УНИВЕРЗИТЕТ У НИШУ
МЕДИЦИНСКИ ФАКУЛТЕТ

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ЕКСТРАЋЕЛИЈСКЕ ВЕЗИКУЛЕ КАО БИОМАРКЕРИ АКТИВНОСТИ БОЛЕСТИ КОД ВАСКУЛИТИСА УДРУЖЕНИМ СА АНТИТЕЛИМА ПРОТИВ ЦИТОПЛАЗМЕ НЕУТРОФИЛА

ДОКТОРСКА ДИСЕРТАЦИЈА

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Data on Doctoral Dissertation

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Title:	Extracellular Vesicles as Biomarkers of Disease Activity in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis

Abstract:

The purpose of this scientific investigation was to evaluate the degree of inflammation and disease activity using activity markers on extracellular vesicles in patients with ANCA-positive vasculitis, and to compare findings with healthy controls. The research was conducted in collaboration with Karolinska University Hospital, Solna, Sweden, within ERASMUS plus exchange program between University of Niš and Karolinska Institute. Data and biological samples were obtained from the VASKA cohort, which included patients with active (defined as BVAS≥1, infection excluded) and inactive ANCA vasculitis, i.e. in remission (BVAS=0). Age- and sex-matched healthy individuals served as control subjects. Concentration and phenotype of extracellular vesicles in plasma were assessed by flow cytometry, in both active and inactive disease, and compared to healthy controls. In addition, the pro-coagulant properties of extracellular vesicles were examined through their effect on thrombin formation, using a modified method based on Calibrated Automated Thrombogram (CAT) assay. The results demonstrated significantly elevated levels of extracellular vesicles bearing PTX3, HMGB1, TWEAK and components of complement system (C3a and C5a) in patients with active disease compared to those in remission and with healthy subjects. This study demonstrates that circulating extracellular vesicles are significantly elevated in active AAV and correlate with clinical disease activity (BVAS). These findings support their potential role as biomarkers for disease monitoring.

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Наслов:	Екстрaћелијске везикуле као биомаркери активности болести код васкулитиса удруженим са антителима против цитоплазме неутрофила

Резиме:	Циљ овог научног истраживања је процена степена запаљења и активности болести код пацијената са АНЦА позитивним васкулитисом, у односу на здраве особе, а помоћу маркера болести локализованим на екстрaћелијским везикулама. Испитивање је спроведено у сарадњи са Каролинска Универзитетском болницом у Штокхолму, Шведска, у оквиру ЕРАСМУС плус пројекта размене студената и наставника докторских академских студија између Универзитета у Нишу и Каролинска Института. Коришћени су подаци и узорци из базе података ВАСКА, која укључује пацијенте са активним (дефинисан као БВАС≥1, у одсуству инфекције) и неактивним АНЦА васкулитисом, тј. у ремисији (БВАС=0). Као контролни, коришћени су узорци здравих особа, одговарајуће старосне и полне дистрибуције. Процењивали смо број и фенотип екстрaћелијских везикула у плазми помоћу метода флоуцитометрије, током активне болести и ремисије, у поређењу са здравим контролама. Додатно, испитиван је прокоагулатни утицај екстрaћелијских везикула на стварање тромбина, применом модификованог метода заснованог на Калибрисаном аутоматизованом тромбограму. У овом истраживању је показано статистички значајно повећање нивоа екстрaћелијских везикула које на својој површини експримирају следеће параметре: PTX3, HMGB1, TWEAK и компоненте система комплемента Ц3а и Ц5а, код пацијената са васкулитисом у поређењу са здравим особама, одн. у поређењу са пацијентима у

	ремисији. Такође су показани повишени нивои циркулишућих екстраћелијских везикула код пацијената са активним васкулитисом у поређењу са пацијентима у ремисији и здравим особама. Осим тога, пронађена је корелација са активношћу болести мерене БВАС-ом, што наводи да ове везикуле могу бити потенцијални биомаркер активности болести код васкулитиса удруженим са антителима против цитоплазме неутрофила.
Научна област:	Медицинске науке
Научна дисциплина:	Педијатрија, Имунологија, Реуматологија
Кључне речи:	Екстраћелијске везикуле, васкулитиси удружени са антителима против цитоплазме неутрофила, биомаркери, активност болести
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ABSTRACT

Background

Extracellular vesicles (EV), previously termed as microparticles, are irregularly shaped submicron membrane-derived structures, generated from the plasma membrane during cell activation, early stages of apoptosis, or in response to cellular stress. Although theoretically all eukaryotic cells can release extracellular vesicles, those originating from circulating blood cells—platelets, erythrocytes, and leukocytes—as well as vascular or endothelial cells, have been studied most extensively. They are involved in diverse biological functions, including cell-to-cell communication, protein transport, regulation of apoptosis and inflammation, and processes such as haemostasis, thrombosis, angiogenesis, and vascular tone. Their molecular composition – membrane fragments, cytoplasmic components, enzymes, and nucleic acids, reflects their cellular source, but also is influenced by environmental factors and the processes that led to their formation.

Vasculitis associated with circulating antineutrophil antibodies (ANCA) is a heterogeneous systemic condition defined by necrotizing vasculitis of small- and medium-sized blood vessels, often accompanied by granulomatous infiltrates rich in neutrophils or eosinophils. The pathogenic process is strongly driven by ANCA-mediated neutrophil activation.

Aim

The study aimed to investigate whether activity markers expressed on extracellular vesicles can reflect inflammation and disease activity in patients with ANCA-positive vasculitis, in comparison with healthy controls.

Methods and Results

The research was carried out in collaboration with Karolinska University Hospital, Solna, Sweden, through the ERASMUS plus project of PhD students and teachers mobility accomplished between University of Niš and Karolinska Institute. We utilized data and samples from VASKA database, including patients with active (defined as BVAS≥1, in absence of infection) and inactive ANCA vasculitis, i.e. in remission (BVAS=0). Healthy controls were matched for age and sex. EV number and phenotype in plasma were determined by flow cytometry, in both active and inactive disease, compared to controls. Additionally, the procoagulant properties of extracellular vesicles were examined through their effect on thrombin generation, using a modified method based on Calibrated Automated Thrombogram (CAT – assay).

The results demonstrated significantly elevated levels of extracellular vesicles carrying PTX3, HMGB1, TWEAK and components of complement system (C3a and C5a) in patients with active disease compared to those in remission and with healthy subjects.

Conclusions

Our findings show that levels of circulating extracellular vesicles are significantly elevated in patients with active ANCA-associated vasculitis, and correlate with clinical disease activity assessed by BVAS. These findings support potential role of these vesicles as biomarkers for disease monitoring.

Key words:

Extracellular Vesicles, ANCA-Associated Vasculitis, biomarkers, disease activity

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"Gutta cavat lapidem, non vi sed saepe cadendo." *(The drop hollows the stone, not by force, but by falling often.)*

To all of you—this work is as much yours as it is mine.

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LIST OF ABBREVIATIONS

AAV	ANCA Associated Vasculitis
ACR	American College of Rheumatology
ANCA	Anti-neutrophil Cytoplasm Antibody
ATP	Adenosine-5'-triphosphatase
BVAS	Birmingham Vasculitis Activity Score
CAT	Calibrated Automated Thrombogram
cANCA	cytoplasmic ANCA
CD	Cluster of Differentiation
CHCC	Chapel Hill Consensus Conference
CRP	C-reactive Protein
CYC	Cyclophospahamide
DNA	Deoxyribonucleic Acid
DCVAS	Diagnostic and Classification Criteria for Vasculitis Study
eGFR	estimated Glomerular Filtration Rate
ESR	Erythrocyte Sedimentation Rate
EGPA	Eosinophilic Granulomatosis with Polyangiitis
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
EUVAS	European Vasculitis Society
EV	Extracellular Vesicles
GPA	Granulomatosis with polyangiitis
H3Cit	Citrulinated Hystone 3
HMGB1	High Mobility Group Box 1
ISEV	International Society for Extracellular Vesicles
IgG	Immunoglobulin G
MPO	Myeloperoxidase
MPA	Microscopic polyangiitis
NETs	Neutrophil Extracellular Traps
NTA	Nanoparticle Tracking Analysis
pANCA	perinuclear ANCA
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PR3	Proteinase 3
PTX3	Pentraxine 3
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
RT	Room Temperature
SD	Standard Deviation
sTWEAK	Solubile Tumor Necrosis Factor-like Weak Inducer of Apoptosis
TF	Tissue Factor
TNF-α	Tumor Necrosis Factor-alpha
VTE	Venous Thromboembolic Events

