricht University Medical Center (MUMC), Maastricht, The Netherlands, were included as previously reported¹. Presenting patients without typical findings of COVID-19 on computed tomography, such as diffuse ground glass opacities and/or bilateral consolidations, and not confirmed by reverse transcriptase polymerase chain reaction of nasopharyngeal swab and/or sputum (i.e., SARS-CoV-2 RNA with a cycle threshold value <40) were excluded. Patients were categorized into mild (not admitted to the hospital), moderate (admitted; requiring up to 5 L/min oxygen support), and severe (admitted; requiring more than 5 L/min oxygen support, or requiring invasive ventilation, and/or COVID-19 related fatal

Chapter 5

courses). Blood samples were obtained at the time of presentation at the emergency department or intensive care unit (ICU) and whenever available during follow-up (i.e., every 5 [±2 days]). After 30 minutes of clotting, serum tubes were centrifuged at 1885 g for 10 minutes at room temperature (RT). Citrated blood was immediately centrifuged at 2000 g for 10 minutes at RT. Subsequently, all samples were aliquoted and stored at -80 degrees Celsius. The study has been approved by the appropriate local ethics committee (medical ethical committee aZM/UM, Maastricht, The Netherlands. Reference number 2020-1315), with a waiver of informed consent. All methods were carried out in accordance with relevant guidelines and regulation.

Data collection

Clinical characteristics and findings, as well as outcomes (i.e., disease severity, lengths of hospitalization and ventilation, thrombotic events, ICU admission, 28 days in-hospital mortality), were retrieved from electronic patient files.

Annexin A1

AnxA1 was quantified in the serum by an enzyme-linked immunosorbent assay (ELISA; Scientific Hemostasis, Franconville, France). Briefly, rabbit anti-human recombinant AnxA1 pAbs (5 μ g/mL diluted in a 0.05 M carbonate buffer at pH 9.6) were coated on microtiter plates overnight at RT and washed. Serum samples were diluted 1:5 in 0.05 M PBS 0.15 M NaCl, 1% BSA, and 0.1% Tween 20 (PBST), incubated for 1 h at RT, and washed. Next, peroxidase labeled anti-AnxA1 pAbs (40 μ g/mL diluted in PBST) were incubated for 1 h at RT, after which TMB/H2O2 substrate (Neogen, Lansin, MI) was added for 15 min at RT. The reaction was stopped with 0.5 M sulfuric acid and AnxA1 was quantified in duplicate at 450 nm.

Soluble complement 5a

Desarginated complement 5a (C5a) was quantified in plasma by ELISA (Quidel, San Diego, CA), according to the manufacturer's instructions.

Von Willebrand factor antigen

Von Willebrand factor antigen (vWF:Ag) was quantified in plasma as previously described¹.

Statistical analysis

Depending on normality and equal variances, continuous variables are indicated as median (interquartile range [IQR]) or mean (±standard deviation [SD]). Differences were

assessed by Mann Whitney U tests, unpaired sample t tests, Kruskal Wallis tests, or oneway ANOVA, as appropriate. Fisher's exact test was used for categorical variables. Correlations between AnxA1 and inflammatory and endothelial markers were calculated using the Spearman's rank correlation coefficient. To investigate associations between baseline AnxA1 (per ten units) and clinical outcomes (i.e., thrombotic events, ICU admission and 28 days in-hospital mortality), univariable and multivariable logistic regressions were performed. Depending on the clinical outcome, the multivariable analysis was adjusted for other factors influencing the clinical outcome. Results were presented as odds ratio (OR) with 95% confidence interval (CI) and area under the receiver operating characteristic curve (AUC). Linear mixed-effects models were run to investigate the effects of longitudinal AnxA1 on clinical outcomes. Statistics were performed with IBM SPSS Statistics (version 28) and GraphPad Prism (version 9). Statistical significance was assumed for a *P*-value <0.05.

RESULTS

Patient population

Overall, AnxA1 was assessed in 220 out of 228 (96%) patients with COVID-19; 8 patients were excluded because of insufficient sampling. The baseline characteristics of the included patients stratified by disease severity are depicted in **Table 5.1**.

The median duration from the onset of symptoms to presentation was 7 (IQR, 5-12) days; 48 (22%), 68 (31%), and 104 (47%) patients presented with mild, moderate, and severe COVID-19, respectively. The comorbidities did not differ between the groups. In accordance with the national recommendations during the first wave, 135 (61%) were treated with hydroxychloroquine¹². Patients were not routinely treated with immunosuppressive drugs, such as, dexamethasone and/or interleukin 6 inhibition; 7 (3%) patients were treated with low-dose prednisolone, that is, <20 mg/d, because of comorbidities.

Annexin A1

The AnxA1 levels are depicted in **Figure 5.1**. The mean and median AnxA1 levels were 16,8 (SD, ±8,5) ng/mL and 14.9 (IQR, 10.4-22.4) ng/mL, respectively, in 58 samples from healthy donors from the Central Diagnostic Laboratory, MUMC (donated before 2019). The AnxA1 reference value of \leq 33,8 ng/mL was based on the mean (+2 x SD). Elevated levels of AnxA1 were found in 85 (39%) out of 220 patients at baseline; there were 11 (23%), 28 (41%), and 46 (44%) patients with mild, moderate, and severe disease, respectively. At presentation, AnxA1 was significantly higher in the moderate (30.1 [IQR,

5

16.0-42.0] ng/mL; *P*<0.001) and severe cases of COVID-19 (28.9 [IQR, 17.3-53.6] ng/mL; *P*<0.001) compared to the healthy controls (14.9 [IQR, 10.4-22.4] ng/mL). AnxA1 in the mild cases was also lower compared to the severe cases (20.4 [IQR, 11.8-32.2] ng/mL; *P*=0.045). Notably, AnxA1 tended to increase over time in patients with COVID-19 admitted to the hospital (**Figure 5.1b** and **Supplementary 5.1**).

	Normal	Mild	Moderate	Severe	Overall P
	Range	(<i>n</i> =48)	(<i>n</i> =68)	(<i>n</i> =104)	
M/F		25/23	43/25	76/28+	0.037
Age, yr.		62 (±16)	70 (±12)‡	69 (±12)†	0.002
Days from illness onset		7 (5-11)	7 (5-14)	7 (5-14)	0.963
SBP, mmHg		129 (±16)	137 (±22)	138 (±25)	0.068
DBP, mmHg		80 (±12)	79 (±12)	80 (±14)	0.885
Heart rate, bpm		90 (75-100)	90 (80-100)	95 (80-110) ^{*, †}	0.043
Body temperature, °C	≤37.9	37.7 (±0.9)	38.1 (±1.0)‡	38.1 (±1.0) ⁺	0.021
Fever, n (%)		14 (30)	39 (58)‡	53 (60)+	0.002
Medical history					
Hypertension, n (%)		15 (31)	27 (40)	34 (33)	0.951
Diabetes, n (%)		11 (23)	11 (16)	25 (24)	0.673
CVA, n (%)		7 (15)	11 (16)	18 (17)	0.736
Cardiac disease, n (%)		13 (27)	24 (35)	31 (30)	0.899
COPD/asthma, n (%)		6 (13)	16 (24)	11 (11)	0.418
None, n (%)		12 (25)	15 (22)	27 (26)	0.804
Laboratory parameters					
Platelets, ×10 ⁹ /L	130-350	195 (164-292)	202 (143-260)	211 (168-246)	0.795
Leukocytes, ×10 ⁹ /L	3.5-11.0	6.0 (5.4-8.4)	6.3 (4.7-8.7)	7.4 (5.8-9.9)*	0.009
Neutrophils, ×10 ⁹ /L	1.4-7.7	4.6 (3.6-6.2)	5.0 (3.4-7.3)	5.9 (4.7-8.1)*	< 0.001
Lymphocytes, ×10 ⁹ /L	1.1-4.0	1.1 (0.7-1.6)	0.8 (0.6-1.2)‡	0.7 (0.5-1.1)*	0.002
NL-ratio		4.6 (2.8-6.6)	6.0 (3.9 – 9.0)‡	8.7 (5.3 – 12.2)*, †	< 0.001
AST, U/L	<35	38 (27-56)	49 (37-64) [‡]	55 (40-80)*	< 0.001
LDH, U/L	<250	256 (205-339)	328 (266-451)‡	451 (358-595) ^{*, †}	< 0.001
Serum creatinine, µmol/L	60-115	83 (62-106)	88 (75-119)	91 (73-120)	0.254
Albumin, g/L	32.0-47.0	34 (31-38)	33 (30-36)	29 (26-32) ^{*, +}	< 0.001
CRP, mg/L	<10	56 (16-95)	66 (39-123)	98 (54-174) ^{*, +}	< 0.001
C5a, ng/mL	≤21.1	15.3 (9.0-25.4)	21.8 (16.2-30.7)‡	21.8 (10.8-30.7)+	0.025
High C5a, n/N		25/41	50/56‡	70/95*	0.005
AnxA1, ng/mL	≤33.8	20.4 (11.8-32.2)	30.1 (16.0-42.0)	28.9 (17.3-53.6)+	0.025
High AnxA1, n (%)		11 (23)	28 (41)‡	46 (44)+	0.023

Table 5.1 Baseline characteristics of 220 patients with COVID-19.

C5a was not measured in all patients because of insufficient sampling. Continuous variables were presented as mean (±standard deviation) or median (interquartile range) as appropriate. Differences between groups were analyzed by unpaired sample t test, Mann Whitney U test or Kruskal Wallis. Differences in categorical variables were analyzed by chi square test or Fisher's exact test when appropriate; significant differences between patients groups: severe versus *moderate or †mild disease; moderate versus ‡mild disease. *Abbreviations*: SBP, systolic blood pressure. DBP, diastolic blood pressure. CVA, cerebrovascular accident. NL-ratio, neutrophil lymphocyte ratio. COPD, chronic obstructive pulmonary disease. AST, aspartate transaminase. LDH, lactate dehydrogenase. CRP, C-reactive protein. C5a, complement 5a. AnxA1, Annexin A1.



Figure 5.1 AnxA1 (Log10 ng/mL) at baseline in healthy controls, mild, moderate and severe patients with COVID-19 (A) and during follow-up in patients with moderate and severe COVID-19 (B). Scatter plots with depicted medians and interquartile ranges for AnxA1. *P*-values are calculated with the Kruskal-Wallis test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. *Abbreviation*: AnxA1, Annexin A1.

Annexin A1, inflammation, and endothelial damage

The characteristics and markers of inflammation, as well as vWF:Ag, a marker of endothelial damage, in the patients with normal and elevated AnxA1 at baseline are presented in **Figure 5.2** and **Supplementary 5.2**. Elevated levels of AnxA1 were associated with higher white blood cell counts (7.8 [IQR, 6.0-10.1] vs. 6.3 [IQR, 4.9-8.5] ×109/L; *P*=0.001), neutrophil counts (6.0 [IQR, 4.4-8.2] vs. 4.9 [IQR, 3.5-6.8] ×109/L; *P*=0.004), CRP (94 [IQR, 57-168] vs. 70 [IQR, 27-116] mg/L; *P*=0.002), C5a (23.3 [IQR, 16.5-32.4] vs. 17.9 [IQR, 10.1-27.0] ng/mL; *P*=0.007), and vWF:Ag (462 [IQR, 346-591] vs. 356 [IQR, 251-457] %; *P*<0.0001) compared to the levels of AnxA1 within the normal range. Lymphocytes and neutrophil-to-lymphocyte ratio (NLR) were not associated with AnxA1.

The Spearman's rank correlation coefficients between AnxA1 and the markers of inflammation and endothelial damage at baseline were assessed (**Supplementary 5.3**). Only weak positive correlations between AnxA1 and leukocyte count (r=0.309; P<0.0001), neutrophil count (r=0.297; P<0.0001), CRP (r=0.227; P=0.001), and vWF:Ag (r=0.315; P<0.0001) were found. Lymphocytes (r=0.160; P=0.026), C5a (r=0.190; P=0.008) and NLR (r=0.082; P=0.457) also correlated poorly with AnxA1.



Figure 5.2 Markers of inflammation and endothelial activation in COVID-19 patients with normal and elevated AnxA1 levels (>33,8 ng/mL) at baseline. Scatter plots with depicted medians and interquartile ranges for normal and high AnxA1. *P*-values are calculated with Mann Whitney U test.

P*<0.05, *P*<0.01, ****P*<0.001, *****P*<0.0001. *Abbreviations*: AnxA1, Annexin A1. NL-ratio, neutrophil lymphocyte ratio. CRP, C-reactive protein. vWF:Ag, von Willebrand factor antigen.

AnxA1 and clinical outcomes

The median number of days from the onset of symptoms to presentation was 9 (IQR, 7-14) days for patients with elevated AnxA1 at baseline and 7 (IQR, 5-10) days for patients with normal AnxA1 (*P*<0.001). The AnxA1 levels appeared to be the highest in patients with thrombotic events and/or those admitted to the ICU (**Figure 5.3**).



Figure 5.3 AnxA1 levels (Log10 ng/mL) in patients with COVID-19 with and without thrombotic events (TE) (A), ICU admitted or not (B), and deceased or not during hospitalization (C). Scatter plots with depicted medians and interquartile ranges for AnxA1. *P*-values are calculated with Mann Whitney U test. **P*<0.05, ***P*<0.01. *Abbreviations*: AnxA1, Annexin A1. TE, thrombotic events. ICU, intensive care unit.

The logistic regression analysis showed that increasing AnxA1 (per ten units) was associated with an increased risk of thrombotic events in patients with COVID-19 (OR 1.064 [95% CI 1.003-1.129]; P=0.040), as did the multivariable analysis (OR 1.067 [95% CI 1.002-1.135]; P=0.042) after adjustment for sex (**Table 5.2** and **Supplementary 5.4**). Increasing levels of AnxA1 (per ten units) did not show an increased risk of ICU admission (OR 1.052 [95% CI 0.997-1.111]; P=0.065) or 28 days in-hospital mortality (OR 1.050 [95% CI 0.982-1.122]; P=0.115).

