# Inflammation and Hypercoagulability in Anti-neutrophil Cytoplasmic Antibody associated Vasculitis

# 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 complement-neutrophil-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 activated[1].

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 H[2], the classical pathway is directly activated by IgM and IgG antibody immune complexes[3], 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 2[4]. 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 C5a[7]. 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 histones 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 severity[8-10].

Since DNA fragments and histones released from NETs can trigger the intrinsic coagulation pathway[11], 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:α1antitrypsin (FXIa:α1AT), 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:α1AT, and FIXa:AT, at baseline in plasma of 30 patients with COVID-19[12]. 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-19[13,14]. 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 thrombogenicity[8]. Of importance, FXII was found to co-localize with NETs on lung biopsies and improved clearance of NETs reduced activation of FXII in vitro[15], 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-19[16]. 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:α1AT 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-19[12]. 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 cells[18]. Indeed, SARS-CoV-2 infection resulted in platelet hyperactivity that directly correlated with mortality and an altered platelet transcriptome[19]. Platelet-monocyte interactions trigger TF expression in patients with severe COVID-19[20]. Furthermore, platelet-neutrophil aggregates were confirmed in microthrombi containing NETs in lung biopsies of deceased patients with COVID-19[13]. 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-19[21]. 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 thrombin[22] and can cleave prothrombin *in vitro*[23]. 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 C5a[26]. 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-19[27]. 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.

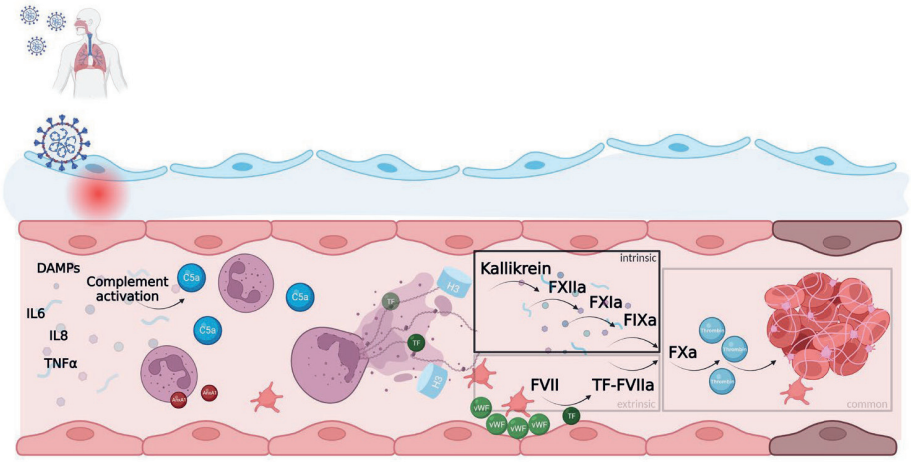


**Figure 7.1** Links between hyperinflammation and hypercoagulability in COVID-19. The illustration summarizes the pathophysiological mechanisms described in this thesis that contribute to hyperinflammation and hypercoagulability after SARS-CoV-2 infection in patients with severe COVID-19. *Abbreviations*: DAMPs, damage-associated molecular patterns. IL, interleukin. TNFα, 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-neutrophil-coagulation 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 mortality[28-30]. 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, 2020[31]. 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 patients[32]. 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 outcomes[33]. 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-19[34]. For example, MASP-2 inhibition by the lectin pathway inhibitor narsoplimab improved survival in a small case series[35]. 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 eculizumab[36], whereas vilobelimab was effective in inhibiting C5a levels[37]. 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 cells[38], 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 inflammation[39-41]. 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 AAV[44] and renal expression of AnxA1 was higher in AAV patients with an improved kidney recovery after treatment[45]. 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 time[47]. 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-19[48]. 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-19[49]. 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 damage[52]. 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 AAV[54-56]. 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 AAV[59]. Additionally, activated platelets and DNA fragments released during the process of NETs formation activated the intrinsic coagulation pathway via FXII directly[60]. 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 pro-coagulable state[51]. Opportunistic infections might additionally trigger inflammation and coagulation; i.e., cytomegalovirus infection directly causes endothelial cell damage and subsequently thrombosis[61]. 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-neutrophil-coagulation 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 AAV[62]. 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

that the intrinsic rather than the extrinsic coagulation pathway 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 effects[63]. 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.

# 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 complement-neutrofiel-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 geanalyseerd. Een verhoogde stollingsneiging, aangegeven door 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

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 FVIIa-tissue 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:$\alpha$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. D-dimeren, 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

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.