1. INTRODUCTION

1.1. Extracellular vesicles

1.1.1. History and terminology of extracellular vesicles

Extracellular vesicles (EV), previously termed as microparticles, are irregularly shaped nano-sized structures, with various diameter from 100 to 1000 nm (0 - 0,1 μm). Back in 1967, Peter Wolf initially described them as "platelet dust", responsible for procoagulant effect of high speed centrifugated plasma and originated from platelets. [1] Over the past two decades, our knowledge about these submicron vesicles has gradually expanded. In 2011 International Society for Extracellular Vesicles (ISEV) was formed and a few years ago they recommended using the generic term "extracellular vesicles" for "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus". [2] Now we know that these vesicles can be released by any eukaryotic cell in extracellular milieu under normal and pathological conditions. They are typically subdivided into microvesicles, exosomes and apoptotic bodies, based on their size and mechanisms of origin. [2] Extracellular vesicles may be distinguished from other circulating particles (including exosomes and apoptotic bodies) on the basis of size, composition, surface markers, origin, mechanism of release and methods for detection. [3,4] Table 1 and Figure 1 below provide an overview of principal similarities and differences between certain subtypes of extracellular vesicles regarding biogenesis, composition, markers and biological functions.

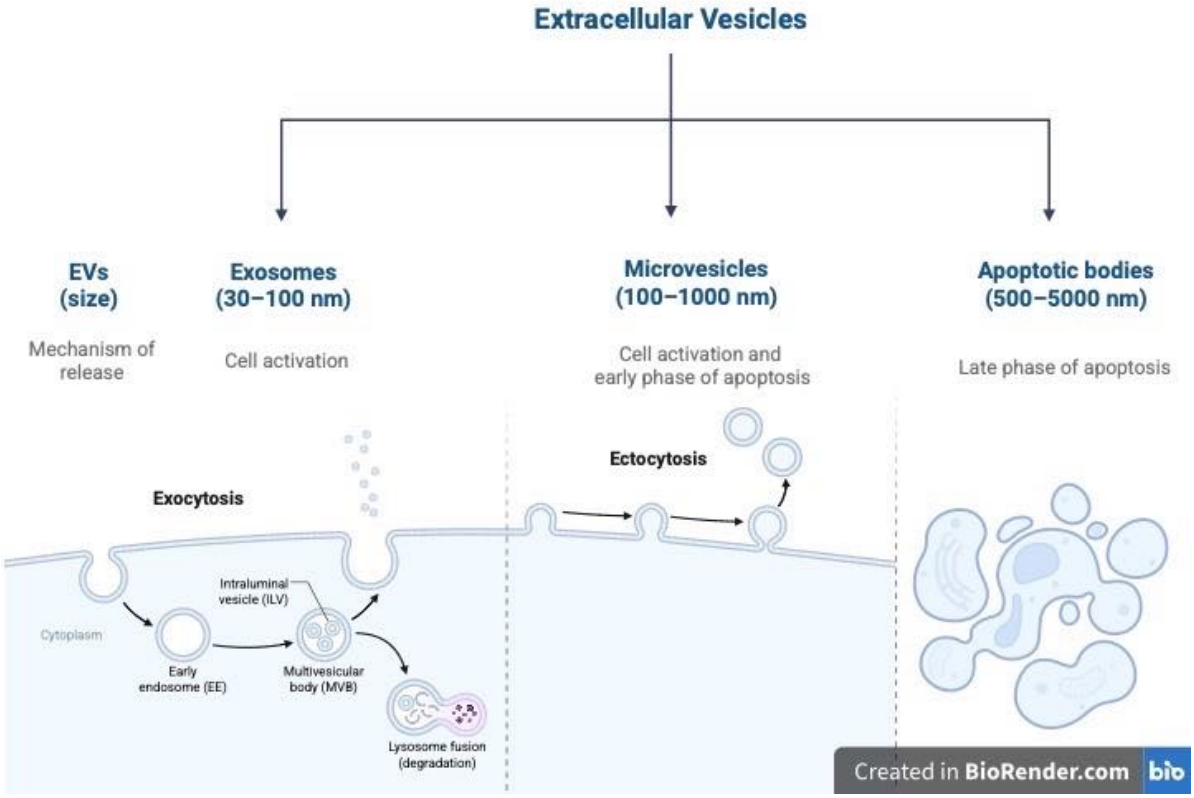


Figure 1. Schematic presentation of extracellular vesicles: Exosomes (30–100 nm) are generated via the endosomal pathway, formed as intraluminal vesicles within multivesicular bodies (MVBs) and secreted into the extracellular space through exocytosis. Microvesicles (100–1000 nm) are produced by outward budding (ectocytosis) of the plasma membrane during cell activation or early stages of apoptosis. Apoptotic bodies (500–5000 nm) are released from cells undergoing late apoptosis and contain cytoplasmic fragments, nuclear material, and organelles. While they differ in size and biogenesis, all EV subtypes carry bioactive cargo—including proteins, lipids, and nucleic acids—that can mediate intercellular communication and serve as potential disease biomarkers. *Created with BioRender.com*

Table 1. Comparative characteristics of extracellular vesicle subtypes, including their size, origin, cargo, surface markers, functions, and biomedical relevance

Characteristic	Extracellular vesicles		
	Microvesicles	**Exosomes**	**Apoptotic bodies**
Size	30-1000 nm	30-150 nm	500-5000 nm
Origin	Direct outward budding from plasma membrane	Intraluminal budding of endosomal membranes (MVBs)	Cell fragmentation during apoptosis
Release mechanism	Cytoskeletal contraction and membrane shedding; during cell activation and early phase of apoptosis	Exocytosis of MVBs; during cell activation	Apoptotic disassembly, blebbing; during late phase of apoptosis
Surface markers	Integrins, selectins, CD40L	CD9, CD63, CD81, Alix, TSG101	Phosphatidylserine, histones, annexin V positivity
Cargo	Proteins, lipids, mRNA, miRNA	Proteins, lipids, miRNA, mRNA, DNA	Cellular organelles, nuclear fragments, chromatin
Functions	Intercellular communication, inflammation, coagulation	Targeted signalling, immune regulation, RNA delivery	Clearance of apoptotic debris, immune tolerance or activation
Detection models	Flow cytometry, NTA, EM	Western blot (CD63, CD81), EM, NTA	Flow cytometry, microscopy, annexin V binding
Clinical relevance	Disease biomarkers, therapeutic vectors	Diagnostic and therapeutic potential (drug delivery, RNA carriers)	Indicators of apoptosis, involved in autoimmunity and inflammation

Abbreviations: EVs – extracellular vesicles; MVB – multivesicular body; EM – electron microscopy; NTA – nanoparticle tracking analysis; miRNA – microRNA; mRNA – messenger RNA; DNA – deoxyribonucleic acid; CD9/CD63/CD81 – tetraspanins; TSG101 – tumor susceptibility gene 101; Alix – ALG-2 interacting protein X; PS – phosphatidylserine.

1.1.2. Composition of extracellular vesicles

The surface of extracellular vesicles contains variable spectrum of antigens, receptors and adhesion molecules, specific to the precursor cell (allowing identification of cellular origin by using labelled antibodies) and enabling complex interaction with target cells. However, circulating vesicles also have the ability to adopt some of the molecules from the circulation. Afterall, besides of membrane components, extracellular vesicles contain cytoplasmic and nuclear material too, that resembles parental cell. [5] (Figure 2.)