		1 1	
	OR (95% CI)	P value	AUC (95% CI)
Univariable			
Thrombotic events	1.064 (1.003-1.129)	0.040	0.638 (0.535-0.741)
ICU admission	1.052 (0.997-1.111)	0.065	0.611 (0.531-0.691)
28 days mortality	1.050 (0.982-1.122)	0.115	0.627 (0.514-0.740)
Multivariable*			
Thrombotic events*	1.067 (1.002-1.135)	0.042	0.729 (0.629-0.829)
ICU admission#	1.043 (0.967-1.125)	0.280	0.759 (0.690-0.828)
28 days mortality±	0.993 (0.923-1.068)	0.851	0.765 (0.690-0.839)

Table 5.2 Binomial logistic regression was used to evaluate the effects increasing AnxA1 (per ten unis) alone (univariable) and together with other predictors (multivariable) on the likelihood of thrombotic events, ICU admission, and 28 days mortality, respectively.

*Adjusted for sex. #Adjusted for CRP (mg/L), sex, hypertension and cardiac disease as comorbidities. ±Adjusted for CRP (mg/L), age in years and diabetes as comorbidity. *Abbreviation*: ICU, intensive care unit.

Next, we ran linear mixed models to investigate differences in the dynamics of AnxA1 over time for several clinical outcomes (**Figure 5.4** and **Supplementary 5.5**). The predicted AnxA1 at baseline tended to be higher in the patients with thrombotic events and, ICU admission, as well as in non-survivors; however, this was without statistical significance. Interestingly, the AnxA1 tended to increase more steeply over time in the patients without adverse events, with a statistically significant rise only in patients without thrombotic events (P=0.048).



Figure 5.4 The predicted AnxA1 (ng/mL) at baseline and over time stratified by patients with and without thrombotic events using linear mixed models. Over time, AnxA1 levels increased significantly in patients without thrombotic events (*P*=0.048).

DISCUSSION

In this study, we demonstrated that AnxA1 is significantly increased at presentation and during hospital admission in patients with moderate and severe COVID-19. This increase is associated with elevated markers of inflammation and endothelial damage. Clinically, elevated AnxA1 at presentation predicts an increased risk of thrombotic events in patients with COVID-19.

Elevated levels of AnxA1 may reflect the pro-inflammatory state of patients with moderate and severe COVID-19. AnxA1 is a pro-resolving mediator of inflammation and may counteract hyperinflammation. Indeed, AnxA1 was associated with markers of inflammation, including the potent anaphylatoxin C5a, and endothelial damage. AnxA1 exerts its anti-inflammatory properties by activating the FPR2 receptor on neutrophils, thereby increasing vascular neutrophil detachment, decreasing neutrophil transmigration to the target site and improving apoptotic neutrophil phagocytosis^{4,7}.

AnxA1 did not differ between the patients with moderate and severe COVID-19 at presentation and over time despite that several markers of inflammation, i.e., CRP and NLR, were higher in the severe cases. The capacity of secreting higher levels of AnxA1 to buffer the pro-inflammatory state is eventually exceeded in severe patients with COVID-19. Unfortunately, little is known about the feedback loops of extracellular AnxA1 secretion during excessive inflammation in humans. Based on our observations, potential therapeutic benefits for severe COVD-19 patients may arise through pharmacologically increasing FPR2 signaling by administering human recombinant AnxA1 or its FPR2-binding peptide (Ac2-26)^{13,14}.

Next, we found that higher levels of AnxA1 were associated with poor clinical outcome. Particularly, AnxA1 at presentation predicted the risk of thrombotic events after correction for sex. We also estimated the longitudinal changes in AxnA1 in relation to clinical outcomes with linear mixed models. AnxA1 tended to increase more steeply over time in the patients without adverse outcomes. The association between AnxA1 and adverse outcomes might reflect a reciprocal increase in AnxA1 in patients with excessive hyperinflammation. The notion that AnxA1 tends to increase less steeply over time in patients with adverse outcomes may reflect, once again, the saturated buffer capacity of AnxA1 in these patients. With respect to thrombotic events, it is unknown whether AnxA1 is directly involved in the regulation of the coagulation cascade and platelet activation or indirectly in thrombus formation by reducing neutrophil participation¹⁵. However, it is well recognized that COVID-19-induced hypercoagulability is linked to excessive inflammation via neutrophil extracellular trap formation and complement activation, which triggers the intrinsic pathway of coagulation^{1,16,17}. Increased neutrophil extracellular trap formation may, therefore, be accompanied by the release of intracellular AnxA1.

93

5

One study previously reported decreased AnxA1 in patients with severe COVID-19 compared to moderate cases and controls¹¹. A cut-off value of 17.2 ng/mL for AnxA1 was suggested as a predictive biomarker for ICU admission, which was in contrast to our findings. There were differences between both cohorts. Moderate and severe cases were defined slightly differently in the studies. Moreover, the patients included in the cited study were predominantly female, particularly in the severe group (66%), which is uncommon for severe COVID-19¹⁸. The actual impact of sex differences on AnxA1, however, is unknown. Our study included a significantly higher number of patients combined with longitudinal measurements, underpinning the robustness of our observations.

Our study has several limitations. First, the detection of AnxA1 in serum could lead to an overestimation of the actual AnxA1 levels, particularly in patients with higher neutrophil counts. AnxA1 is stored in granules of neutrophils¹⁹ and, through centrifugation, intracellular AnxA1 could be released and artificially increase the serum fraction. However, we found only a weak correlation between AnxA1 and neutrophils in our cohort, making it unlikely that this effect would significantly affect our observations. Secondly, the sample size of the follow-up samples is limited.

In conclusion, we demonstrate that the pro-resolving mediator of inflammation, AnxA1, is increased in the sera of patients with moderate and severe COVID-19 and is associated with adverse clinical outcomes. We believe that these findings reflect an insufficient increase in AnxA1 as a response to the excessive hyperinflammation in these patients. Future studies should evaluate whether hyperinflammation in COVID-19 patients can be diminished by targeting the FPR2 through the administration of human recombinant AnxA1 or Ac2-26 peptide.

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SUPPLEMENTALS

Supplementary 5.1 Median AnxA1 (interquartile range) of moderate and severe patients with COVID-19 during the course of disease. AnxA1 did not significantly change over time between moderate and severe patients.

		Moderate		Severe	P-value
AnxA1, ng/mL	п	median (IQR)	п	median (IQR)	
Baseline	68	30.1 (16.0-42.0)	104	28.9 (17.3-53.6)	0.583
≤7 days	31	27.3 (13.8-49.9)	65	39.1 (23.4-60.3)	0.102
8–14 days	9	37.5 (32.0-55.2)	50	39.6 (27.4-64.8)	0.891

Differences between groups were analyzed by the Mann Whitney U test. *Abbreviation*: AnxA1, Annexin A1.

	Normal	Normal AnxA1	Elevated AnxA1	Overall P
	Range	(<i>n</i> =135)	(<i>n</i> =85)	
M/F		90/45	54/31	0.634
Age, yr.		67 (±14)	70 (±12)	0.098
Days from illness onset		7 (5-10)	9 (7-14)	< 0.001
Vitals				
SBP, mmHg		132 (±21)	142 (±23)	< 0.001
DBP, mmHg		79 (±13)	81 (±13)	0.305
Heart rate, bpm		90 (80-103)	86 (77-100)	0.095
Body temperature, °C	≤37.9	38.0 (±1.0)	38.0 (±1.0)	0.818
Fever, n (%)		69 (55)	37 (49)	0.002
Medical history				
Hypertension, n (%)		48 (36)	28 (33)	0.691
Diabetes, n (%)		26 (19)	21 (25)	0.337
CVA, n (%)		17 (13)	13 (15)	0.570
Cardiac disease, n (%)		44 (33)	24 (28)	0.496
COPD/asthma, n (%)		20 (15)	13 (15)	0.923
None, n (%)		31 (23)	23 (27)	0.492
Disease severity				
Mild, n (%)		37 (27)	11 (13)	0.011
Moderate, n (%)		40 (30)	28 (33)	0.605
Severe, n (%)		58 (43)	46 (54)	0.107

Supplementary 5.2 Baseline characteristics of patients with normal and elevated AnxA1.

Continuous variables were presented as mean (±standard deviation) or median (interquartile range) as appropriate. Differences between groups were analyzed by the unpaired sample *t* test or Mann Whitney U test. Differences in categorical variables were analyzed by the chi square test. *Abbreviations*: AnxA1, Annexin A1. SBP, systolic blood pressure. DBP, diastolic blood pressure. CVA, cerebrovascular accident. COPD, chronic obstructive pulmonary disease.

	AnxA1 (ng/mL)	AnxA1 (ng/mL)	
	Spearman's r	P value	
CRP (mg/L)	0.227	0.001	
Leukocytes (×109/L)	0.309	< 0.001	
Neutrophils (x10 ⁹ /L)	0.297	< 0.001	
Lymphocytes (x10 ⁹ /L)	0.160	0.026	
NLR	0.082	0.457	
C5a (ng/mL)	0.190	0.008	
vWF:Ag (%)	0.315	< 0.001	

Supplementary 5.3 Correlations between AnxA1 and inflammatory markers and vWF:Ag at baseline in patients with COVID-19.

Spearman rank correlation coefficients were used. *Abbreviations*: AnxA1, Annexin A1. CRP, C-reactive protein. NLR, neutrophil-lymphocyte ratio. C5a, complement 5a. vWF:Ag, von Willebrand factor antigen.

Supplementary 5.4 Univariable regression models for predictors of thrombotic events, ICU admission, and 28 days mortality in patients with COVID-19.

Thrombotic events

Male sex and the risk for developing a thrombotic event during the course of COVID-19 (OR 4.867 [95% CI 1.415-16.733]; P<0.012).

ICU Admission

Sex (male) (OR 2.163 [95% CI 1.117-4.189]; *P*= 0.022), CRP (mg/L) (OR 1.008 [95% CI 1.005-1.012]; *P*<0.0001) and hypertension (OR 0.462 [95% CI 0.239-0.895]; *P*=0.022) and cardiac disease (OR 0.455 [95% CI 0.228-0.907]; *P*=0.025) as comorbidities were risk factors for ICU admission in patients with COVID-19.

28 days mortality

Sex (male) (OR 2.056 [95% CI 1.023-4.131]; *P*=0.043), CRP (mg/L) (OR 1.004 [95% CI 1.001-1.008]; *P*=0.015) and diabetes as comorbidity (OR 3.551 [95% CI 1.771-7.120]; *P*<0.0001) were risk factors for 28 days in-hospital mortality in patients with COVID-19.

Abbrevations: ICU, intensive care unit. CRP, C-reactive protein.

Supplementary 5.5 Linear mixed models calculated mean predicted AnxA1 levels at baseline and over time for thrombotic events, ICU admission and 28 days in-hospital survival.

	AnxA1	
	Estimate (95% CI)	P value
Thrombotic events		
Thrombosis	+ 27.8 (-2.7-58.3)	0.074
Over time	+ 5.4 (0.1-10.7)	0.048
Thrombosis*Over time	- 3.2 (-13.6-7.2)	0.537
ICU admission		
ICU admitted	+ 20.5 (-2.6-43.5)	0.082
Over time	+ 2.8 (-5.0-10.5)	0.480
ICU admitted*Over time	+ 2.1 (-7.6-11.7)	0.670
28 Days Mortality		
Non-survivors	+ 7.7 (-17.9-33.3)	0.553
Over time	+ 5.0 (-0.1-10.2)	0.056
Non-survivors*Over time	- 1.4 (-12.4-9.7)	0.806

Abbreviation: AnxA1, Annexin A1. ICU, intensive care unit.



Chapter 6

The intrinsic coagulation pathway plays a dominant role in driving hypercoagulability in antineutrophil cytoplasmic antibody– associated vasculitis

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Blood Adv. 2024;8:1295-1304

ABSTRACT

The risk of venous thrombotic events (VTEs) is increased in patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). However, a detailed understanding of the underlying mechanisms of hypercoagulability is limited. We assessed prospectively different coagulation parameters in 75 patients with active AAV at baseline and after 6 months of follow-up. D-dimers and fibrinogen were increased in most patients at presentation and remained elevated in about half of the patients. Particularly thrombin: antithrombin (T:AT) and activated coagulation factors in complex with their natural inhibitors of the intrinsic coagulation pathway (i.e., activated FXII:C1 esterase inhibitor [FXIIa:C1Inh], FXIa:AT and FXIa:alpha1-antitrypsin [FXIa:α1AT]) were profoundly elevated in patients at baseline. Thrombin formation was dominantly correlated with coagulation factors of the intrinsic pathway (i.e., FXIIa:AT, FXIa:AT, FXIa:α1AT and FXIa:C1Inh) compared to the extrinsic pathway (i.e., FVIIa:AT). Hypercoagulability correlated with higher disease activity, ANCA levels, C-reactive protein, serum creatinine and proteinuria. VTEs were observed in 5 out of 75 (7%) patients within 1 month (IOR, 1-5) after inclusion. Baseline T:AT levels were significantly higher in patients with vs. without VTEs (P=0.036), but other clinical or laboratory markers were comparable between both groups. Hypercoagulability is dominantly characterized by the activation of the intrinsic coagulation pathway and D-dimers in active AAV. The driving factors of hypercoagulability are yet to be studied, but are most likely related to an interplay of increased disease activity, vascular inflammation, and endothelial damage. Future targets for intervention could include inhibitors of the intrinsic coagulation pathway and compounds specifically reducing the hyperinflammatory state.

INTRODUCTION

The risk of a venous thrombotic event (VTE) is higher in patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), with an incidence of up to 14% compared to the general population¹. The risk is highest among patients with active disease involving the skin, lungs, and those with impaired kidney function at presentation. Most VTEs occur during active disease, suggesting that vascular inflammation and/or endothelial damage trigger hypercoagulability^{2,3}. Markers of hypercoagulability and endothelial damage, however, remained elevated in patients with AAV in clinical remission, indicating low-grade inflammation that persists during quiescent disease⁴. Also, impaired fibrinolysis has been demonstrated in a subset of patients with active AAV^{5,6}. However, knowledge of the specific pathophysiological mechanisms that lead to hypercoagulability in patients with AAV and its translation into clinical guidance for identifying patients at risk is limited.