The plasma membrane of these lipid bilayer-enclosed particles, as well as all cells, is made of asymmetrically distributed phospholipids. The outer leaflet is predominantly comprised of uncharged cholinephospholipids (phosphatidylcholine and sphingomyelin), whereas the inner leaflet is formed by aminophospholipids (phosphatidylserine – negatively charged molecule and phosphatidylethanolamine). This membrane asymmetry is regulated by enzymes (flippase, floppase and scramblase), which shuttles phospholipids between the bilayers. The first two are adenosine-5'-triphosphatase (ATP) dependent, while the last enzyme is ATP-independent, but calcium-dependent. [6]

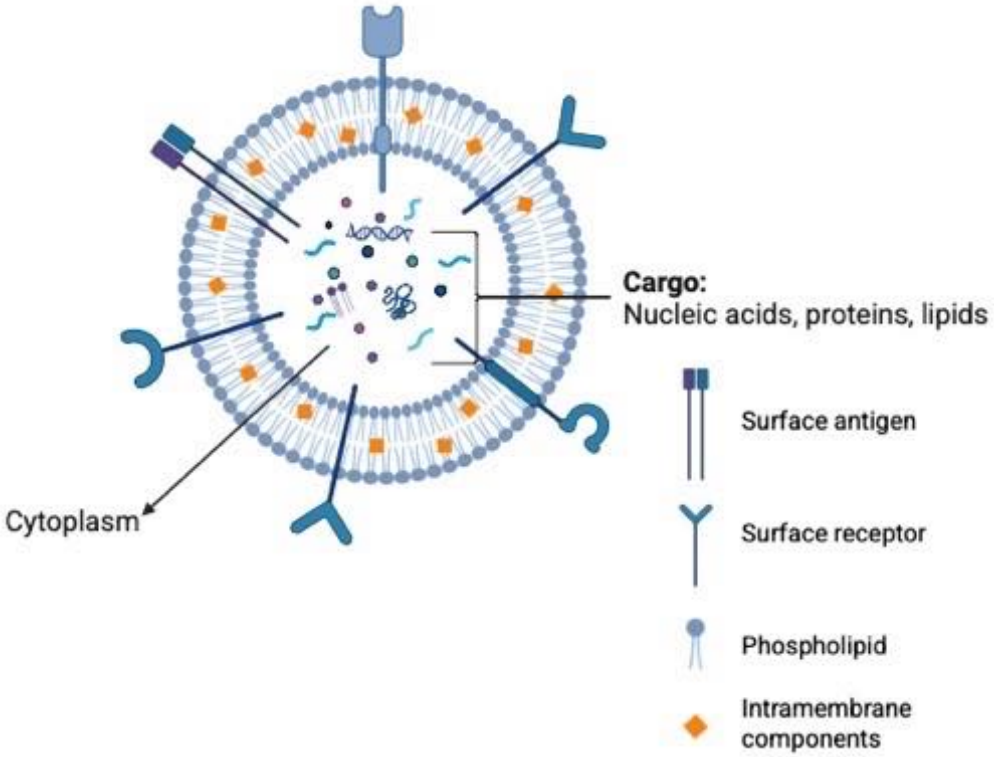


Figure 2. Structural organization of EVs: Extracellular vesicles are enclosed by a lipid bilayer that incorporates phospholipids, surface antigens, surface receptors, and intramembrane components. Their cargo consists of bioactive molecules, including nucleic acids (DNA, mRNA, miRNA), proteins, and lipids, which mediate intercellular communication and reflect the functional state of the cell of origin. The vesicular membrane enables selective packaging and protection of cargo, while external surface markers facilitate interactions with recipient cells. *Created with BioRender.com*

1.1.3. Formation of extracellular vesicles

The main feature in generation of extracellular vesicles is cytoskeletal reorganization and alteration in normal asymmetry of cell membrane phospholipids, in response to a variety of stimuli (during cellular activation, early stages of apoptosis and/or in response to cellular stress). Cell stimulation triggers the increase in intracellular calcium levels leading to inactivation of flippase and enhancing the activities of floppase and scramblase. Consequently, externalization of phosphatidylserine on the outer leaflet of the bilipid membrane layer occurs, as well as outward blebbing of the membrane, causing formation of extracellular vesicles and subsequent shedding into extracellular space by a process of exocytic budding. [7]

1.1.4. Methods for detection of extracellular vesicles

Due to easier methods for detection, in the investigation of the physiology of these small biological particles, the attention has been focused primarily on circulating cell structures (i.e. platelets, erythrocytes or leucocytes), as well as vascular (i.e. endothelial) cells.

Regardless human serum is loaded with various extracellular vesicles, their detection can be challenging due to its heterogeneity and small size. These vesicles can be isolated by several techniques, considering flow cytometry most commonly used, before atomic force microscopy, nanoparticle tracking analysis (NTA) or transmission electron microscopy, depending on the vesicle population. [8] The reasons are mainly its availability and small sample size, as well as advantage of using multiple antibodies simultaneously (by labelling cells or vesicles with a different fluorochrome). Flow cytometry is used for measuring physical properties (size and origin of extracellular vesicles, as well as their cargo) and fluorescence characteristics of single cells while passing through a laser beam. Additionally, this technique can count and simultaneously detect different markers to determine the source of extracellular vesicle. Nevertheless, flow cytometry also has some limitations, i.e. low size sensitivity (limit of detection is around 0.4-0.5 μm due to the laser wavelength 488nm). [9] For that reason, the number and surface expression of the smallest vesicles can be underestimated using this technique.

On the other hand, functional assays, such as CAT (Calibrated Automated Thrombogram) - assay analyse relative enumeration of extracellular vesicles based on measurement of procoagulant or prothrombinase activity. [10]

1.1.5. The role of extracellular vesicles

Extracellular vesicles participate in both pathological and physiological events, such as cell-to-cell communication and protein transfer, regulation of apoptosis and inflammation, stress responses, and processes such as haemostasis, thrombosis, angiogenesis and vascular reactivity, including endothelial cell dysfunction. [11] Their small size and presence in the circulation enable them to mediate both local and long-range signalling, since they are involved in intricate cellular communication. In one hand, they may transfer receptors to the target cell. Also, they may transfer proteins to target cell and release them in their extracellular environment. Even more, they are capable of transporting nucleic acids (such as microRNA, messenger RNA, DNA) from parental to recipient cells, thereby modulating the phenotype or function of the target cells. [12] A distinctive feature of extracellular vesicles is their content of membrane, cytoplasmic and nuclear components, which reflects the characteristics of the parental cell and allows determination of their cellular origin. Their involvement in coagulation and haemostasis, as well as in inflammation and vascular functions (including angiogenesis and endothelial injury) is already well known. Their potential use in human medicine is constantly growing, either as promising candidate for diagnostic markers in various diseases or as a therapeutic delivery vehicle (based on the ability to transfer their cargo) and even for novel vaccines. [13,14,15]

1.1.6. Extracellular vesicles among physiological and various pathophysiologic settings

Extracellular vesicles are normally detectable in low concentrations within healthy individuals' plasma, reflecting their role in physiological processes and cellular homeostasis. In contrast, a significant increase in circulating levels of extracellular vesicles has been consistently observed across a wide spectrum of pathological conditions. [16] This elevation is thought to reflect underlying mechanisms such as cellular activation, apoptosis, oxidative stress, or inflammatory responses, which drive enhanced vesicle release. Consequently, the quantitative and qualitative changes in EV populations not only mirror the degree of tissue or vascular injury but also provide valuable insights into disease mechanisms. These features make EVs particularly attractive as potential biomarkers, offering diagnostic and prognostic relevance in disorders characterized by immune dysregulation, vascular dysfunction, or systemic inflammation. [17] Also, they can be detected in other body fluids, including urine, cerebrospinal fluid, saliva, mother milk and others. [18]

During the last decades prothrombotic and proinflammatory potential of extracellular vesicles are well established and broadly investigated in numerous pathological conditions [19] such as: atherosclerosis [20,21], venous thromboembolism [22], acute coronary syndromes [23], congestive heart failure, hypertension [24], preeclampsia [25], diabetes [26], sepsis [27], cancer [28,29], as well as in many autoinflammatory diseases [30] as in antiphospholipid syndrome [31], systemic lupus erythematosus [32], multiple sclerosis [33] and even in schizophrenia [34] and Covid-19. [35]

Procoagulant property mainly occurs as a consequence of the rich phosphatidylserine content of the extracellular vesicles (exposed in higher density on platelet derived extracellular vesicles than even on activated platelets and showing 50 to 100 folds more procoagulant activity), which acts as one of the essential lipid cofactors for clotting. [36] It promotes thrombin generation by activating the extrinsic coagulation pathway (mediated by tissue factor and factor VII) and by facilitating the binding of positively charged γ-carboxylation domains of clotting factors (FVII, FIX, FX and prothrombin) to negatively charged phosphatidylserine, ultimately resulting in the conversion of prothrombin to thrombin. Furthermore, phosphatidylserine exposure together with tissue factor, noticeably emphasis and augment the procoagulant activity of extracellular vesicles via promoting the activation of the coagulation cascade. [37]

Along with their procoagulant property, extracellular vesicles contribute to endothelial damage in many autoimmune diseases. As seen in systemic vasculitis, activated neutrophils release extracellular vesicles that are expressing myeloperoxidase (MPO) on their surface. Further, activation of MPO-hydrogen peroxide-chloride system leads to endothelial cell dysfunction. [38] Their implication in renal injury of autoimmune kidney diseases was investigated recently, suggesting them as promising biomarkers of renal diseases. [39]

1.2. Anti-neutrophil cytoplasm antibody (ANCA) – associated vasculitis (AAV)

1.2.1. Definition and classification criteria

Anti-neutrophil cytoplasm antibody (ANCA) – associated vasculitis (AAV) represents a heterogeneous group of multisystem diseases characterized by pauci-immune necrotizing inflammation of small- to medium-sized vessels, typically occurring with minimal or absent deposition of immunoglobulins and/or complement. In addition to vasculitic lesions, AAV may present with granulomatous inflammation enriched in neutrophils or eosinophils. Based on clinicopathological features, three major systemic entities are recognized within the spectrum of AAV:

1. granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis),
2. microscopic polyangiitis (MPA), and
3. eosinophilic GPA (EGPA, previously known as Churg Strauss syndrome). [40]

A serological hallmark of AAV is the presence of circulating anti-neutrophil cytoplasm autoantibodies (ANCA), predominantly of the IgG subclass, which are directed towards cytoplasmic components of neutrophils. According to their antigen specificity and target protein, two principal subtypes are recognized: ANCA against proteinase 3 (PR3-ANCA) and ANCA targeting myeloperoxidase (MPO-ANCA). [41] PR3-ANCA is most frequently associated with granulomatosis with polyangiitis (GPA), being detected in up to 80% of patients, whereas MPO-ANCA predominates in microscopic polyangiitis (MPA), where it is present in approximately 70 % of cases. [42] In eosinophilic granulomatosis with polyangiitis (EGPA), ANCA positivity is less frequent, occurring in only a minority of patients, typically with MPO specificity, and is often associated with more pronounced vasculitic manifestations.

The first formal classification criteria for granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA) were established by the American College of Rheumatology (ACR) in 1990. These criteria, however, had notable limitations, particularly the absence of specific definitions for microscopic polyangiitis (MPA) and the lack of incorporation of ANCA testing. To address these gaps, the Chapel Hill Consensus Conference (CHCC) proposed a set of nomenclature and definitions for systemic vasculitides in 1994. [43] These definitions expanded the classification to include MPA and provided clearer descriptions of disease manifestations, helping to unify terminology across clinical and research settings. In 2012, the CHCC definitions were revised to reflect advances in understanding of vasculitis pathogenesis and clinical features. [44] The update incorporated additional distinctions among AAV subtypes, emphasized the role of ANCA specificity, and refined disease descriptors, providing a more precise framework for both clinical practice and research applications (as summarized in Table 2.).

Table 2. Classification criteria for AAV according to 2012 revised CHCC definition

AAV	Key clinical features	Histopathology	ANCA association	Typical organ involvement
GPA	Upper and lower respiratory tract involvement; sinusitis; nasal/oral ulcers; necrotizing glomerulonephritis	Necrotizing granulomatous inflammation	PR3-ANCA positive in ˜80%	Lungs, sinuses, kidneys
MPA	Rapidly progressive glomerulonephritis; purpura	Necrotizing vasculitis without granulomas	MPO-ANCA positive in ˜70%	Kidneys, skin, lungs
EGPA	Asthma, allergic rhinitis, peripheral eosinophilia	Eosinophil-rich granulomatous inflammation	MPO-ANCA positive in minority of cases	Lungs, skin, peripheral nerves

Abbreviations: CHCC - Chapel Hill Consensus Conference; GPA - granulomatosis with polyangiitis; MPA - microscopic polyangiitis; EGPA - eosinophilic granulomatosis with polyangiitis; ANCA - anti-neutrophil cytoplasm autoantibodies; AAV – ANCA associated vasculitis.

More recently, the ACR in collaboration with the European Alliance of Association for Rheumatology (EULAR) has proposed updated classification criteria for each phenotype of AAV. These criteria were developed based on data from the multinational Diagnostic and Classification Criteria for Vasculitis Study (DCVAS), providing a contemporary framework that integrates clinical, serological, and histopathological features to improve diagnostic precision and consistency across diverse patient populations. [45,46,47] A concise overview of these evolving classification criteria is described in table below. (Table 3.)

Table 3. Evolution of classification criteria for AAV from 1990 to 2022

Year	Classification body	Main focus	Key notes	Clinical relevance	Main limitation
1990	ACR	First GPA and EGPA criteria	Focused on clinical features	Provided initial framework for diagnosis	Did not include MPA; ANCA testing not incorporated
1994	CHCC	Nomenclature and definitions for systemic vasculitides	Standardized terminology across vasculitides	Helped unify research and clinical communication	Lacked detailed guidance on ANCA specificity and subtypes
2012	CHCC revised	Updated AAV definitions	Refined disease descriptors; emphasized ANCA	Enabled more precise subtype classification; improved diagnostic accuracy	Some complexity in clinical application; still evolving with emerging data
2022	ACR/EULAR	New classification criteria for each AAV phenotype based on DCVAS study	Integrated clinical, serological and histopathological features	Improved diagnostic precision and consistency across multinational cohorts	Criteria still require validation in diverse populations

Abbreviations: ACR - American College of Rheumatology; CHCC - Chapel Hill Consensus Conference; EULAR - European Alliance of Association for Rheumatology; DCVAS - Diagnostic and Classification Criteria for Vasculitis Study; GPA - granulomatosis with polyangiitis; MPA - microscopic polyangiitis; EGPA - eosinophilic granulomatosis with polyangiitis; ANCA - anti-neutrophil cytoplasm autoantibodies; AAV – ANCA associated vasculitis.

Building on the classical definitions of AAV subtypes, the most recent ACR/EULAR 2022 classification criteria provide a structured and quantitative approach to phenotype-specific diagnosis.

Table 4 summarizes the key elements of these criteria for granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA), highlighting the weighted clinical, serological, and histopathological features that inform classification. By integrating modern diagnostic considerations, these criteria complement the traditional subtype definitions presented in Table 2 and offer a standardized framework applicable across diverse patient populations and research settings. [48,49]

Table 4. Key elements of ACR/EULAR 2022 clasification criteria for AAV

Subtype	Main criteria domains	Key features	ANCA association	Clinical/diagnostic notes
GPA	Weighted clinical, serological and histopathological items	Upper/lower respiratory involvement, necrotizing granulomatous inflammation, PR3-ANCA	PR3-ANCA emphasized	Incorporates scoring system; granulomatous features are heavily weighted
MPA	Clinical features and serology	Small-vessel necrotizing vasculitis, rapidly progressive glomerulonephritis	MPO-ANCA emphasized	Criteria focus on renal involvement and small-vessel manifestations
EGPA	Clinical, laboratory and histopathology	Asthma, eosinophilia, eosinophil-rich vasculitis	MPO-ANCA included if present	Emphasizes asthma and eosinophilia; vasculitic manifestations

Abbreviations: ACR - American College of Rheumatology; EULAR - European Alliance of Association for Rheumatology; GPA - granulomatosis with polyangiitis; MPA - microscopic polyangiitis; EGPA - eosinophilic granulomatosis with polyangiitis; ANCA - anti-neutrophil cytoplasm autoantibodies; AAV – ANCA associated vasculitis.

1.2.2. AAV epidemiology

ANCA-associated vasculitis represents a rare autoimmune disease, with an overall annual incidence in Europe ranging from 15 to 20 new cases per million and estimated prevalence varying between 46 and 250 cases per million. [50,51] Epidemiological studies have reported these varying incidence and prevalence rates across different regions, reflecting differences in geography, genetics,

population characteristics and specific AAV subtypes. [52,53] Geographic variation in the incidence of ANCA-associated vasculitis is illustrated in Figure 3, highlighting regions with the highest, intermediate, and lowest reported rates worldwide. A recent population-based study in Oslo observed an increase in AAV incidence, with a shift from granulomatosis with polyangiitis (GPA) to microscopic polyangiitis (MPA) under the ACR/EULAR 2022 classification criteria. [54]