We therefore investigated the coagulation cascade in a well-defined prospective cohort of patients with active AAV, using state-of-the-art assays to test for activation of the intrinsic and extrinsic pathways of coagulation, at presentation and clinical remission.

METHODS

Patient population

This study was conducted with patients with active AAV included in a prospective observational cohort between April, 2019, and April, 2022 at the Maastricht University Medical Center, Maastricht, The Netherlands. Patients with AAV were defined according to the revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides⁷. Patients with granulomatosis and polyangiitis (GPA) or microscopic polyangiitis (MPA) were included, whereas those with eosinophilic GPA were excluded. Patients with de novo AAV or major relapse of the disease who needed immunosuppressive treatment for the induction of a remission according to the EULAR/ERA-EDTA recommendations were included⁸. Patients provided written informed consent. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and approved by the appropriate ethics committee (reference no. 2020_007).

Data and sample collection

Demographic, clinical, laboratory, radiographic and therapeutic data were prospectively assessed. Blood samples were collected on 3.2% trisodium citrate and on K2-EDTA (BD Vacutainer®) at the time of presentation prior to remission induction treatment and after

6 months when a clinical remission was achieved. Blood was processed immediately, centrifuged at 2000 g for 10 minutes at room temperature and stored at -80 degrees Celsius until testing.

Outcome assessment

Disease activity was assessed according to the Birmingham Vasculitis Activity Score v3 (BVAS)⁹. Clinical remission was defined as a BVAS of 0 and a glucocorticoid dosage of less than 7.5 mg/d. Major relapse was defined as recurrent AAV (BVAS >0) requiring the initiation of high-dose glucocorticoids with cyclophosphamide and/or rituximab.

Measurement of coagulation

The activated partial thromboplastin time (aPTT) was measured using Dade® Actin® FSL Activated PTT Reagent (Siemens Healthcare Diagnostics), prothrombin time (PT) was measured using Dade® Innovin® Reagent (Siemens Healthcare Diagnostics), fibrinogen levels were determined with Multifibren® U Reagent (Siemens Healthcare Diagnostics) and D-dimer was measured using INNOVANCE® D-Dimer Kits (Siemens Healthcare Diagnostics). All assays were performed according to the manufacturer instruction using a BCS® System (Siemens). Prothrombin fragment 1+2 was measured using EnzygnostTM F1+2 ELISA kits (Siemens Healthineers) by following the manufacturer instructions. Activated coagulation factors in complex with their natural inhibitors (i.e., activated FVII:antithrombin [FVIIa:AT], FXIIa:AT, FXIIa:C1 esterase inhibitor [FXIIa:C1Inh], FXIa:AT, FXIa:alpha1-antitrypsin [α 1AT], FXIa:C1Inh, FIXa:AT, FVIIa:AT, epresents activation of the extrinsic pathway, FXIIa:AT, FXIIa:C1Inh, FXIa:AT, FXIIa:AT, FXIIa:C1Inh, FXIa:AT, FXIIa:AT, FXIIA:

Statistical analysis

Continuous variables were presented as mean (±standard deviation [SD]) or median (interquartile range [IQR]) and compared using the independent samples t-test or Mann Whitney U test as appropriate. Categorical variables, expressed as numbers (percentages), were compared using the chi-square test. Paired variables were compared using the Wilcoxon signed-rank test or McNemar's test as appropriate. In case of missing data, a complete-case analysis was conducted. Correlations between coagulation parameters were assessed with Spearman's rank correlation coefficient. Univariable (and multivariable) logistic regression was used to assess associations between clinical characteristics, routine laboratory markers and the measured coagulation factors on VTEs. Statistics were performed using IBM SPSS Statistics version 28 (IBM Corp.,

Armonk, NY) and GraphPad Prism version 9 (GraphPad Software, San Diego, CA). *P* values below 0.05 were considered significant.

RESULTS

Patient characteristics

The baseline characteristics of the 75 included patients with active AAV are depicted in **Table 6.1**. Of these patients, 53 (71%) and 22 (29%) were diagnosed with GPA and MPA, respectively. At presentation, 40 (53%) patients had de novo AAV. Five (7%) patients had a history of VTE, including 3 with deep venous thrombosis (DVT), 1 with pulmonary embolism (PE), and 1 with concomitant DVT and PE. At presentation, 3 (4%) patients were treated with therapeutic anticoagulation for atrial fibrillation (*n*=3) and 1 patient received prophylactic anticoagulation (*n*=1). Median follow-up was 27 months (IQR, 18-36). All patients achieved complete remission within 6 months of treatment. During follow-up, 18 (24%) patients experienced a major relapse of AAV after a median of 17 months (IQR, 12-24). Seven (10%) patients died after a median of 7 months (IQR, 4-13); none of the patients deceased on the background of thrombosis.

	Normal range	N=75
Male, (%)		47 (63)
Age in years, (±SD)		63 (±14)
BMI, (IQR)	18.5-25	25.8 (24.1-29.3)
Diagnosis, (%)		
MPA		22 (29)
GPA		53 (71)
ANCA, (%)		
MPO		31 (41)
PR3		42 (56)
Double positive		2 (3)
De novo presentation, (%)		40 (53)
BVAS, (±SD)		14 (±5)
Organ involvement, (%)		
General		44 (59)
Cutaneous		6 (8)
Mucous membranes		4 (5)
ENT		40 (53)
Chest		37 (50)
Cardiovascular		2 (3)
Abdominal		0 (0)
Renal		50 (67)
Nervous system		8 (11)

Table 6.1 Baseline characteristics of patients with active antineutrophil cytoplasmic antibodyassociated vasculitis at presentation.
Table 6.1 (continued)

	Normal range	N=75
Remission-induction scheme, (%)		
RTX		40 (54)
RTX/CYC		16 (21)
CYC		19 (25)
Prednisone ≤5 mg in days, (IQR)		105 (77-140)
Anticoagulation use, (%)		4 (5)
Previous thrombotic events, (%)		5 (7)
Laboratory parameters		
Hemoglobin, (±SD)	8.2-11.0 / 7.3-9.7 mmol/L	7.2 (±1.2)
Platelet, (IQR)	130-350 x10 ⁹ /L	332 (264-387)
Leukocytes, (IQR)	3.5-11.0 x10 ⁹ /L	8.5 (6.8-12.1)
Serum creatinine, (IQR)	60-115 μmol/L	154 (95-234)
CRP, (IQR)	<10 mg/L	20 (7-45)
IgG, (±SD)	7.0-16.0 g/L	11.7 (±3.3)
ANCA level, (IQR)	_	70 (30-120)

Abbreviations: SD, standard deviation. IQR, interquartile range. BMI, body mass index. MPA, microscopic polyangiitis. GPA, granulomatosis with polyangiitis. ANCA, anti-neutrophil cytoplasmic antibody. MPO, myeloperoxidase. PR3, proteinase 3. BVAS, Birmingham vasculitis activity score. ENT, ear, nose and throat. RTX, rituximab. CYC, cyclophosphamide. CRP, C-reactive protein. IgG, immunoglobulin G.

Coagulation markers at baseline and after 6 months

Table 6.2 shows coagulation markers during active and quiescent disease in patients with AAV. D-dimer levels above the upper limit of normal were found in 56 (79%) and 31 (55%) patients at presentation and remission (*P*=0.035), respectively. Fibrinogen was elevated at presentation in most patients (*n*=66, 92%), which decreased after 6 months (*n*=37 [66%]; *P*=0.003). T:AT, an activation marker of the common pathway, as well as FXIIa:C1Inh, FXIa:AT and FXIa: α 1AT, markers of the intrinsic coagulation pathway, were increased in a large number of patients at presentation. Other complexes and in particular FVIIa:AT, a marker of the extrinsic coagulation pathway, were elevated only in 10-17% of the patients. The observations point towards profound activity of coagulation factors from the intrinsic coagulation pathway. The coagulation factors remained stable or decreased after 6 months of follow-up.

	Normal	n	Baseline	n	Follow-up	Overall P
	range					
D-dimers, µg/mL	< 0.5	72	1.2 (0.6-2.6)	56	0.6 (0.3-1.0)	0.010
High D-dimers, (%)			56 (79)		31 (55)	0.035
Fibrinogen, g/L	1.7-4.0	72	6.4 (5.2-8.6)	56	4.7 (3.7-5.7)	< 0.001
High Fibrinogen, (%)			66 (92)		37 (66)	0.003
PT, sec	10-12	72	8.8 (8.3-9.5)	56	8.4 (8.2-8.9)	0.003
Prolonged PT, (%)			6 (8)		3 (5)	0.625
APTT, sec	28-32	72	27 (25-30)	56	26 (24-29)	0.019
Prolonged APTT, (%)			9 (12)		5 (9)	0.453
FVIIa:AT, pM	≤ 2641	72	760 (480-897)	56	615 (378-857)	0.100
High FVIIa:AT, (%)			7 (10)		0 (0)	0.125
FXIIa:AT, pM	≤ 118	75	57 (36-79)	55	47 (32-67)	0.521
High FXIIa:AT, (%)			11 (15)		5 (9)	1.000
FXIIa:C1Inh, pM	≤ 2166	75	3859 (3565-4276)	55	3895 (3502-4152)	0.269
High FXIIa:C1Inh, (%)			74 (100)		53 (96)	0.500
FXIa:AT, pM	≤ 43	72	48 (25-92)	56	51 (23-82)	0.629
High FXIa:AT, (%)			40 (56)		31 (55)	0.687
FXIa:α1AT, pM	≤ 29	72	26 (15-39)	56	14 (0.4-22)	< 0.001
High FXIa:α1AT, (%)			30 (42)		4 (7)	0.003
FXIa:C1Inh, pM	≤ 514	72	71 (71-204)	56	71 (71-71)	0.048
High FXIa:C1Inh, (%)			12 (17)		2 (4)	0.125
FIXa:AT, pM	≤ 472	72	257 (197-361)	56	246 (187-321)	0.506
High FIXa:AT, (%)			11 (15)		6 (11)	1.000
FXa:AT, pM	≤ 279	72	176 (152-204)	56	190 (161-210)	0.993
High FXa:AT, (%)			8 (11)		1 (2)	0.125
T:AT, μg/L	≤ 4.7	72	2.8 (1.8-5.8)	56	1.7 (1.4-2.8)	0.005
High T·AT. (%)			20 (28)		4(7)	0.065

Table 6.2 Coagulation parameters at presentation with active disease and at follow-up after 6 months of treatment in patients with active antineutrophil cytoplasmic antibody associated vasculitis.

Wilcoxon signed-ranks test was used for continuous variables and McNemar's test was used for categorical variables. Green color indicates general coagulation tests, blue color indicates marker(s) of the extrinsic pathway, yellow color indicates markers of the intrinsic pathway, and grey color indicates markers of the common pathway. *Abbreviations*: PT, Prothrombin time. aPTT, activated partial thromboplastin time. FVIIa, activated FVII. AT, antithrombin. FXIIa, activated factor XII. C1Inh, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. FXa, activated factor X. T:AT, thrombin in complex with antithrombin.

Associations between activated coagulation factors at baseline

The dynamics between the different coagulation factors were explored by calculating the Spearman rank correlation coefficients (**Figure 6.1**). There was a strong positive correlation between T:AT and D-dimers (r=0.73, P<0.001) and a moderate positive correlation between T:AT and FXIIa:AT (r=0.35, P=0.003), FXIa:AT (r=0.33, P=0.004), FXIa: α 1AT (r=0.42, P<0.001), and FXIa:C1Inh (r=0.46, P<0.001), pointing to activation of

the intrinsic pathway of coagulation. No correlation was found between T:AT and activation of the extrinsic pathway of coagulation (FVIIa:AT; *r*=0.19, *P*=0.103).



Figure 6.1 Correlation matrix between the different coagulation factors and D-dimers in patients with active AAV. Spearman rank correlation coefficients were used. *indicates a *P* value <0.05. *Abbreviations*: FVIIa, activated FVII. AT, antithrombin. FXIIa, activated factor XII. C1Inh, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. FXa, activated factor X. T:AT, thrombin in complex with antithrombin.

Next, we focused on patients with elevated T:AT and D-dimers to better understand hypercoagulability among these patients in particular (**Table 6.3**). Patients with increased T:AT showed higher levels of FXIIa:AT (*P*=0.003), FXIa: α 1AT (*P*=0.007), FXIa:C1Inh (*P*<0.001), and FIXa:AT (*P*<0.001) as compared to those with normal T:AT, indicating activation of the intrinsic pathway of coagulation. T:AT was associated with D-dimers. Notably, D-dimers also were associated with activation of the intrinsic pathway of coagulation as reflected by higher levels of FXIIa:AT (*P*=0.005), FXIa: α 1AT (*P*=0.007), and FIXa:AT (*P*<0.001). No relevant correlation was found between FVIIa:AT and T:AT or D-dimers, indicating that hypercoagulability is associated with activation of the intrinsic rather than extrinsic pathway of coagulation.

	Elevated T:AT	Normal T:AT	Overall P	Elevated D-	Normal D-	Overall P
				dimers	dimers	
	n=20	n=52		n=56	n=16	
FVIIa:AT, pM	859 (658-2627)	654 (413-876)	0.020	773 (515-919)	591 (385-856)	0.169
FXIIa:AT, pM	72 (57-423)	47 (35-68)	0.003	62 (43-97)	36 (29-59)	0.005
FXIIa:C1Inh, pM	3870 (3492-6105)	3844 (3565-4131)	0.266	3904 (3565-4303)	3827 (3547-4114)	0.332
FXIa:AT, pM	83 (27-186)	44 (24-78)	0.084	51 (24-92)	41 (26-96)	0.935
FXIa:α1AT, pM	39 (23-172)	24 (14-33)	0.007	29 (19-45)	16 (1-25)	< 0.001
FXIa:C1Inh, pM	269 (71-2842)	71 (71-71)	< 0.001	71 (71-329)	71 (71-71)	0.062
FIXa:AT, pM	446 (297-1529)	231 (190-287)	< 0.001	279 (215-383)	191 (139-284)	0.006
FXa:AT, pM	185 (165-330)	175 (151-198)	0.145	176 (152-209)	176 (152-193)	0.583
T:AT, µg/L	-	-	-	3.3 (2.1-8.5)	1.7 (1.2-2.1)	< 0.001

Table 6.3 Coagulation parameters of patients with elevated versus normal T:AT and D-dimers at presentation.