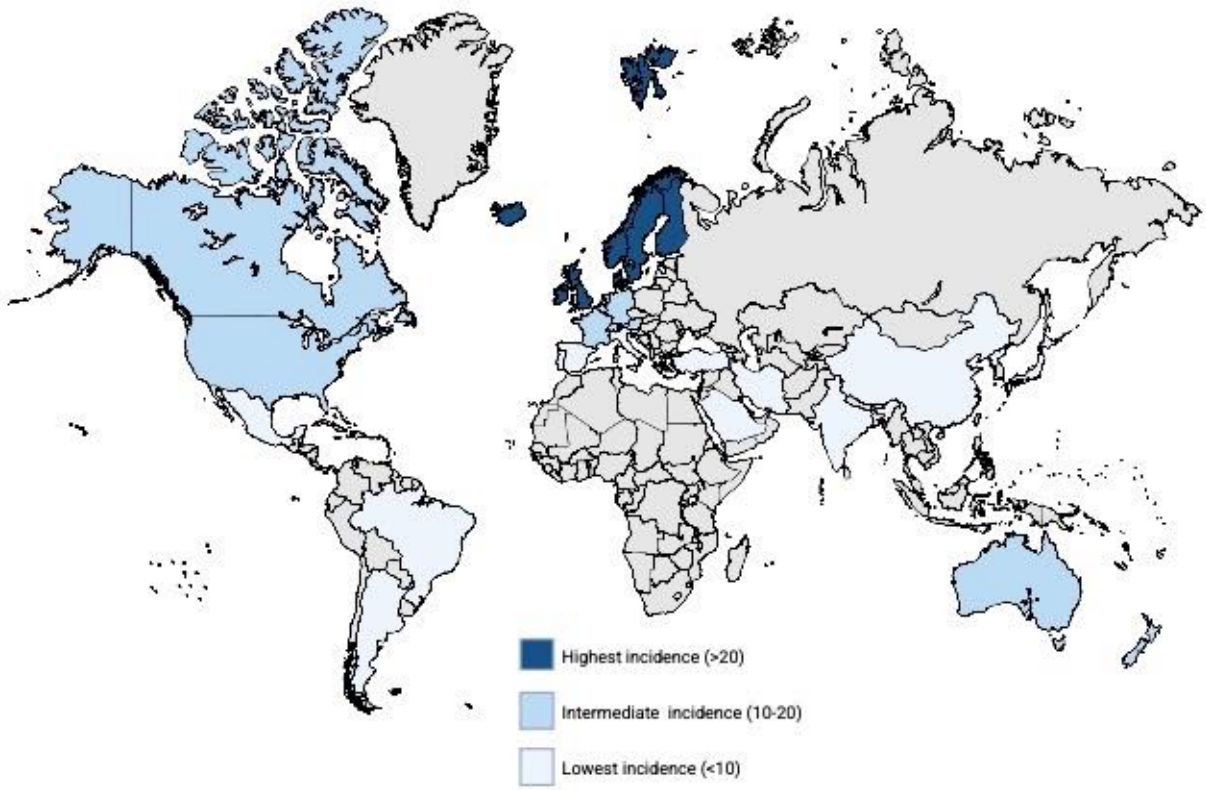


Figure 3. Schematic world map of AAV epidemiology: This map illustrates the global distribution of AAV, showing variability in incidence across regions. The highest incidence (>20 per million person-years) is reported in Northern Europe, particularly Scandinavia and the United Kingdom. Intermediate incidence (10–20 per million person-years) is observed in Central/Western Europe, North America, and Oceania, whereas lower incidence (<10 per million person-years) has been documented in Southern Europe, Asia, and most developing regions. *Created with BioRender.com.*

Although AAV can manifest at any age, its frequency notably increases with advancing age, peaking in older adults. The gender distribution is generally balanced, with some studies suggesting a slight female predominance. [55] These epidemiological patterns underline the importance of considering demographic factors when evaluating disease burden and designing population-based studies.

1.2.3. Clinical presentation in AAV

AAV exhibits a wide spectrum of organ involvement, ranging from limited disease to severe forms with multiorgan failure, as summarized in Figure 4 below. The most commonly affected organ

systems include the upper and lower respiratory tract (presented with epistaxis, sinusitis, otitis media, and even pulmonary nodules or diffuse alveolar haemorrhage…), the kidneys (typically manifested as pauci-immune noncrescentic glomerulonephritis, potentially progressing to end-stage renal failure), the skin (presented with different skin rashes) and the peripheral nerves (commonly presenting as mononeuritis multiplex or sensory peripheral neuropathies). Nonspecific systemic inflammatory symptoms, such as fever, myalgia and arthralgia, induced by high levels of circulating inflammatory cytokines, are usually observed at disease onset or during exacerbations. [56,57] Therewithal, necrotizing inflammation and fibrinoid necrosis of vessel walls lead to more specific clinical signs of vasculitis, which depend on the affected vessels i.e. organs and overall disease activity. [58]

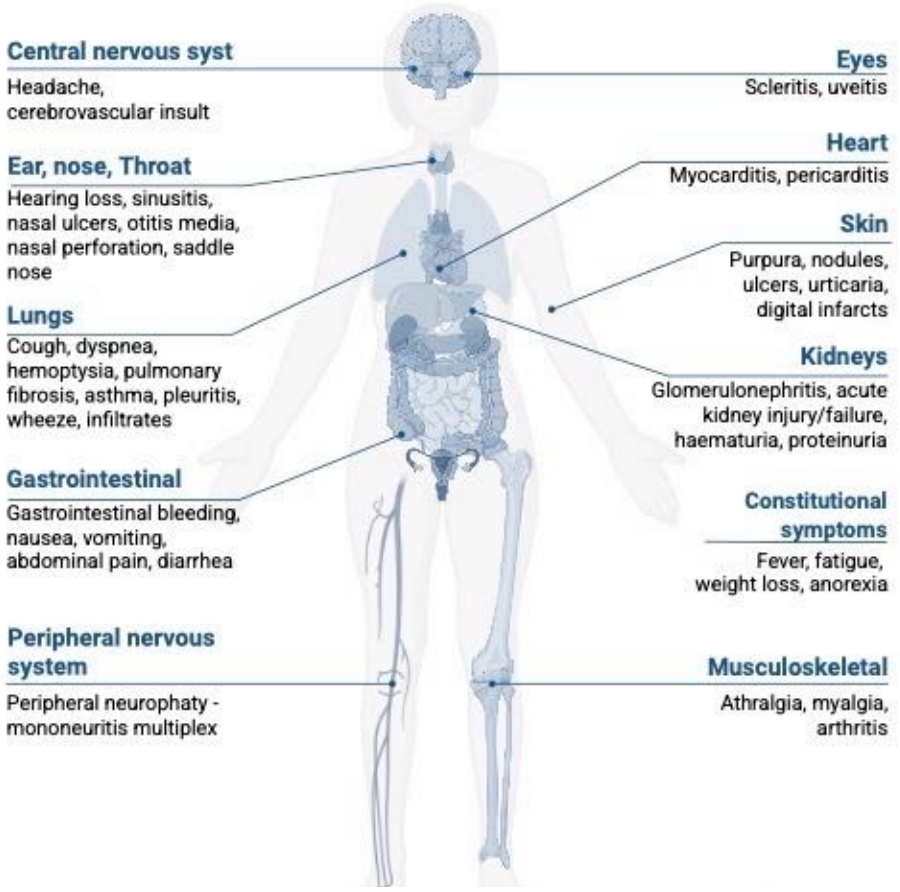


Figure 4. Schematic presentation of potential clinical manifestations and organ involvement in AAV. *Created with BioRender.com*

1.2.4. AAV diagnosis and scoring disease activity

The evaluation of disease activity and monitoring of AAV traditionally relies on the Watts classification system. [59] However, improved classification was recently proposed by ACR/EULAR [45,46,47] and it comprises of the combination of three different components - clinical features, histopathology and serology (serial measurements of ANCA), as it was explained in detail in previous

section. The Birmingham Vasculitis Activity Score (BVAS) remains a validated instrument for assessing disease severity and guiding therapeutic decisions. Despite these advances, the diagnostic gold standard continues to involve invasive procedures—most notably tissue biopsy—with histopathological confirmation of vasculitis, as recommended by current clinical guidelines. [60] Initially, ANCA detection was performed using indirect immunofluorescence (IIF), which enabled classification into two major staining patterns: perinuclear (p-ANCA), typically associated with myeloperoxidase (MPO) specificity and frequently linked to microscopic polyangiitis (MPA), and cytoplasmic (c-ANCA), generally directed against proteinase 3 (PR3) and predominantly observed in granulomatosis with polyangiitis (GPA). Subsequently, enzyme-linked immunosorbent assay (ELISA) was introduced to allow antigen-specific identification of ANCA subtypes, and remains widely employed in clinical practice. [61] Although ANCA testing remains a cornerstone in the diagnostic evaluation of AAV, its utility as a standalone marker of disease activity remains debated. Studies have yielded inconsistent findings regarding its sensitivity and specificity for distinguishing active disease from remission, with ANCA-negative cases and ANCA positivity in non-vasculitic conditions further complicating clinical interpretation. [62,63,64,65] The diagnostic performance of ANCA testing is limited by suboptimal sensitivity and specificity, as their presence has been documented in various non-vasculitic conditions, including inflammatory bowel disease, rheumatic disorders, and hepatic pathologies. Moreover, ANCA reactivity often lacks specificity for MPO, PR3, or elastase antigens. A further diagnostic challenge arises from the subset of AAV patients who are ANCA-negative, underscoring the fact that ANCA positivity is neither exclusively indicative of AAV nor essential for its diagnosis. [66] Consequently, ANCA test results must always be interpreted in conjunction with clinical findings. Inflammatory markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), procalcitonin, and other systemic indicators of inflammation are commonly elevated but remain nonspecific. Given these limitations, there is a pressing need to identify novel biomarkers that can more accurately reflect vascular injury and guide both diagnosis and therapeutic monitoring.

The Birmingham Vasculitis Activity Score (BVAS) is an internationally recognized, standardized tool designed to quantify disease activity in patients with systemic vasculitis. The instrument encompasses 56 clinical features organized across nine organ-specific domains, generating a cumulative score ranging from 0 to 63, where higher values reflect greater disease burden. Only new or worsening manifestations are recorded, ensuring that the score reflects active inflammation rather than chronic damage. [67] Originally published in 1994 [68], the BVAS has undergone successive revisions to enhance its clinical utility, with the current third iteration (BVAS v3.0) introduced in 2009 and still in use. [69] (Figure 13) According to the EUVAS/EULAR definition, clinical remission is achieved when the BVAS score is 0, indicating complete absence of

active disease, whereas a score ≥1 denotes ongoing disease activity, which may range from mild to refractory forms requiring treatment escalation.

While BVAS serves as a standardized tool for capturing active disease manifestations, it does not account for irreversible damage that accumulates over time as a result of ongoing inflammation or treatment-related toxicity. To address this limitation, the Vasculitis Damage Index (VDI) was developed by the European Vasculitis Study Group (EUVAS) as a complementary instrument aimed at assessing permanent organ damage in patients with systemic vasculitis. [70] In contrast to activity-based indices, the VDI records damage items that persist for at least three months and are no longer attributed to active disease. It includes 64 predefined entries across 11 organ systems, offering a structured framework for quantifying long-term morbidity. The VDI has demonstrated prognostic value for patient outcomes, including mortality, and is widely used in both clinical research and longitudinal patient monitoring. However, because it does not account for the severity or functional impact of specific damage, the Combined Damage Assessment (CDA) index was developed as an expanded scoring tool that integrates both physician-reported and patient-centered domains. [71] The CDA offers a more nuanced and comprehensive evaluation of disease burden, and has shown improved sensitivity in detecting clinically meaningful but often underrecognized long-term sequelae of ANCA-associated vasculitis.

1.2.5. AAV aetiology

Despite ongoing debate and incomplete understanding of the precise etiopathogenesis of AAV, it is widely accepted that disease development results from a complex interplay between genetic predisposition [72] and environmental triggers. Environmental contributors may include microbial agents—most notably *Staphylococcus aureus* [73]—certain pharmacological agents such as propylthiouracil [74], inhalation of silica dust [75], and immune system dysregulation. These factors are believed to act synergistically to initiate disease onset and promote the formation of ANCA autoantibodies.

The role of genetic

Although ANCA-associated vasculitis (AAV) is not classified as an inherited disorder and exhibits rare familial clustering, multiple genetic associations have been identified—particularly within the major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) region. These associations span both class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, -DP) loci. [72] Early genome-wide association studies (GWAS) revealed a clear genetic distinction between granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA), notably

implicating the HLA-DPB1 locus as a primary genetic determinant for GPA susceptibility. [76] Subsequent studies confirmed that genetic predisposition to AAV is not only subtype-specific (i.e. PR3-ANCA vs. MPO-ANCA), but also population-dependent. For instance, in Scandinavian cohorts, HLA-DRB1*04:04 was strongly associated with MPO-ANCA vasculitis, whereas two independent risk loci within HLA-DPB1 were linked to PR3-ANCA-positive disease. [77] In East Asian populations, several studies have confirmed the role of HLA-DQ alleles in MPO-AAV pathogenesis. One study from China identified a high-risk haplotype (HLA-DQA103:02–DQB103:03), which showed strong linkage disequilibrium ($r^2 = 0.69$) and a significant association with MPO-AAV. [78] Another investigation highlighted the HLA-DPB1*04 allele as a strong risk factor for GPA, particularly in PR3-ANCA-positive patients. [79] Importantly, this allele has also been linked to increased risk of relapse and correlated with disease activity. [80] In Japanese patients, the HLA-DRB109:01–DQA103:02–DQB1*03:03 haplotype has similarly been associated with MPA and MPO-ANCA positivity, further emphasizing the role of HLA-DQ polymorphisms in shaping ethnic and serotype-specific susceptibility. [81] Collectively, these findings support the hypothesis that genetic predisposition to AAV is polygenic, heterogeneous, and varies significantly across populations.

The role of environment

Environmental exposures are increasingly recognized as significant contributors to the development of AAV. [82] Among these, occupational exposure to silica dust has been consistently associated with elevated disease risk, as confirmed by epidemiological studies and meta-analyses that demonstrate a dose–response relationship. [75] Silica particles are known to activate neutrophils, promote the release of proteolytic enzymes, and induce the formation of neutrophil extracellular traps (NETs), all of which contribute to vascular endothelial injury. [83] In addition to occupational hazards, ambient air pollution has emerged as a relevant risk factor for several autoimmune and rheumatic conditions, including AAV, likely through mechanisms involving systemic inflammation and enhanced autoimmune reactivity. [84]

Chronic infections have also been implicated in AAV pathogenesis. *Staphylococcus aureus*, particularly in cases of persistent nasal colonization, is associated with increased relapse rates in granulomatosis with polyangiitis (GPA), suggesting a role in disease maintenance and reactivation. [73,85] Viral triggers are under ongoing investigation: Epstein–Barr virus has been hypothesized as a molecular mimicry agent [86,87], while isolated case reports have described temporal associations between AAV onset and infections with SARS-CoV-2 [88], parvovirus B19 [89], and other viral pathogens.

Drug-induced AAV represents another well-documented subtype, with antithyroid agents—especially propylthiouracil—being the most frequently implicated. [74] However, numerous additional medications have been identified in retrospective analyses, including hydralazine, minocycline, and levamisole-contaminated cocaine, among others. [90] These cases typically resolve following cessation of the triggering agent. In rare instances, vaccines—such as influenza and SARS-CoV-2—have been temporally associated with AAV onset or relapse [91,92], although causal relationships remain unproven and warrant further investigation.

1.2.6. AAV pathogenesis

Although the precise mechanisms underlying the pathogenesis of AAV remain incompletely defined, experimental and clinical evidence supports a model involving a pathogenic triad: ANCAs, neutrophils, and the complement system. [93] These components interact in a reciprocal manner to initiate and propagate vascular inflammation. ANCAs bind to their target antigens—primarily proteinase 3 (PR3) or myeloperoxidase (MPO)—on the surface of primed neutrophils, resulting in their activation. This leads to a respiratory burst, degranulation, and the release of neutrophil extracellular traps (NETs), collectively contributing to endothelial injury. [58] Concurrently, activated neutrophils trigger the alternative complement pathway, generating C5a, a potent chemoattractant and neutrophil activator. C5a further primes neutrophils and sustains the inflammatory cascade, thereby establishing a self-amplifying feedback loop. [93] This loop is central to the development of necrotizing small-vessel vasculitis and will be discussed in more detail in the following sections. (Figure 5.)