The Mann-Whitney U test was used for continuous variables. Blue color indicates marker(s) of the extrinsic pathway, yellow color indicates markers of the intrinsic pathway, and grey color indicates markers of the common pathway. *Abbreviations*: FVIIa, activated FVII. AT, antithrombin. FXIIa, activated factor XII. C1Inh, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. FXa, activated factor X. T:AT, thrombin in complex with antithrombin.

Associations between clinical and laboratory findings and coagulation at baseline

The Spearman rank correlation coefficients between the different parameters and T:AT as well as D-dimers are presented in **Supplementary 6.1**. Age in years positively correlated with T:AT (r=0.29, P=0.013) and D-dimers (r=0.39, P<0.001), respectively. D-dimers positively correlated with BVAS (r=0.28, P=0.017), PT (r=0.33, P=0.004), serum creatinine (r=0.30, P=0.012), C-reactive protein (CRP; r=0.39, P<0.001), ANCA levels (r=0.29, P=0.016), and proteinuria (r=0.37, P=0.002). There was a negative correlation between D-dimers and hemoglobin (r=-0.24, P=0.044) and eGFR (r=-0.36, P=0.003). These findings point towards association between higher inflammation, disease activity and hypercoagulability in patients with active AAV.

Thromboembolic events

VTEs occurred in 5 (7%) out of 75 patients (DVT, *n*=3; PE, *n*=2) after a median of 1 month (IQR, 1-5) after presentation (**Table 6.4**). None of them had a prior VTE. No betweengroup differences in terms of routine clinical parameters at presentation and outcomes measures, that is, relapse rate and survival, were found. T:AT levels were higher in patients with VTEs (8.8 [IQR, 3.3-14.7] vs. 2.5 [IQR, 1.7-5.4] μ g/L; *P*=0.036). None of the patients with VTEs presented with a SARS-CoV-2 infection (**data not shown**).

No significant difference was found between-groups when using logistic regression analyses to assess the prognostic value of routine clinical parameters and activated coagulation factors in relation to VTEs (**data not shown**).

Chapter 6

\mathbf{I}_{α}	Table 6.4	Baseline characteristics of	patients with and	without thrombosis
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	Normal range	Thrombosis	No Thrombosis	Overall P
	8	n=5	n=70	
Male, (%)		3 (60)	44 (63)	0.898
Age in years, (IQR)		71 (64-77)	63 (55-72)	0.126
BMI, (IQR)	18.5-25	27.1 (23.8-29.2)	25.7 (24.2-29.3)	0.887
Diagnosis, (%)		(, , , , , , , , , , , , , , , , , , ,	~ /	0.588
MPA		2 (40)	20 (29)	
GPA		3 (60)	70 (71)	
ANCA, (%)				0.176
MPO		3 (60)	28 (40)	
PR3		1 (20)	41 (59)	
Double positive		1 (20)	1 (1)	
De novo presentation, (%)		4 (80)	36 (51)	0.216
BVAS, (IQR)		17 (13-21)	14 (12-17)	0.212
Organ involvement, (%)				
General		5 (100)	39 (56)	0.052
Cutaneous		0 (0)	6 (9)	0.495
Mucous membranes		0 (0)	4 (6)	0.583
ENT		2 (40)	38 (54)	0.536
Chest		2 (40)	35 (51)	0.643
Cardiovascular		0 (0)	2 (3)	0.702
Abdominal		0 (0)	0 (0)	-
Renal		4 (80)	46 (66)	0.513
Nervous system		2 (40)	6 (9)	0.029
Remission-induction scheme, (%)				0.320
RTX		3 (60)	37 (53)	
RTX/CYC		2 (40)	14 (20)	
CYC		0 (0)	19 (27)	
Prednisone ≤5 mg in days, (IQR)		75 (60-95)	106 (79-140)	0.087
Previous thrombotic events, (%)		0 (0)	5 (7)	0.530
Laboratory parameters				
Hemoglobin, (IQR)	8.2-11.0 /	7.9 (6.9-8.9)	7.2 (6.2-8.0)	0.182
	7.3-9.7 mmol/L			
Platelet, (IQR)	130-350 x10 ⁹ /L	283 (237-441)	333 (264-388)	0.377
Leukocytes, (IQR)	3.5-11.0 x10 ⁹ /L	10.3 (7.0-12.7)	8.5 (6.8-11.8)	0.585
Serum creatinine, (IQR)	60-115 µmol/L	95 (81-225)	154 (96-224)	0.376
CRP, (IQR)	<10 mg/L	11 (3-90)	23 (7-44)	0.956
IgG, (IQR)	7.0-16.0 g/L	9.7 (8.6-14.4)	11.9 (9-13.6)	0.534
ANCA level, (IQR)		64 (34-81)	70 (30-120)	0.713
Coagulation parameters				
D-dimers, (IQR)	≤4.9 µg/mL	3.3 (1.2-15.7)	1.1 (0.6-2.5)	0.053
Fibrinogen, (IQR)	1.7-4.0 g/L	5.8 (3.2-7.4)	6.6 (5.2-8.5)	0.176
PT, (IQR)	10-12 sec	8.6 (8.1-10.5)	8.8 (8.3-9.5)	0.610
APTT, (IQR)	28-32 sec	29 (23-35)	27 (25-30)	0.825
FVII:AT, (IQR)	≤2641pM	641 (433-824)	770 (480-915)	0.406
FXIIa:AT, (IQR)	≤118 pM	63 (35-97)	55 (36-79)	0.788
FXIIa:C1Inh, (IQR)	≤2166 pM	3784 (3659-4617)	3874 (3565-4252)	0.755
FXIa:AT, (IQR)	≤43 pM	30 (19-67)	49 (25-95)	0.358
FXIa:α1AT, (IQR)	≤29 pM	40 (25-68)	25 (15-37)	0.166
FXIa:C1Inh, (IQR)	≤514 pM	71 (71-199)	71 (71-262)	0.968
FIXa:AT, (IQR)	≤472 pM	296 (232-304)	253 (197-364)	0.833
FXa:AT, (IQR)	≤279 pM	146 (121-171)	177 (155-206)	0.033
T:AT, (IOR)	≤4.7 ug/L	8.8 (3.3-14.7)	2.5(1.7-5.4)	0.036

	Normal range	Thrombosis	No Thrombosis	Overall P
	0	n=5	n=70	
Outcome measures				
Relapse during follow-up		2 (40)	16 (23)	0.386
Mortality during follow-up		0 (0)	7 (10)	0.458

Table 6.4 (continued)

The Mann-Whitney U test was used for continuous variables and the chi-square test was used for categorical variables. *Abbreviations:* SD, standard deviation. IQR, interquartile range. BMI, body mass index. MPA, microscopic polyangiitis. GPA, granulomatosis with polyangiitis. EGPA, eosinophilic granulomatosis with polyangiitis. ANCA, anti-neutrophil cytoplasmic antibody. MPO, myeloperoxidase. PR3, proteinase 3. BVAS, Birmingham vasculitis activity score. ENT, ear, nose and throat. RTX, rituximab. CYC, cyclophosphamide. CRP, C-reactive protein. IgG, immunoglobulin G. PT, Prothrombin time. aPTT, activated partial thromboplastin time. FVIIa, activated FVII. AT, antithrombin. FXIIa, activated factor XII. C1Inh, C1 esterase inhibitor. FXIa, activated factor XI. α 1AT, α 1-antitrypsin. FIXa, activated factor IX. FXa, activated factor X. T:AT, thrombin in complex with antithrombin.

DISCUSSION

In this prospective study, we uniquely characterized hypercoagulability in detail in a well characterized cohort of patients with AAV. We observed that activated coagulation factors of the intrinsic and extrinsic coagulation pathway are elevated in a subset of patients with active disease, while several remained stable and others decreased after 6 months of follow-up. T:AT formation and increased D-dimers were predominantly associated with activation of the intrinsic coagulation pathway during active disease and linked to higher disease activity and inflammation.

Patients with AAV are at an increased risk of developing VTEs due to a hypercoagulable state, especially during active disease. This is indicated by elevated levels of D-dimers and fibrinogen, as well as by increased levels of activated coagulation factors with their natural inhibitors in our study. Our findings extend previous studies that have reported on abnormal D-dimers and fibrinolysis in patients with active AAV^{13,14}. After achieving remission, not all patients showed an improvement in their pro-thrombotic state as assessed by the levels of coagulation markers after 6 months. This is in line with *Hilhorst* and colleagues' findings on sustained hypercoagulability in patients with AAV in remission, indicated by an increased endogenous thrombin potential as well as elevated FVIII and tissue factor pathway inhibitor levels⁴. Ongoing low-grade inflammation and subsequent endothelial damage may be causative factors in these patients, even though they were assumed to be in remission in terms of clinical and laboratory markers.

To better understand these observations, we further assessed activated coagulation factors in complex with their natural inhibitors of the intrinsic and extrinsic coagulation pathways. During active disease, we found that thrombin formation was more

profoundly associated with the intrinsic coagulation pathway, specifically with the complexes formed by FXIIa and FXIa with their natural inhibitors. Particularly, patients with elevated T:AT or D-dimers showed higher levels of activated coagulation factors in the intrinsic coagulation pathway compared to patients with normal T:AT or D-dimers. These findings point towards an important role of the intrinsic coagulation pathway in hypercoagulability in patients with active AAV.

Clinically, higher disease activity and inflammation, as reflected by higher BVAS, ANCA levels and CRP at presentation, as well as more severe kidney disease, as reflected by increased serum creatinine and spot urinary protein excretion, were associated with higher D-dimers. Our data are in line with previous publications, indicating that elevated D-dimers are correlated with inflammation, disease activity and renal impairment^{13,14}. It is known that an impaired kidney function is associated with increased markers of coagulation^{15,16}, implying that renal involvement is an independent risk factor of hypercoagulability in AVV.

A detailed understanding of the factors driving hypercoagulability in AAV is currently limited, but the majority of VTEs are observed during active disease, pointing towards an interaction between proinflammatory stimuli, complement activation, platelet activation, and endothelial damage. For example, neutrophil extracellular traps (NETs) formation has been associated with hypercoagulability in other conditions^{12,17}. The interplay of activated platelets and negatively charged DNA fragments decorated in NETs activates FXII leading to thrombin formation¹⁸. Higher levels of tissue factor (TF) are also associated with mediators released during NETs formation¹⁹. In AAV, several studies implicated that NETs formation is abundant in patients with active disease²⁰⁻²². NETs were found to co-localize with thrombi in a patient with MPA²³, suggesting a direct involvement of NETs formation in the process of thrombosis in AAV. In this context, complement 5a (C5a) primed neutrophils of patients with active AAV released NETs expressing high levels of TF that induced thrombin formation^{24,25}. Our findings are in harmony with these observations; as hypercoagulability during active AAV disease may be driven by excessive inflammation, endothelial damage, and platelet activation, leading to activation of both the intrinsic (i.e., via FXII and FXI) and extrinsic (i.e., via TF release) pathways of coagulation. Inflammation and endothelial damage typically improve with the initiation of remission-induction therapy, resulting into a reduction of the hypercoagulable state observed in most but not all of the patients with AAV in our cohort.

In this study, 7% of the patients experienced VTEs with an average time of 1 month (IQR, 1-5) after inclusion. Patients who developed VTEs had higher T:AT levels at presentation, suggesting that hypercoagulability was present before the clinical significance of thrombosis was identified in these individuals. The significantly higher FXa:AT complexes seen in patients without VTEs are most likely not clinically meaningful since the median and IQRs were below the cut-off <279 pM. Previous studies

have reported higher BVAS, MPO-ANCA positivity, skin or pulmonary involvement and impaired kidney function as risk factors for the development of VTEs in AAV^{1,26}. The lack of distinguishable clinical or laboratory features between patients with and without VTEs in our study may be attributed to the relatively small number of patients who developed VTEs during follow-up.

Our study has several limitations. First, due to the ongoing nature of our prospective observational study and the impact of the COVID-19 pandemic on patient follow-up, we were unable to assess coagulation factors in all patients during follow-up. On the other hand, our study represents the first prospective detailed analysis of the coagulation cascade in a well-defined cohort of patients with AAV, with extensive assessments conducted over time. Second, the enrollment of patients partially occurred during the COVID-9 pandemic. It is worth noting that hypercoagulability has been reported to be present and persistent in patients with COVID-19^{10,27}. None of our patients, however, presented with a SARS-CoV-2 infection prior to sampling. Third, due to COVID-19 related travel restrictions, blood sampling after 6 months of follow-up was not possible in all patients, yet they all remained included in the study. Given that most insights are derived from the baseline assessments, the impact of the missing follow-up data on the study's conclusion is limited.

In conclusion, patients with active AAV have an increased hypercoagulable state, characterized by elevated levels of coagulation factors in complexes with their natural inhibitors, especially those linked to the intrinsic coagulation pathway, as well as D-dimers. Although the precise mechanisms underlying hypercoagulability in AAV are not fully understood, it is most likely related to an interplay of higher disease activity, excessive vascular inflammation, and endothelial damage. To reduce the risk of VTEs in AAV, potential targets for intervention include the intrinsic coagulation pathway, including novel FXIa inhibitors, and specific inhibition of neutrophil activation, such as through C5a (receptor) inhibition.