Pathogenicity of ANCA

Anti-neutrophil cytoplasmic antibodies (ANCAs) are predominantly immunoglobulin G (IgG) class autoantibodies that target antigens located within cytoplasmic granules of neutrophils and lysosomes of monocytes. The two principal antigenic targets are proteinase 3 (PR3-ANCA) and myeloperoxidase (MPO-ANCA), which define the serological subtypes of AAV. [94] MPO is a heme-containing peroxidase enzyme involved in innate immune defence, primarily by catalysing the formation of reactive oxygen species during phagocytosis. It is stored in azurophilic granules and can be translocated to the cell surface upon neutrophil activation. In contrast, PR3 is a serine protease primarily localized in primary granules but also constitutively expressed on the surface of resting neutrophils, particularly in PR3-ANCA-positive individuals. Owing to its localization in secretory vesicles and its membrane accessibility, PR3 is more readily mobilized than MPO. Functionally, PR3 contributes to extracellular matrix degradation and proteolytic processing at sites of inflammation. [58]

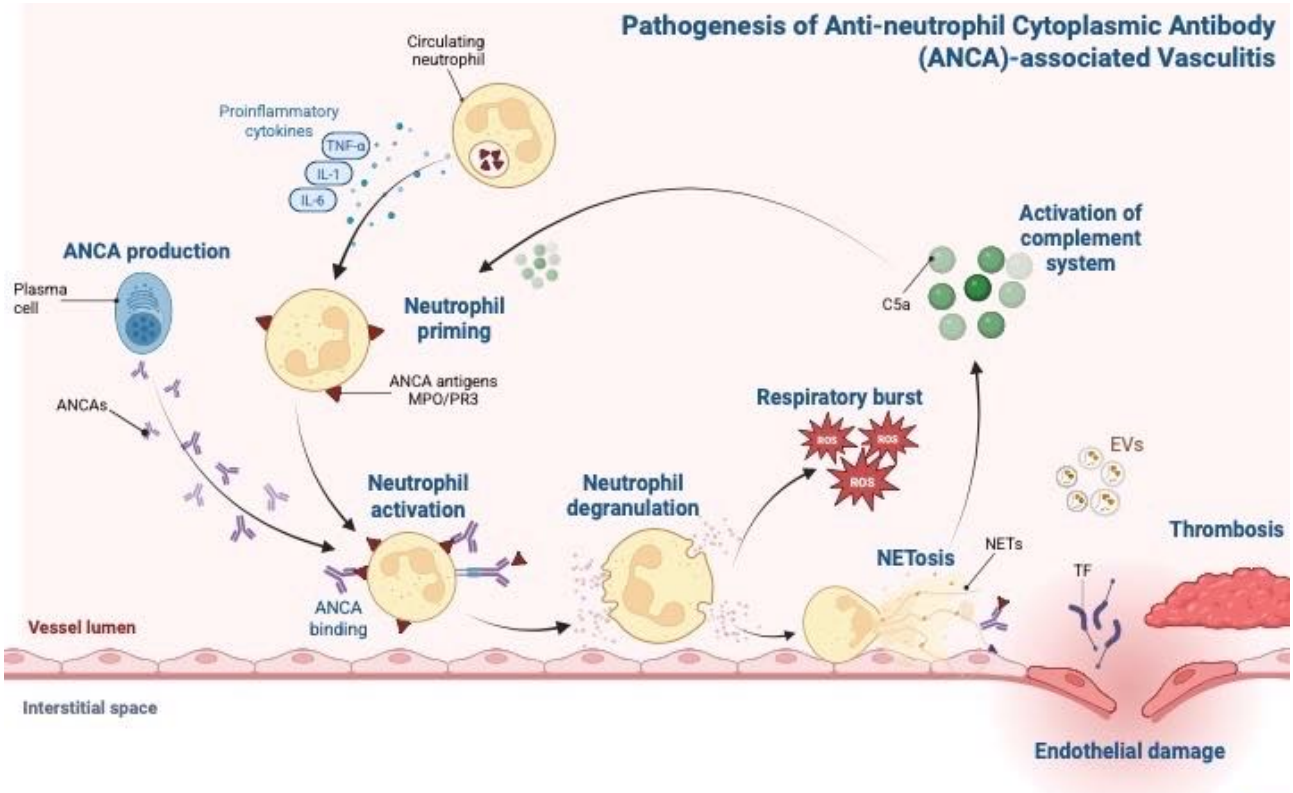


Figure 5. Schematic presentation of AAV pathogenesis: Priming neutrophils by proinflammatory cytokines (e.g., TNF-α, IL-1, IL-6) leads to increased surface expression of ANCA antigens (MPO/PR3). Binding of ANCAs to primed neutrophils results in their activation, followed by degranulation, respiratory burst, and the release of neutrophil extracellular traps (NETs). Activation of alternative complement pathway, particularly via C5a, further amplifies this process in a positive feedback loop. Extracellular vesicles (EVs), NETs, and tissue factor (TF) contribute to endothelial injury and thrombosis, which represent hallmarks of vascular inflammation in ANCA-associated vasculitis. *Created with BioRender.com*

The presence of ANCAs in patient serum remains a central biomarker for the diagnosis of ANCA-associated vasculitis, particularly when correlated with clinical and histological features. [95] However, the precise mechanisms underlying their initial development are not fully understood. Low-titer, low-avidity ANCA-like autoantibodies have been detected in healthy individuals, where they may contribute to immunological homeostasis. [96] In contrast, pathogenic ANCA responses in AAV appear to arise from a breakdown in peripheral immune tolerance, potentially triggered by microbial agents—most notably *Staphylococcus aureus* via molecular mimicry [97,98]—as well as certain drugs (e.g. propylthiouracil, hydralazine, levamisole), impaired regulatory T- and B-cell function, or aberrant antigen expression. [58,99]

ANCAs have also been identified in individuals with other non-vasculitic autoimmune and inflammatory conditions, including inflammatory bowel disease, cutaneous polyarteritis nodosa, IgA vasculitis, and autoimmune hepatitis, indicating that their presence alone is insufficient for disease development. [100] The transition from non-pathogenic to pathogenic ANCA may involve epitope spreading, increased affinity maturation, and co-stimulatory signals from the innate immune system. [101] The pathogenicity of ANCA has been supported by both clinical and experimental data. A

notable case report described transplacental transmission of anti-MPO antibodies from mother to neonate, resulting in neonatal microscopic polyangiitis with pulmonary haemorrhage and renal involvement. [102] Moreover, several animal studies have provided compelling evidence: anti-MPO IgG antibodies induced necrotizing vasculitis and crescentic glomerulonephritis when passively transferred to mice. [103,104,105]

Despite this, several questions remain unresolved. A subset of patients with biopsy-confirmed AAV lacks detectable ANCA by standard immunoassays [106], raising the possibility of assay insensitivity, the presence of antigen–antibody complexes that mask detection or antibody sequestration. Additionally, ANCA titres often do not correlate consistently with disease activity. [63] One hypothesis is that not all ANCA are equally pathogenic [107]; rather, only those targeting specific immunodominant epitopes may trigger neutrophil activation and vasculitis. [108] While data from Kettritz *et al.* [109] and others strongly support a pathogenic role for ANCA, further studies are needed to refine the distinction between pathogenic and non-pathogenic autoantibodies by refining both immunologic assays and mechanistic criteria.

Priming and activation of neutrophils

Neutrophils play a central role in the pathogenesis of ANCA-associated vasculitis, serving as key effector cells in vascular injury. Sun *et al*. [110] recently summarized a multi-step mechanism by which ANCAs activate neutrophils and trigger endothelial damage. This concept aligns with the earlier work of Jennette and Falk [58], who emphasized ANCA-induced neutrophil activation as the initiating step of vasculitis. The proposed model is supported by a wealth of *in vitro* and *in vivo* data (briefly listed in their review [111]), as well as clinical observations in AAV patients. [112]

The process begins with the priming of neutrophils by low concentrations of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-18, and granulocyte-macrophage colony-stimulating factor (GM-CSF), often secreted by activated monocytes. This priming induces the translocation of ANCA antigens - namely PR3 and MPO - from cytoplasmic granules to the cell surface. Once exposed, these antigens become accessible to circulating ANCAs, which bind via their F(ab')2 regions, while their Fc fragments engage neutrophil Fcγ receptors. This dual interaction results in full neutrophil activation.

Activated neutrophils undergo a respiratory burst, releasing reactive oxygen species (ROS), along with degranulation that liberates cytotoxic proteases such as elastase, cathepsin G, and MPO. These proteases contribute to endothelial injury and are considered even more destructive than ROS. [105,113] Moreover, activated neutrophils release neutrophil extracellular traps (NETs)—web-like structures composed of decondensed chromatin and granule proteins—that propagate inflammation by exposing intracellular autoantigens, amplifying ANCA production, and activating the complement

system, particularly the alternative pathway via C5a generation. [114] This forms a self-perpetuating inflammatory loop that promotes further neutrophil recruitment, endothelial damage, and microvascular thrombosis. [115] (Figure 5)

Interestingly, similar mechanisms may occur extravascularly: primed neutrophils encountering tissue-bound ANCAs may initiate localized necrotizing inflammation, believed to represent the first phase in granuloma formation in GPA. [58] Walulik *et al*. [116] additionally emphasized that NETs not only serve as pro-inflammatory effectors but also act as structural scaffolds for immunothrombosis, directly contributing to small vessel occlusion and organ damage in AAV. These insights highlight NETs as potential diagnostic and therapeutic targets, as further discussed in Section 1.3.1. regarding extracellular vesicle biomarkers.