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SUPPLEMENTALS

	T:AT		D-dimers	
-	Spearman's r	P value	Spearman's r	P value
Age in years	0.29	0.013	0.39	<0.001
AAV type	0.03	0.818	- 0.05	0.672
ANCA type	0.13	0.273	0.03	0.795
De novo diagnosis	0.07	0.541	0.03	0.831
BVAS	0.10	0.419	0.28	0.017
Fibrinogen	- 0.06	0.600	0.12	0.333
PT	0.21	0.072	0.33	0.004
APTT	- 0.19	0.106	0.05	0.652
Hemoglobin	- 0.19	0.330	- 0.24	0.044
Platelet	0.18	0.136	0.17	0.164
Leukocytes	0.22	0.071	0.11	0.383
Serum creatinine	0.11	0.369	0.30	0.012
eGFR	- 0.23	0.066	- 0.36	0.003
CRP	0.214	0.076	0.39	<0.001
IgG	0.04	0.759	0.14	0.271
ANCA level	0.16	0.185	0.29	0.016
Protein excretion	0.2	0.095	0.37	0.002

Supplementary 6.1 Correlation matrix between clinical and laboratory findings and coagulation in patients with active AAV.

Spearman rank correlation coefficients were used. *Abbreviations:* AAV type, either microscopic polyangiitis or granulomatosis with polyangiitis. ANCA type, either myeloperoxidase or proteinase 3. BVAS, Birmingham vasculitis activity score. PT, Prothrombin time. aPTT, activated partial thromboplastin time. CRP, C-reactive protein. IgG, immunoglobulin G.





Chapter 7

General discussion, summary, and future perspectives

Manuscript in preparation (review article)

GENERAL DISCUSSION, SUMMARY, AND FUTURE PERSPECTIVES

The thesis aimed to better understand hypercoagulability and the potential link to hyperinflammation observed in antineutrophil cytoplasmic antibody-associated vasculitis (AAV). The risk of venous thrombotic events (VTEs) is increased in patients with AAV. However, knowledge of the underlying mechanisms driving hypercoagulability and its translation into clinical guidance for identifying patients at risk is limited. Since the risk of VTEs is the highest in patients with active AAV, excessive neutrophilic inflammation, complement activation, neutrophil extracellular traps (NETs) formation and vascular damage likely play an essential role. We therefore initiated a prospective observational study of patients with active AAV in 2019. Then, coronavirus disease 2019 (COVID-19) emerged in the Netherlands. Early observations of dysregulated immune responses and a high percentage of VTEs in patients with severe COVID-19 triggered us to hypothesize a link between these observations similar to what we assumed in AAV. There was an urgent need to unravel the underlying driving factors of inflammation and hypercoagulability and to identify potential therapeutic targets for patients with COVID-19. Due to the highly dynamic and rapid spread of SARS-CoV-2, we conducted a prospective cohort study in early 2020 involving over 200 patients to study mechanisms and potential therapeutic targets in COVID-19. As a result, the objective of this thesis shifted to gain a deeper understanding of the link between hyperinflammation and hypercoagulability in COVID-19 and to apply the lessons learned to AAV. In this chapter, the findings and implications of the thesis will be discussed in the context of the recent literature and suggestions for future research are presented.

Neutrophils and the intrinsic coagulation pathway are potential drivers of COVID-19

In **Chapter 2**, we provided evidence for the pathogenic role of the complementneutrophil-coagulation axis in COVID-19, demonstrating in a large prospective cohort of 228 patients with COVID-19 that as disease severity increased, the anaphylatoxin complement 5a (C5a) was generated, neutrophils underwent NETs formation, and the intrinsic pathway of coagulation was strongly activated¹.

C5a is a circulating proteolytic fragment of the complement system and increased levels indicate complement activation. We measured C5a levels in the plasma of patients with SARS-CoV-2 infection presenting at the emergency department. C5a was elevated in 153 (76%) out of 201 patients with COVID-19 and the highest levels were found in hospitalized patients, i.e., moderate and severe COVID-19, compared to patients with a mild course of disease. These findings indicate that as the disease severity of COVID-19 advances, the complement system becomes increasingly activated. Our observations



were confirmed and extended in numerous studies. Indeed, later studies found that SARS-CoV-2 infection is associated with the activation of all complement pathways. The alternative pathway is activated by the competition between SARS-CoV-2 and factor H², the classical pathway is directly activated by IgM and IgG antibody immune complexes³, and the lectin pathway is activated by the interaction of SARS-CoV-2 spike proteins with mannose-binding lectin, ficolin 2, collectin 11 and mannose-binding protein-associated serine protease 2⁴. Other studies also found that complement activation is directly linked to disease severity in COVID-19. For example, increased C5b-9, C5a, and C4d levels are associated with respiratory failure and a severe course of COVID-19^{3,5,6}.

C5a is a potent anaphylatoxin that promotes inflammation by attracting neutrophils and other cells to the site of infection and by stimulating the release of cytokines like tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-8. The subsequent attraction, priming, and activation of neutrophils initiate an amplification loop involving complement and neutrophil activation, resulting in more generation of C5a7. Under certain conditions, this feedback loop contributes to an excessive pro-inflammatory response, ultimately leading to an uncontrolled release of NETs by neutrophils. We found that NETs formation was abundant in patients with COVID-19. First, the presence of extracellular histores H3 (H3) was assessed by Western blot in the plasma and sputum of patients with COVID-19. Extracellular H3 was detected in patients with moderate and severe COVID-19 but not in those with mild disease. Importantly, H3 was citrullinated in 73% of the patients, indicating that H3 originated from NETs. Citrullinated H3 was also found in sputum samples obtained from nine patients with respiratory failure, providing evidence that NETs formation occurs within the lungs of mechanically ventilated patients with COVID-19. Finally, we investigated whether patients with COVID-19 had circulating factors that induce NETs formation by incubating neutrophils from healthy donors with patients' serum. With the samples from all nine patients with severe COVID-19, NETs formation as indicated by positive staining for DNA, citrullinated H3, neutrophil elastase, and myeloperoxidase (MPO) was visualized by immunofluorescence microscopy. In contrast, serum samples from mild and moderately ill patients did not display NETs formation. Other studies were in line with our observations; citrullinated H3 and MPO-DNA complexes, another circulating marker of NETs, were elevated in patients with COVID-19 and correlated with complement activation and disease severity⁸⁻¹⁰.

Since DNA fragments and histones released from NETs can trigger the intrinsic coagulation pathway¹¹, we hypothesized that the complement activation and NETs formation observed in patients with severe COVID-19 substantially contributed to hypercoagulability in COVID-19. We therefore assessed activated coagulation factors of the intrinsic pathway in complex with their natural inhibitors over time. Hypercoagulability indicated by elevated levels of thrombin:antithrombin (T:AT) was found in 131 (60%) out of 217 patients with COVID-19. We demonstrated that the intrinsic coagulation pathway is highly activated, as indicated by increased levels of

plasma kallikrein:C1 esterase inhibitor (PKa:C1INH), FXIa:a1antitrypsin (FXIa:a1AT), FXIa:antithrombin (FXIa:AT), and FIXa:AT, in patients with COVID-19. Notably, upstream activation of the intrinsic pathway was observed in most patients with COVID-19, but activation of the downstream mediators, i.e., FXIa:AT, FIXa:AT, and T:AT, were predominantly associated with severe COVID-19. These complexes remained elevated in patients with severe COVID-19 during follow-up. Our findings were confirmed in a small cohort study, showing activation of the intrinsic cascade, i.e., FXIIa:C1INH, kallikrein:C1INH, FXIa:C1INH, FXIa: α 1AT, and FIXa:AT, at baseline in plasma of 30 patients with COVID-19¹². In summary, we concluded that hypercoagulability in COVID-19 was substantially driven by complement activation, NETs formation and activation of the intrinsic coagulation pathway, particularly in patients with a severe disease course. Several studies confirmed the presence of mediators derived from NETs together with endothelial damage and thrombosis in organ biopsies of deceased patients with COVID-1913,14. Indeed, markers of complement and NETs formation were directly linked to each other in patients with COVID-19 and in vitro C5a inhibition disrupted NETs driven thrombogenicity⁸. Of importance, FXII was found to co-localize with NETs on lung biopsies and improved clearance of NETs reduced activation of FXII in vitro¹⁵, highlighting that activation of the intrinsic coagulation pathway is directly linked to NETs formation in patients with COVID-19.

A drawback of our study was that we could not better look into the extrinsic coagulation pathway to understand the interplay between both coagulation pathways in COVID-19. In Chapter 3, we therefore extended the analysis by assessing markers of the extrinsic coagulation pathway and linking T:AT and vWF:antigen to adverse clinical outcomes in patients with COVID-1916. Levels of free FVIIa and FVIIa:AT, a marker of circulating FVIIa-tissue factor (TF) complexes, were not statistically different between patients with COVID-19 and remained stable over time. Moreover, T:AT strongly correlated with FXIa:AT and FIXa:AT but not with free FVIIa and FVIIa:AT, indicating that thrombin formation was substantially driven via the intrinsic coagulation pathway. C-reactive protein (CRP) correlated positively with FXIa:α1AT, FIX:AT, T:AT, and vWF:Antigen (vWF:Ag), again linking activity of the intrinsic coagulation pathway and endothelial damage to excessive inflammation in COVID-19. Next, we tested the prognostic value of the coagulation factors on intensive care unit [ICU] admission, thrombosis and mortality. Increased levels of FXIa: α 1AT and T:AT at baseline were associated with an increased risk for ICU admission. This was in line with another study, indicating that FXIa:AT is associated with progressive respiratory abnormalities in COVID-1912. T:AT levels were also associated with an increased risk of thrombosis, but none of the evaluated markers had a prognostic value for in-hospital mortality at 28 days, confirming previous observations^{6,17}. Finally, we assessed the prognostic value of the coagulation factors and vWF:Ag over time in those patients admitted to the hospital using linear mixed models. We found that ongoing vascular damage, reflected by increased vWF:Ag levels over time,



was associated with ICU admission and mortality, whereas activation of the intrinsic pathway reflected hypercoagulability in COVID-19.

Our findings regarding the dysregulated complement-neutrophil-coagulation axis as a driving force behind the pathophysiological mechanisms in COVID-19 represent only a small portion of the intricate interplay between SARS-CoV-2 infection and the host defense mechanisms responsible for the development of a severe disease course. Numerous studies have contributed significant insights into comprehending the underlying mechanisms involved in COVID-19 that extend beyond the scope of this thesis. For example, platelet hyperactivation is also recognized as a feature of hypercoagulability in COVID-19 leading to enhanced platelet aggregation. phosphatidylserine exposure, and platelet interactions with other cells¹⁸. Indeed, SARS-CoV-2 infection resulted in platelet hyperactivity that directly correlated with mortality and an altered platelet transcriptome¹⁹. Platelet-monocyte interactions trigger TF expression in patients with severe COVID-1920. Furthermore, platelet-neutrophil aggregates were confirmed in microthrombi containing NETs in lung biopsies of deceased patients with COVID-1913. Vascular endothelial cell activation and injury with a subsequent release of Weibel-Palade bodies containing ultra-large vWF multimers that interacted with platelets and the coagulation cascade further contribute to a procoaguable state in COVID-19²¹. From an evolutionary perspective, the coagulation cascade directly cross-talks with the complement system in the concept of immunothrombosis, thereby contributing to the overactivity of the complement-neutrophil-coagulation axis. Mannose-associated serine protease 1 (MASP-1) shows homology with thrombin²² and can cleave prothrombin in vitro23. TF expression on endothelial cells and monocytes is also directly stimulated by C5a^{24,25}. Vice versa, FXIIa and kallikrein have been linked to cleavage of C3 and C5, thus leading to the generation of C5a²⁶. However, these specific interactions between the coagulation and complement system have yet to be documented in COVID-19. Finally, specific patient characteristics including age, sex, diabetes, hypertension, obesity, and chronic kidney disease have been reported as predisposing risk factors for a severe course of COVID-1927. These factors may negatively affect the quality of the immune response against SARS-CoV-2, leading to impaired viral clearance, excessive inflammation, vascular damage, and thrombosis.



Figure 7.1 Links between hyperinflammation and hypercoagulability in COVID-19. The illustration summarizes the pathophysiological mechanisms described in this thesis that contribute to hyperinflammation and hypercoagulability after SARS-CoV-2 infection in patients with severe COVID-19. *Abbreviations*: DAMPs, damage-associated molecular patterns. IL, interleukin. TNF α , tumor necrosis factor α . C5a, Complement 5a. AnxA1, Annexin A1. TF, tissue factor. H3, histone H3. vWF, von Willbrand factor.

Complement 5a inhibition is safe and effective in reducing the risk of a severe course of COVID-19

We hypothesized that interfering with the dysregulated complement-neutrophilcoagulation axis could improve the adverse events observed in COVID-19 even before it was recognized that immunosuppressants like dexamethasone and IL-6 inhibition are effective in preventing the progression to severe COVID-19 and reducing the in-hospital mortality²⁸⁻³⁰. Based on our previous observations, C5a inhibition was as an attractive target because it can potentially mitigate inflammation and tissue damage caused by neutrophils without impairing C5b formation or other components of the immune system necessary for an effective microbial clearance. We tested the hypothesis in Chapter 4 by conducting an exploratory, open-label, phase 2 randomized controlled trial in collaboration with colleagues of the Amsterdam UMC between March and May, 2020³¹. The safety and potential benefits of inhibiting C5a with vilobelimab, a monoclonal antibody selectively blocking C5a, was explored in 30 patients with severe COVID-19. The primary outcome, i.e., change in the ratio of partial pressure of arterial oxygen to fractional oxygen concentration in inspired air, was not different between the treatment and control group at day five after inclusion. However, C5a inhibition appeared safe and showed benefits in reducing renal impairment, severe-grade pulmonary embolisms and



mortality. The findings were limited by the open-label design and the small number of included patients. To overcome these restrictions, we participated in a multicenter, double-blind, phase 3 randomized placebo-controlled trial with 368 included patients³². The study aimed to evaluate the efficacy of vilobelimab in addition to standard of care in improving survival outcomes in patients with COVID-19 requiring invasive mechanical ventilation. Vilobelimab significantly reduced the all-cause mortality at 28 days in the predefined analysis without site-stratification (HR 0.67, 95% CI 0.48-0.96; P=0.027), leading to a significant absolute risk reduction of 11% in mortality. In contrast, C5a receptor 1 (C5aR1) inhibition by avdoralimab, evaluated in a large randomized controlled phase 3 trial with 207 hospitalized patients with COVID-19, did not improve clinical outcomes³³. Differences in the observed efficacy between the two studies may be reflected by differences in the SARS-CoV-2 variants during the study, in patient characteristics, and potentially in C5a effector mechanisms. By blocking C5a, vilobelimab is targeting both the C5aR1 and C5aR2 whereas avdoralimab is interfering only with C5a - C5aR1 signaling. Thus, adverse inflammatory effects mediated via C5aR2 could potentially contribute to differences in the outcomes of the two studies. Interventional studies also investigated various other target of the complement system in COVD-19³⁴. For example, MASP-2 inhibition by the lectin pathway inhibitor narsoplimab improved survival in a small case series³⁵. Upstream inhibition of C5 by eculizumab showed improvements in survival but was associated with a higher rate of severe (infectious) adverse events. However, ravulizumab, another C5 inhibitor, failed to improve survival in a phase 3 randomized controlled trial with 201 invasive or non-invasive ventilated patients with COVID-19. The limited clinical effectiveness of upstream C5 inhibition might be related to an insufficient reduction of C5a levels previously observed with eculizumab³⁶, whereas vilobelimab was effective in inhibiting C5a levels³⁷. Notably, most of the studies had limitations in the sample size and study design. In addition, the investigation of therapeutic targets in COVID-19 has been heavily influenced by the rapid and ongoing evolution of SARS-CoV-2 variants, as well as the introduction of effective vaccinations and the use of immunosuppressants such as glucocorticosteroids and IL-6 inhibitors.

Taken together, our findings have supported the hypothesis that targeting circulating C5a is effective and favorable compared to other targets within the complement system for treating patients with severe COVID-19. This was recognized by the Food and Drug Administration that issued an emergency use authorization of vilobelimab for treating patients with COVID-19 requiring invasive mechanical ventilation in April 2023.

Annexin A1 is elevated and associated with adverse clinical outcomes in COVID-19

Given the critical role of excessive pro-inflammatory stimuli in the pathogenesis of COVID-19, substantial efforts have been dedicated to elucidating the precise underlying

mechanisms and potential targets for intervention. Investigating anti-inflammatory mechanisms and identifying targets to attenuate severe COVID-19 without the drawbacks of immunosuppressive therapies may also hold significant potential. Modulating excessive inflammation while preserving immune competence may mitigate the adverse effects of immune dysregulation. Annexin A1 (AnxA1) belongs to the annexin superfamily and acts as a pro-resolving mediator of inflammation. AnxA1 has been found intracellularly in various tissues and immune cells³⁸, and exhibits biochemical activity once it is externalized. It plays a role in the adhesion and migration of leukocytes. Particularly, AnxA1 enhances L-selectin shedding by neutrophils, thereby preventing the transendothelial migration of leukocytes to the site of inflammation³⁹⁻⁴¹. AnxA1 is also involved in the down-regulation of the inflammasome, T-cell activity, and wound healing^{42,43}. Interestingly, proteomic analysis showed that AnxA1 dysregulation was also found in neutrophils from patients with AAV⁴⁴ and renal expression of AnxA1 was higher in AAV patients with an improved kidney recovery after treatment⁴⁵. AnxA1 is an interesting option for clinical interventions because its anti-inflammatory effects are transduced through the formyl peptide receptor 2 (FPR2), which can be therapeutically targeted by human recombinant AnxA1 or FPR2-binding peptide (Ac2-26)⁴⁶.

In Chapter 5, we investigated the role of AnxA1 in inflammation, vascular damage and clinical outcomes in COVID-19 by assessing AnxA1 levels in the serum of 220 patients from our COVID-19 cohort using enzyme-linked immunosorbent assay (ELISA) at presentation and over time47. Compared to healthy controls, AnxA1 levels were significantly higher in patients with moderate (P<0.001) and severe COVID-19 (P<0.001) at presentation. AnxA1 levels tended to increase in admitted (i.e., moderate and severe) patients over time. Elevated AnxA1 levels were linked to increased markers of inflammation (i.e., CRP [P < 0.002] and C5a [P=0.007]) and endothelial damage (vWF:Ag [P<0.001]). Since AnxA1 is known as a pro-resolving mediator of inflammation, the elevated levels of AnxA1 in hospitalized patients may indicate a reciprocal feedback mechanism in response to the excessive inflammation and endothelial damage observed in COVID-19. Although markers of inflammation were higher in patients with severe compared to moderate COVID-19, AnxA1 levels did not differ between both groups at presentation and over time. This finding might indicate that the capacity to externalize more AnxA1 to buffer inflammation is eventually exhausted in patients with severe COVID-19. We also found that AnxA1 levels correlated with unfavorable clinical outcomes, specifically demonstrating that higher baseline levels of AnxA1 were predictive of an increased risk of thrombotic events in patients with COVID-19. The direct role of AnxA1 in regulating the coagulation system is unknown. However, we showed in the previous chapters that excessive inflammation with complement activation and NETs formation is critically linked to hypercoagulability in COVID-19^{1,16}. Only one other study also reported on AnxA1 in COVID-1948. In contrast to our findings, decreased AnxA1 levels were observed in patients with severe COVID-19. Furthermore, low AnxA1



levels were found to be predictive of the risk of ICU admission. The study differed from our study in both methodological and epidemiological aspects. It had included a high percentage of female patients (66%) with a severe course of disease which is highly uncommon for COVID-19⁴⁹. Different ELISA kits for the measurement of AnxA1 were used as well. Finally, our study included a considerably larger number of patients with longitudinal AnxA1 measurements, strengthening the reliability and robustness of our findings.

In conclusion, our findings support a role for an inadequate homeostasis of AnxA1 in hospitalized patients with COVID-19, especially in those cases with a severe course of disease. Although this must be confirmed in future studies, a potential therapeutic benefit for patients may arise through pharmacologically increasing FPR2 signaling by administering human recombinant AnxA1 or Ac2-26.

The intrinsic coagulation pathway plays a dominant role in driving hypercoagulability in Antineutrophil Cytoplasmic Antibody–Associated Vasculitis

AAV is with striking homology to COVID-19 also associated with excessive neutrophilic inflammation, complement activation, NETs formation and vascular damage. Moreover, patients with AAV have a higher incidence of VTEs than the general population, especially during active disease. We evaluated the intrinsic and extrinsic coagulation pathways by uniquely assessing activated coagulation factors in complex with their natural inhibitors and general clinical coagulation markers in a prospective cohort of 75 patients with AAV during active disease and after six months during remission in Chapter 6. Most patients had increased levels of D-dimers and fibrinogen at presentation, indicating hypercoagulability during active disease, which is in line with previous studies^{50,51}. Hypercoagulability generally improved after treatment with immunosuppressants, although this was not observed in all patients after six months of follow-up. One study previously showed that AAV patients in remission persisted in a hypercoagulable state, which might be attributed to ongoing low-grade inflammation and vascular damage⁵². By dissecting the coagulation cascade in more detail, we found that activated coagulation factors associated with the intrinsic coagulation pathway. Specifically FXIIa and FXIa in complex with their natural inhibitors were predominantly increased in patients with active AAV. Furthermore, patients with increased D-dimers or T:AT levels were associated with higher activity of the intrinsic coagulation pathway. These observations indicated that a strong activation of the intrinsic coagulation pathway characterized hypercoagulability in active AAV.

Although the exact mechanisms driving hypercoagulability were not studied here, disease activity and systemic inflammation, as reflected by higher Birmingham Vasculitis Activity Scores, ANCA levels, kidney injury and CRP, were associated with elevated

D-dimers in this thesis and in other studies^{50,51,53}. These observations might point towards immunothrombosis via the complement-neutrophil-coagulation axis as a driver of hypercoagulability in AAV. Comparable to what was observed in COVID-19. complement activation and NETs formation is abundant in AAV⁵⁴⁻⁵⁶. C3a and C5a levels were elevated in patients with active disease and C5a induced neutrophil activation through C5aR signaling^{57,58}. NETs from C5a primed neutrophils expressed high levels of TF and induced thrombin formation; NETs were also found to colocalize with thrombi in AAV⁵⁹. Additionally, activated platelets and DNA fragments released during the process of NETs formation activated the intrinsic coagulation pathway via FXII directly⁶⁰. There are numerous other factors contributing to hypercoagulability in AAV that, however, were beyond the scope of this thesis. As a result of endothelial cell damage, vWF antigen levels and vWF as well as FVIII activity were elevated in AAV, further increasing the procoagulable state⁵¹. Opportunistic infections might additionally trigger inflammation and coagulation; i.e., cytomegalovirus infection directly causes endothelial cell damage and subsequently thrombosis⁶¹. Taken together, hypercoagulability is most likely driven by excessive inflammation, NETs formation, endothelial damage, and platelet activation in AAV, leading to a dominant activation of the intrinsic coagulation pathway via FXII and FXI and less extensively to activation of the extrinsic coagulation pathway via the release of TF.

Future studies should focus on whether interfering with the complement-neutrophilcoagulation axis could ameliorate hypercoagulability in AAV. Similar to what we have learned from COVID-19, targeting the complement system might offer promising opportunities for the treatment of AAV. The C5aR1 inhibitor avacopan has been proven effective as a prednisone-sparing agent in patients with AAV⁶². It would be interesting to investigate its potential effects on improving hypercoagulability and reducing the risk of VTEs in these patients. Additionally, targeting the intrinsic coagulation pathway directly with novel FXIa inhibitors might also be a promising asset against the adverse events of hypercoagulability in AAV and should be further explored.

In conclusion, we found that hypercoagulability is predominantly characterized by activation of the intrinsic coagulation pathway in patients with active AAV. The driving factors of hypercoagulability are yet to be studied but are most likely related to an interplay of increased disease activity, vascular inflammation, and endothelial damage. Targets for intervention could include inhibitors of the intrinsic coagulation pathway and specific inhibition of neutrophil activation, such as through C5aR inhibition.

Summary and Future Perspectives

In summary, we found that complement activation, NETs formation and vascular damage are key features of inflammation and drive hypercoagulability via the intrinsic coagulation pathway in COVID-19 (**Chapter 2**). Subsequently, we showed in more detail



intrinsic rather than the extrinsic coagulation pathway that the defined hypercoagulability in COVID-19 (Chapter 3). Thrombin formation and vascular damage were important markers of disease severity. VTEs, and mortality in these patients. Based on the observations that complement and neutrophil activation are key, we provided evidence that C5a inhibition by vilobelimab was safe and a highly potential therapeutic target for patients with severe COVID-19 (Chapter 4). AnxA1 is another potential target for the treatment of COVID-19. This pro-resolving mediator of inflammation was increased and associated with adverse clinical outcomes in patients with COVID-19 (Chapter 5). Finally, we showed that patients with active AAV were also in a hypercoagulable state that was predominantly linked to the activation of the intrinsic coagulation pathway (Chapter 6). Similar to what we have learned from COVID-19, driving factors of hypercoagulability in AAV are likely related to an interplay of complement activation, NETs formation, and vascular damage, pointing to C5a inhibition or inhibitors of the intrinsic coagulation pathway. We have also learned from the experience of COVID-19 that it is crucial not to narrow our focus on a problem solely to our own field of expertise (i.e., immunology). Instead, we have learned to broaden our perspective with colleagues from other fields (i.e., coagulation, biochemistry, infectious diseases, and intensive care) to seek comprehensive answers collectively. Much like the complement and coagulation systems in immunothrombosis, (patho)physiological responses are not isolated to a single aspect of human biology but are intricately interconnected within responsive networks.

The work presented in this thesis contributes to our understanding of the underlying pathophysiological mechanisms of hyperinflammation and hypercoagulability in COVID-19 and AAV. The triangular relationship of complement activation, NETs formation and activation of the intrinsic coagulation pathway are important disease drivers, and thus, potential targets for intervention. We focused on C5a inhibition by vilobelimab, leading to an emergency use authorization of vilobelimab by Food and Drug Administration in patients with severe COVID-19. The insights of this thesis also bring up new questions. The patients included in phase 3 trial with vilobelimab were required to be mechanically ventilated before intervention was initiated. From an immunological perspective, the timing of C5a inhibition at an earlier stage of disease may have a more significant impact on mitigating hyperinflammation and tissue damage, translating into prevention of disease progression and mortality in even more patients with COVID-19. On the other hand, this would also increase the number needed to treat and health care costs. Addressing this concern could involve investigating predictive markers that identify patients who would benefit the most from C5a inhibition. The findings of this thesis offer the opportunity to investigate other potential treatment strategies in infectious and inflammatory conditions as well. Extracellular histones released during NETs formation exert strong cytotoxic and prothrombotic effects⁶³. The dynamics of extracellular histones and potential benefits of anti-histones targeted therapies should be further explored, not only in COVID-19 but also in other infectious diseases like bacterial sepsis or influenza. Anti-inflammatory targeted strategies through pharmacologically increasing AnxA1/FPR2 signaling by administering human recombinant AnxA1 or its Ac2-26 could also be a novel and beneficial approach in these patients. The strong link between hyperinflammation and activation of the intrinsic coagulation pathway in COVID-19 and AAV identified in this thesis indicates a role for targeting hypercoagulability in these and similar conditions. Rather than relying on prophylactic low molecular weight heparins, the use of intrinsic coagulation pathway blockers like FXIa inhibitors can be promising. For AAV, however, it is important to explore better discriminative clinical markers that help us to accurately identify and target patients with the highest risk of VTEs. In this line, the role of C5a inhibition on hyperinflammation and hypercoagulability in AAV is an interesting field of investigation since C5aR1 inhibition with avacopan is currently recommended for the treatment of patients with AAV.

As of 2023, the focus on COVID-19 and its devasting consequences has receded into the background. This does not mean that the findings of this thesis are not relevant. SARS-CoV-2 is still circulating, and the emergence of new variants represents an ongoing potential threat that COVID-19 could evolve into a more severe disease once again. SARS-CoV-2 also continues to pose a substantial health risk, particularly for vulnerable and immunosuppressed individuals worldwide. The lessons learned from this thesis also contribute to our understanding of the pathophysiological responses and interplay between the immune and coagulation systems in other diseases such as AAV. These findings not only suggest (potential) novel treatment approaches but should also be explored in the context of more diseases in the future.



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Addendum

Samenvatting Impact paragraph Dankwoord Curriculum Vitae List of publications

SAMENVATTING

Het onderzoek beschreven in dit proefschrift was gericht op het bestuderen van een verhoogde stollingsneiging (hypercoagulabiliteit) en de potentiele relatie met overmatige ontsteking in antineutrofiele cytoplasmatische antistoffen-geassocieerde vasculitis (AAV) met behulp van een prospectieve observationele studie die in 2019 gestart was. Toen dook het coronavirusziekte 2019 (COVID-19) op in Nederland. Vroege observaties van een ontregeld immuunsysteem en een hoog aantal veneuze trombo-embolieën (VTEs) bij patiënten met ernstige COVID-19 deden ons vermoeden dat er een verband was tussen ontsteking en een verhoogde stollingsneiging, vergelijkbaar met wat we aannemen bij AAV. Er was een grote behoefte om de onderliggende pathofysiologie snel te ontrafelen en om potentiele therapeutische doelen te identificeren voor patiënten met COVID-19. Als gevolg hiervan verschoof het doel van dit proefschrift naar het verkrijgen van een beter begrip van de relatie tussen overmatige inflammatie en hypercoagulabiliteit bij COVID-19 en het toepassen van de geleerde lessen op AAV.

In **Hoofdstuk 1** worden de belangrijkste onderwerpen van dit proefschrift geïntroduceerd. Het geeft een overzicht over het complement systeem en het stollingssysteem en de evolutionaire interacties tussen beide systemen, waaronder ook immunothrombose valt. Verder worden de relevante inzichten van COVID-19 beschreven inclusief een mogelijke rol voor de anti-inflammatoire mediator Annexine A1 (AnxA1) in COVID-19. Daarna wordt een introductie gegeven over de bestaande kennis van AAV met ook een overzicht van de stollingsproblematiek bij patiënten met AAV. Tenslotte worden de hoofdlijnen en doelstellingen van dit proefschrift uitgelegd.

In Hoofdstuk 2 wordt bewijs geleverd voor de schadelijke rol van de complementneutrofiel-stolling as in COVID-19. In een grote prospectieve cohortstudie van 228 patiënten met COVID-19 werd aangetoond dat naarmate de ziekte ernstiger wordt, complement 5a (C5a) levels stijgen, neutrofiel extracellulaire netten (NETs) gevormd worden en de intrinsieke stollingscascade sterk wordt geactiveerd. C5a was verhoogd bij 153 (76%) van de patiënten en voornamelijk bij patiënten met matige en ernstige COVID-19. Deze bevindingen geven aan dat naarmate de ernst van COVID-19 toeneemt, het complement steeds meer wordt geactiveerd. Daarnaast werd op verschillende manieren aangetoond dat de vorming van NETs overvloedig is bij patiënten met ernstige COVID-19. DNA fragmenten en histonen die tijdens de vorming van NETs vrij komen kunnen de intrinsieke stollingscascade activeren. Daarom hebben wij geactiveerde stollingsfactoren gebonden aan hun natuurlijke remmers van de intrinsieke route in de loop van de tijd Een verhoogde stollingsneiging, aangegeven door geanalyseerd. verhoogde trombine: antitrombine (T:AT) levels, werd in 131 (60%) van de patiënten met COVID-19 gevonden. De stollingsfactoren van de intrinsieke route waren verhoogd en een
Addendum

verhoogde activatie was geassocieerd met de ernst van de ziekte. Deze bevindingen suggereren dat de verhoogde stollingsneiging in COVID-19 grotendeels door complement activatie, NETs formatie en activatie van de intrinsieke stollingscascade aangedreven wordt. Toekomstige studies moeten verder onderzoeken of bijvoorbeeld remming van het complement systeem of interventie op het niveau van NETs formatie de ontstekingsreactie en een ernstig ziektebeloop bij COVID-19 kunnen verminderen (*zie Hoofdstuk 4*). Daarnaast is een nadeel van deze studie dat de extrinsieke stollingscascade niet onderzocht werd om nog beter de dynamiek van het geactiveerde stollingssysteem bij COVID-19 te begrijpen.

In Hoofdstuk 3 wordt daarom door aanvullend de markers van de extrinsieke stolling te analyseren nog meer in detail de stollingscascade in hetzelfde COVID-19 cohort onderzocht. De levels van vrij FVIIa en FVIIa:AT, een marker van circulerende FVIIatissue factor complexen, verschilden statisch niet tussen de patiënten met COVID-19 en veranderden ook niet in de loop van de tijd. Het werd bevestigd dat de verhoogde stollingsneiging vanuit de intrinsieke maar niet extrinsieke stollingscascade aangedreven werd. C-reactive protein vertoonde een positieve correlatie met geactiveerde stollingsfactoren van de intrinsieke cascade, T:AT en von Willebrandfactor:Antigen (vWF:Ag), wat opnieuw de activiteit van de intrinsieke stollingscascade en endotheel schade koppelt aan overmatige ontsteking bij COVID-19. Vervolgens werd de prognostische waarde van de geactiveerde stollingsfactoren gebonden aan hun natuurlijke remmers en vWF:Ag gemeten en gerelateerd aan intensive care unit (IC) opname, trombose en mortaliteit. Verhoogde FXIa: α 1AT en T:AT levels bij presentatie waren geassocieerd met een verhoogd risico op een IC opname en verhoogde T:AT levels waren ook met een verhoogd risico op trombose geassocieerd. Tenslotte werd de prognostische waarde van de stollingsfactoren en vWF:Ag in de loop van de tijd met behulp van lineair mixed models beoordeeld bij patiënten die in het ziekenhuis opgenomen waren. Voortdurende endotheel schade, weerspiegeld door stijgende vWF:Ag levels in de loop van de tijd, waren geassocieerd met IC opname en mortaliteit, terwijl activatie van de intrinsieke stollingscascade op een verhoogde stollingsneiging bij COVID-19 wees. Op basis van deze bevinding moet verder worden onderzocht of remmers van de intrinsieke stollingscascade, zoals FXIa remmers, effectief kunnen zijn in het verminderen van trombose bij ernstig ziekte patiënten met COVID-19.

In **Hoofdstuk 4** wordt samen met onderzoekers van het Amsterdam UMC de hypothese getest dat remming van C5a de ontstekingsreactie en endotheel schade kan verminderen en in potentie het ernstige beloop bij COVID-19 kan voorkomen. Hiervoor werd de veiligheid en mogelijke voordelen van vilobelimab, een monoclonaal antilichaam gericht tegen C5a, bij 30 patiënten met ernstig COVID-19 in een exploratieve, open-label, fase 2 gerandomiseerde klinische studie onderzocht. De verandering in de verhouding van de

partiele druk van arteriële zuurstof tot de fractie zuurstofconcentratie in de ingeademde lucht verschilde niet tussen de behandelings- en controlegroep op dag 5 na opname. C5a inhibitie was veilig en vertoonde voordelen bij het verminderen van nierschade, longembolieën en mortaliteit. De bevindingen werden beperkt door het kleine aantal geïncludeerde patiënten, maar gaf aanleiding tot verder onderzoek met een grootschalige fase 3 klinische studie.

In **Hoofdstuk 5** wordt de rol van AnxA1 bij ontsteking, endotheel schade en klinische uitkomsten bij COVID-19 onderzocht door AnxA1 levels in het serum van 220 patiënten uit het COVID-19 cohort te bepalen bij de eerste presentatie en longitudinaal. AnxA1 levels waren significant hoger in patiënten met een matig en ernstig ziektebeloop en leken in de loop van de tijd nog door te stijgen. De hoogte van AnxA1 was gekoppeld aan ontstekingsmarkers, zoals CRP en C5a, en aan endotheel schade, namelijk vWF:Ag. Daarnaast voorspelden hogere AnxA1 levels een verhoogd risico op trombose bij patiënten met COVID-19. Aangezien Anx1 een anti-inflammatoire mediator is kunnen de hogere levels van AnxA1 bij de opgenomen patiënten wijzen op een feedback-mechanisme als reactie op de overmatige ontsteking en endotheel schade bij COVID-19. Het is interessant om te onderzoeken of de capaciteit om meer AnxA1 te externaliseren om ontstekingen te dempen uiteindelijk wordt overschreden bij patiënten met ernstige COVID-19 en of farmacologische interventie door middel van humane recombinante AnxA1 of Ac2-26 dus voordelig zou kunnen zijn.

In **Hoofdstuk 6** wordt een vertaalslag van de bevinding uit dit proefschrift van COVID-19 naar AAV gemaakt. Het mechanisme achter de verhoogde stollingsneiging bij AAV werd onderzocht in een prospectieve cohortstudie. Algemene klinische stollingsmarkers en geactiveerde stollingsfactoren gebonden aan hun natuurlijke remmers werden hiervoor gemeten bij 75 patiënten met actieve ziekte en na 6 maanden tijdens remissie. Ddimeren. fibrinogeen en geactiveerde stollingsfactoren van de intrinsieke stollingscascade, namelijk FXIIa en FXIa gebonden aan hun natuurlijke remmers, waren voornamelijk verhoogd bij patiënten met actieve AAV en bleven bij een deel van de patiënten zelfs nog na 6 maanden verhoogd. Patiënten met een verhoogde stollingsneiging, namelijk hoge D-dimeer of T:AT levels, vertoonden een verhoogde activatie van de intrinsieke stollingscascade. Hoewel de exacte mechanismen die de verhoogde stollingsneiging bij AAV aansturen in dit hoofdstuk niet volledig bestudeerd werden, waren ziekteactiviteit en ontsteking, zoals weergegeven door een verhoogde Birmingham Vasculitis Activity Score, ANCA levels, nierschade en CRP, geassocieerd met verhoogde D-dimeren. Toekomstige studies moeten verder onderzoeken of inderdaad de complement-neutrofiel-stolling as net als bij COVID-19 ook een belangrijke rol speelt in het aandrijven van de verhoogde stollingsneiging bij AAV. Ook hier biedt zich C5a remming door middel van avacopan aan, een C5aR1 remmer die bij patiënten

Addendum

met AAV inmiddels is toegelaten, om deze interactie verder te onderzoeken. Daarnaast wijzen de inzichten van dit hoofdstuk in de richting van interventie door middel van bijvoorbeeld FXIa remmers om de activiteit van de van de intrinsieke stollingscascade en dus het trombose risico bij patiënten met actieve AAV te beperken.

Tot slot worden in **Hoofdstuk 7** de inzichten van dit proefschrift samengevat en in de context van de bestaande wetenschappelijke literatuur kritisch bediscussieerd. Daarnaast worden de getrokken lessen van COVID-19 uit dit proefschrift besproken en vertaald naar AAV. De relevantie van deze bevindingen wordt dan ook in een toekomstig perspectief geplaatst en er worden suggesties voor vervolgonderzoeken gemaakt.

IMPACT PARAGRAPH

The World Health Organization declared COVID-19 as a pandemic on 11th of March 2020. The consequence was a global crisis with a tremendous impact on public health, social life, and economies. Evidence based treatment strategies were scarce at the beginning of the pandemic but substantial scientific and financial efforts led to a stepwise improvement in the understanding of the pathogenesis and therapeutic options for COVID-19.

Our research group entered the scientific COVID-19 rollercoaster early when SARS-CoV-2 made its debut in the Netherlands. As a team of immunologists, we were puzzled by the excessive systemic inflammation and damaged pulmonary vasculature observed in patients with severe COVID-19. We noticed striking homology with the pathogenesis of autoimmune diseases like AAV in which autoantibody and complement driven adverse neutrophilic responses (i.e., excessive NET formation) lead to vasculature inflammation, thrombosis and end-organ damage. The hypothesis was that SARS-CoV-2 elicited a dysregulated innate immune response with excessive complement activation and NETs formation that was linked to hypercoagulability in patients with severe COVID-19. The research presented in this thesis showed that, indeed, hypercoagulability in COVID-19 was associated with complement activation and NETs formation via the intrinsic pathway of coagulation. These findings were later confirmed and extended by other groups. The insights gained were socially and economically meaningful because they improved the understanding of the pathophysiology of COVID-19 and implicated a role for the inhibition of the complement system or the intrinsic coagulation pathway (i.e., plasma kallikrein and FXIa blocker) for the treatment of severe COVID-19. Indeed, as reported in this thesis C5a inhibition with vilobelimab was safe and had potential benefits on clinical outcomes in a phase two randomized controlled trial. The follow-up phase three randomized controlled trial confirmed these findings, leading to an emergency use authorization of vilobelimab for the treatment of patients with severe COVID-19 in 2023. Next, the conceptual insights gained on AnxA1 in patients with COVID-19 may present a novel anti-inflammatory treatment approach in infectious or auto-immune diseases, potentially avoiding the adverse side effects associated with currently used immunosuppressive drugs. Taken together, the findings described in this thesis indicated a successful application of translational basic science research resulting into a clinical intervention within a time frame of about three years and also directly impacted patients with COVID-19 and the health care system.

The thesis also aimed to better understand the link between adverse immune responses and hypercoagulability cross-sectionally in AAV. As previously outlined, one hypothesis was that comparable adverse (innate) immune responses and coagulation abnormalities might drive the course of disease in COVID-19 and AAV. Particularly during active disease, the number of thrombotic events is increased in patients with AAV. However, the underlying mechanisms that contribute to hypercoagulability in AAV and how to prevent them are incompletely understood. Thrombotic events in AAV pose significant risks to patients' well-being and the health care system, as they can be potentially lifethreatening and lead to long-term comorbidities. Consequently, gaining a better understanding of hypercoagulability in AAV is important for improved clinical management and patient outcomes. This thesis describes that hypercoagulability is, similarly to COVID-19, linked to activity of coagulation factors of the intrinsic coagulation pathway in patients with AAV. The driving mechanisms were not investigated but might also be linked to excessive complement and neutrophil activation leading to vascular inflammation and endothelial damage. The findings point towards inhibitors of coagulation factors of the intrinsic coagulation pathway or potentially complement inhibition as potential therapeutic targets to reduce the risk of thrombotic events in AAV.

Future directions

More than three years later, following the implementation of effective vaccination programs combined with a rather benign evolution of SARS-CoV-2 variants, the pandemic has come to an end. This does not mean that the impact of the findings described in this thesis are no longer relevant. SARS-CoV-2 is still present and there remains a potential risk that a new variant could lead to a severe course of disease. The insights obtained from this thesis regarding the pathophysiological mechanisms and the efficacy of C5a inhibition in COVID-19 can provide valuable guidance in mitigating the adverse effects in the event of a resurgence of a SARS-CoV-2 variant. This is particularly important for high-risk patients for whom COVID-19 can still be fatal and those who may progress to severe COVID-19 despite receiving widely recommended immunosuppressants like glucocorticosteroids or IL-6 inhibitors. Future studies should investigate the potential harms and benefits of combining vilobelimab with the previously mentioned immunosuppressants in COVID-19. Additionally, it would be valuable to explore whether vilobelimab could be considered as the first choice in selected patients with severe COVID-19. The lessons learned that the innate immune system is linked to the coagulation system and defines disease severity in COVID-19 (and AAV) should be applied and studied in other (infectious) diseases like influenza or bacterial sepsis. This includes further research on vilobelimab's efficacy in reversing systemic inflammation (and hypercoagulability) in conditions associated with sepsis, considering the unfavorable outcomes of glucocorticosteroid use in most of the sepsis studies in the past.

The exact driving mechanisms of hypercoagulability in AAV remain to be discovered. This thesis indicated a dominant contribution of the intrinsic coagulation cascade in the process of hypercoagulability in AAV. Future studies should address the underlying driving factors that result into the activation of the coagulation system. Since the risk of thrombotic events is the highest during active disease, again, overactivation of the complement system, neutrophils and NETs formation are potential targets to focus on besides endothelial and vascular damage. Clinically, it is difficult to identify the patients at risk for a thrombotic event, future studies should explore predictors of hypercoagulability and whether the prophylactic use of anticoagulants can be preventive in these patients. The results of this thesis point towards a potential role for inhibitors of coagulation factors of the intrinsic pathway to prevent VTEs. Furthermore, C5aR1 inhibition with avacopan is now accessible for the treatment of patients with AAV. It would be interesting to explore whether C5aR1 or C5a inhibition with vilobelimab (the results of a phase 2 randomized controlled trials in AAV are in preparation) is associated with an improvement of the observed coagulation abnormalities and a reduced risk of thrombotic events.

DANKWOORD

Met dit dankwoord beëindig ik mijn proefschrift en wil ik mijn collega's en dierbaren erkennen die mij gedurende deze reis ongelooflijk hebben gesteund.

Dr. van Paassen, Pieter, ik zal ons eerste gesprek op jouw kantoor ergens in 2017 nooit vergeten; jij gaf mij het vertrouwen dat het wisselen van specialisatie naar de klinische immunologie en allergologie de juiste keuze was. Later in 2019 zag jij al wat ik toen nog niet direct zag, namelijk hoe leuk en belangrijk een promotieonderzoek zou zijn voor mijn toekomstige carrière. Ik heb vele jaren met jou als arts en onderzoeker mogen samenwerken en samen hebben we vol enthousiasme de wonderen in ons vak ontdekt. Jij hebt mij zowel in de kliniek als in de wetenschap opgeleid zonder dat het als een opleiding voelde. Altijd stond je voor me klaar maar gaf je me ook de vrijheid en het vertrouwen om te worden wie ik nu ben. Hoewel onze toekomstplannen anders zijn verlopen dan verwacht, ben ik enorm trots en vooral dankbaar dat ik zoveel jaren aan jouw zijde mocht staan.

Prof. dr. Reutelingsperger, Chris, ik heb het jou nooit verteld, maar jij leerde mij al lang geleden tijdens mijn bachelor moleculaire levenswetenschappen de wetenschappen voordat wij elkaar echt leerden kennen. Tijdens mijn promotieonderzoek heb jij mij vol enthousiasme de wereld van de Annexines laten ontdekken, met als hoogtepunt het Annexine congres in Stockholm. Jij stond altijd klaar met raad en daad, en het was verfrissend om weer eens de problemen vanuit de fundamentele biochemie te benaderen. Jouw vrolijke en toegankelijke begeleiding hebben mij enorm geholpen om het proefschrift tot een goed einde te brengen.

Dr. Damoiseaux, Jan, ik wil je hartelijk bedanken voor de onschatbare rol die je hebt gespeeld tijdens mijn opleiding en promotieonderzoek. Met jouw expertise heb je mij de weg gewezen in het vakgebied van de immunologische diagnostiek, een cruciale pijler in mijn rol als arts. Jouw analytische en gestructureerde benadering van wetenschappelijk onderzoek heeft mij enorm geholpen om mijn promotieonderzoek op een georganiseerde en efficiënte manier af te ronden. Ik waardeer bijzonder dat jij altijd beschikbaar was waardoor jij mij in zo veel momenten van mijn werkzaamheden in Maastricht hebt ondersteund.

Ik wil ook de beoordelingscommissie bestaande uit Prof. dr. Leon Schurgers, Prof. dr. Yvonne Henskens, dr. Abraham Rutgers en Prof. dr. Frank van de Veerdonk hartelijk bedanken voor de tijd en moeite die geïnvesteerd werd in het beoordelen van mijn proefschrift. I want to thank the Target to B consortium. This scientific collaboration comprised brilliant and creative minds whose contributions were crucial to my personal scientific development.

Hartelijke dank aan het team van Prof. dr. Alexander Vlaar van het Amsterdam UMC voor de fijne en belangrijke samenwerking tijdens de coronapandemie met betrekking tot de PANAMO II en III studies.

Beste Prof. dr. ten Cate, Hugo, dr. Spronk, Henri, en dr. Nagy, Magdi, ik wil jullie in het bijzonder bedanken. Dankzij jullie expertise en unieke vaardigheden op het gebied van stollingsonderzoek was het mogelijk om het stollingssysteem in detail te onderzoeken bij COVID-19 en AAV patiënten. Jullie wetenschappelijke bijdrage aan dit proefschrift is van grote waarde. Deze samenwerking heeft mijn denken over de samenhang tussen stolling en immunologie substantieel verbeterd. Hopelijk zal de kruisbestuiving tussen het stollings- en auto-immuun onderzoek in Maastricht leiden tot nog meer belangrijke inzichten in de toekomst.

Mijn oprechte dank gaat uit naar alle patiënten die hebben deelgenomen aan de onderzoeken in dit proefschrift. In het bijzonder wil ik mijn dank uitspreken aan de vasculitispatiënten en aan Peter Verhoeven aan de Vasculitis Stichting, die mij onvoorwaardelijk hun vertrouwen schonken. Als arts en onderzoeker heb ik het onvergetelijke voorrecht gehad om een deel van hun leven te mogen begeleiden.

Ik wil graag iedereen van het RNWG lab - Lucienne, Nele, Ruud, Henk en Maarten bedanken. Toen ik als arts in opleiding bij jullie kwam, werd ik meteen hartelijk opgenomen in de groep. Met elk van jullie unieke eigenschappen hebben jullie op verschillende manieren een belangrijke rol gespeeld in mijn wetenschappelijke ontwikkeling. Ik zal de wekelijkse onderzoeksvergaderingen ("gaat die vandaag door of niet?") en de legendarische vrijdagborrels enorm missen. Aangesloten hieraan wil ik mijn gezellige PhD lotgenoten uit de groep bedanken. Joop, ik ben ingestapt in de wetenschappelijke wereld van de vasculitis toen jij al een tijdje bezig was. De gezamenlijke congressen met jou rondom de wereld waren inspirerend en vooral heel erg gezellig. Wij hebben hard gewerkt en samen veel met elkaar gelachen. Renée, toen jij na de semi-arts stage op de afdeling immunologie met jouw wetenschappelijk onderzoek begon, ging alles meteen in de versnelling. Je hebt het prospectieve vasculitis onderzoek verder uitgebouwd en gevormd tot wat het nu is. We hebben veel leuke momenten gedeeld en vulden elkaar uitstekend aan. Rachid, helaas geen vasculitis onderzoeker maar niet minder enthousiast en leuker dan de rest van de groep. Wij hebben veel met elkaar gelachen en vooral de McDonalds en tafeltennis in de nachtdiensten waren hilarisch. Daan, jij bent de meest recente promovendus die bij de groep is aangesloten,

maar vanaf het allereerste moment altijd een superfijne en gezellige toevoeging geweest. Je bent van enorme waarde voor de groep, en ik wil je specifiek bedanken voor jouw uitstekende epidemiologische en R-vaardigheden waarmee je me hebt bijgestaan. En allerlaatst Sjoerd, ik wil je hartelijk bedanken voor je onschatbare bijdrage aan mijn wetenschappelijke ontwikkeling. Ik heb ontzettend veel van je geleerd. Samen hebben we de afgelopen jaren talloze leuke projecten uitgevoerd en fantastische ideeën voor de toekomst in Maastricht bedacht. Toch lag de basis altijd in de goede gesprekken die we hadden, over van alles en nog wat, met een grote pot bier voor ons. Het vervult me met bijzondere trots dat je me tijdens mijn verdediging als paranimf zult steunen.

Beste Chris, Judith en Marjan, jullie hebben mij klinisch opgeleid en ik heb met jullie een fantastische tijd bij de klinische immunologie en allergologie gehad. Op totaal verschillende manieren hebben jullie mij in mijn ontwikkeling als arts geholpen en gesteund, ik wil jullie hiervoor enorm danken. Marjan, wij waren beiden met de opleiding tot klinisch immunoloog en allergoloog bezig toen wij elkaar leerden kennen; het was gezellig om met jou samen deze eerste stappen in de differentiatie te mogen doen. Chris, jij was mijn meester in de allergologie, nu in een ander centrum werkende ben ik zo ontzettend dankbaar voor hoeveel jij mij hebt geleerd. Judith, jij bent voor mij een voorbeeld. Ik heb veel van jou in de kliniek geleerd, daarnaast was ik altijd enorm onder de indruk van jouw onuitputbare power en veerkracht. Wij hebben allemaal veel positieve en negatieve dingen met elkaar doorgemaakt, uitsluitend de positieve ervaringen blijven in herinnering.

Ik wil al mijn collega's van het Maxima Medisch Centrum en Maastricht Universitair Medisch Centrum bedanken, die mij bewust of onbewust hebben geholpen in mijn ontwikkeling als arts, onderzoeker en persoon. Ook wil ik mijn nieuwe collega's van het Rijnstate ziekenhuis bedanken voor het vertrouwen dat ik vanaf het begin mocht ervaren.

Beste Roswitha, Ronald en Marlies, op talloze momenten in mijn leven hebben jullie mij en Elke op zowel bewuste als onbewuste wijze gesteund en geholpen. Ik ben ontzettend dankbaar en trots dat jullie zo'n essentieel onderdeel van mijn leven vormen. Marlies, het is lachen hoe jij op de SEH sneller dan de artsen de juiste immunologische diagnoses bij de patiënten stelt.

Worte sind ungenügend, um meine Gefühle für euch zu beschreiben, daher halte ich es kurz: Meine aufrichtigste Wertschätzung gilt meinen Eltern Gerti und Andreas und meiner Schwerster Christina für die unerschütterliche Unterstützung in allen Aspekten meines Lebens. Euer bedingungsloser Rückhalt, zum Beispiel nach meiner Entscheidung für ein Studium in den Niederlanden, bedeutet mir sehr viel. Ich bin daher auch besonders stolz darauf, dass du, Christina, bei meiner Verteidigung als meine "paranimf" hinter mir stehst.

Tenslotte wil ik de laatste woorden aan mijn echtgenote richten. Lieve Elke, wij hebben elkaar leren kennen tijdens onze studie in Maastricht, en ik ben enorm trots en dankbaar dat je al zo lang aan mijn zijde staat. Wij hebben veel prachtige momenten beleefd, maar helaas ook enkele moeilijke. Als absolute hoogtepunten denk ik zeker aan onze bruiloft en aan de geboorte van onze dochter Charlotte en zoon Lucas, die ons zoveel liefde en vreugde schenken. Ik ben trots op de persoon die jij bent en op alles wat je doet. Jij hebt altijd de familie op de eerste plaats gezet. Zonder jouw onvoorwaardelijke steun had ik nooit daadwerkelijk kunnen bereiken wat wij tot nu toe samen hebben bereikt. Ik ben ongelofelijk dankbaar en blij dat wij elkaar hebben gevonden en dat je bij mij bent.

CURRICULUM VITAE

Matthias Heinrich Busch was born on May 2nd 1987 in Haselünne, Germany. He graduated from the high school Kreisgymnasium St. Ursula in Haselünne in 2006. After obtaining his bachelor's degree in Molecular Life Sciences from Maastricht University in 2010, he was selected for the Medical and Clinical Research master program ("Arts-Klinisch Onderzoeker") at Maastricht University, from which he graduated in 2014. He performed his residency in internal medicine at the department of internal medicine at Máxima Medisch Centrum in Veldhoven and at Maastricht University Medical Center in Maastricht



between 2014 and 2018. Afterwards he began his specialization in allergy and clinical immunology at the Maastricht University Medical Center. During his specialization, he started as a PhD candidate at the department of nephrology and clinical immunology at the Maastricht University Medical Center under supervision of dr. P. van Paassen, prof. C. Reutelingsperger and dr. J. Damoiseaux in 2019. He combined his clinical and scientific passion on antineutrophil cytoplasmic antibody-associated vasculitis, as well as working on COVID-19 research during the pandemic. In 2022 he completed his specialization in internal medicine with focus on allergy and clinical immunology and continued his PhD candidacy and clinical duties in Maastricht. During his time in Maastricht, he participated in national research collaborations like Target to B, contributed to several clinical trials as a sub-investigator, and was board member of the junior Dutch Internal Medicine Society and junior member of the steering committee of the national educational workgroup for allergology and clinical immunology. In 2023, he moved with his family to Arnhem and is currently working as a physician at the department of internal medicine with focus on allergology and clinical immunology at the Rijnstate hospital in Arnhem. He is married to Elke and father of two children.

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