The role of complement system

Although AAV is traditionally characterized by minimal immunoglobulin and complement deposition—thus labelled as 'pauci-immune'—early assumptions excluded a significant role for the complement system in disease pathogenesis. However, pivotal studies in murine models conducted nearly two decades ago overturned this view, demonstrating that activation of the complement cascade, particularly through the alternative pathway, is a key mediator in ANCA-induced inflammation. [114]

Subsequent experimental and clinical investigations have consistently supported this concept. Complement component C5a has emerged as a central effector molecule, acting as a potent chemoattractant and activator of neutrophils via engagement with the C5a receptor (CD88). [93] Activated neutrophils, in turn, propagate further complement activation, establishing a self-amplifying inflammatory loop. This mechanism results in the aggressive necrotizing vasculitis characteristic of AAV. [110,117] The role of this pathway has been confirmed in both *in vitro* and *in vivo* models, as well as in clinical studies, as reviewed by Yuan *et al*. [118] and Sun *et al*. [110]

Key experimental data showed that knockout mice deficient in C3, C5, or factor B are protected from developing AAV, underscoring the essential role of the alternative pathway. [114] Human studies have echoed these findings: A Chinese group of authors reported that renal biopsies in AAV patients with complement deposition show more severe glomerular injury. [119,120] Also, elevated serum levels of C3a, C5a, factor Bb, and the terminal complement complex C5b-9 (membrane attack complex - MAC) have been observed during active disease compared to remission or healthy controls. [121,122]

Importantly, clinical remission has been associated with significant reductions in plasma C3a, C5a, and Bb levels, regardless of ANCA serotype or clinical phenotype. [122] These insights have propelled the development of targeted therapies that inhibit complement activation, particularly

through blockade of the C5a-C5aR axis. Complement fragment C5a represents a potent chemoattractant and immunostimulatory mediator that plays a central role in neutrophil recruitment and activation in the context of AAV. Upon its generation through complement cascade activation, C5a engages specific C5a receptors (CD88) on neutrophils, leading to their full functional activation. These neutrophils subsequently enhance the inflammatory environment by further stimulating the alternative complement pathway, which in turn generates additional C5a. This creates a self-sustaining pro-inflammatory circuit that contributes to ongoing vascular injury. The critical role of C5a-CD88 interaction in disease amplification has positioned the C5a receptor as a promising therapeutic target, particularly in ANCA-associated glomerulonephritis where neutrophil-mediated damage is pronounced. [93,123] The most advanced of these, Avacopan (CCX168), is an oral C5a receptor antagonist. It was initially evaluated in the CLEAR trial (ClinicalTrials.gov, Identifier: NCT01363388), a phase II, multicentre, randomized, double-blind, placebo-controlled study, conducted by EUVAS. Later, the phase III ADVOCATE trial confirmed its efficacy, leading to FDA approval in 2021 for use in AAV patients. [124] Avacopan represents a novel, steroid-sparing approach to treatment, offering both clinical and safety benefits.

The role of B and T cells

In addition to the well-established role of neutrophils in AAV, increasing attention has been directed toward the involvement of adaptive immune cells—particularly B and T lymphocytes—in disease immunopathogenesis. B cells are the primary source of ANCA autoantibodies; however, their contribution extends beyond antibody production. They also participate in antigen presentation, cytokine secretion, and formation of ectopic lymphoid aggregates within inflamed tissues, all of which sustain the autoimmune process and amplify tissue damage.

The pivotal role of B cells is strongly supported by the clinical success of Rituximab, a monoclonal antibody directed against CD20 that effectively depletes mature B lymphocytes. Rituximab is now an established therapeutic agent for both remission induction and maintenance in AAV. Nonetheless, residual or re-emerging B-cell populations following rituximab therapy have been detected in some patients and may be associated with increased risk of disease relapse, even in the absence of detectable ANCA. [125] This suggests that not all B-cell subsets are equally affected by therapy and that autoreactive memory B cells or plasmablasts may persist and retain pathogenic potential.

Moreover, dysregulation of B-cell survival factors—particularly B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL)—has been implicated in sustaining autoreactive B-cell clones in AAV. These cytokines enhance B-cell survival, differentiation, and antibody production, and represent additional immunological targets under active investigation. [126]

Given the therapeutic implications of modulating B-cell responses, the significance of these pathways will be explored in greater detail in the subsequent chapter dedicated to the treatment strategies for AAV.

Some authors are emphasizing the role of T and B lymphocytes in the development of AAV. B cells are producing ANCA, but that is not the only role they are playing in AAV pathogenesis. This is supported by the fact that rituximab, as a monoclonal antibody that recognizes and deletes B lymphocytes, is used in treatment of these patients. And even after this treatment, serum levels of B cells are still increased. [127]

In parallel with B-cell dysfunction, T lymphocytes play an essential role in the pathogenesis of AAV. $CD4^+$ T-helper (Th) subsets, particularly Th17 and T follicular helper (Tfh) cells, contribute to disease activity by promoting B-cell differentiation and sustaining autoantibody production. [128] Elevated levels of Th17 cells and associated cytokines (e.g., IL-17A, IL-21, and IL-23) have been detected in patients with active disease, suggesting their involvement in promoting tissue inflammation. [129] Furthermore, impaired function or reduced numbers of regulatory T cells (Tregs) have been reported, disrupting peripheral immune tolerance and allowing expansion of autoreactive clones. $CD8^+$ cytotoxic T cells may also contribute to endothelial injury through direct cytotoxic mechanisms, although their exact role remains less clearly defined. [130]

The imbalance between effector and regulatory T-cell subsets appears to be a central mechanism underlying persistent immune activation in AAV and offers potential targets for future therapeutic modulation. Moreover, recent advances in immunotherapy have extended beyond monoclonal antibodies. A proof-of-concept study by Lodka *et al.* [131] demonstrated that CD19-targeting chimeric antigen receptor (CAR) T cells can effectively prevent ANCA-induced acute kidney injury in preclinical models. This approach highlights the pathogenic relevance of B cells beyond antibody production and opens promising avenues for highly targeted interventions in refractory AAV.

1.2.7. AAV treatment and monitoring patients

Although the treatment of ANCA-associated vasculitis is not curative, it aims to achieve durable disease control, prevent organ damage, and improve long-term survival. If left untreated, AAV typically follows a fulminant and potentially life-threatening course, particularly in patients with renal or pulmonary involvement. The current therapeutic approach is therefore based on prompt immunosuppressive intervention tailored to disease severity and extent of organ involvement.

Management of AAV is typically divided into two distinct but interdependent phases. The first is induction therapy, whose primary goal is to achieve rapid remission of active inflammation.

This is followed by a maintenance phase, designed to sustain remission and minimize the risk of relapse while reducing treatment-associated toxicity. The precise choice of therapeutic agents depends on several factors, including ANCA subtype, clinical phenotype, comorbidities, and prior treatment response. [132]

Over the past two decades, treatment paradigms have evolved substantially—from conventional cytotoxic regimens to biologic therapies targeting B cells and complement pathways—guided by findings from pivotal clinical trials and recommendations from international consortia such as European Vasculitis Study Group (EUVAS), the European League Against Rheumatism (EULAR) and The Kidney Disease: Improving Global Outcomes (KDIGO).

According to the 2009 management recommendations [133] developed by EUVAS/EULAR, stratifying patients based on disease severity was essential for tailoring induction protocols. These guidelines emphasized the use of more aggressive regimens for life- or organ-threatening manifestations, while proposing less intensive approaches for non-severe disease. In 2015, an updated version of the recommendations was published in collaboration with the European Renal Association–European Dialysis and Transplant Association (ERA-EDTA), expanding guidance on long-term management, renal protection, and biologic therapies. [134] Most recently, in 2021, the first formal American guidelines were jointly released by the American College of Rheumatology (ACR) and the Vasculitis Foundation. [135] These ACR guidelines incorporate data from landmark clinical trials and real-world practice, refining therapeutic pathways based on ANCA serotype, organ involvement, and patient-specific risk factors. A summary of these recommendations is illustrated in the flow chart below.

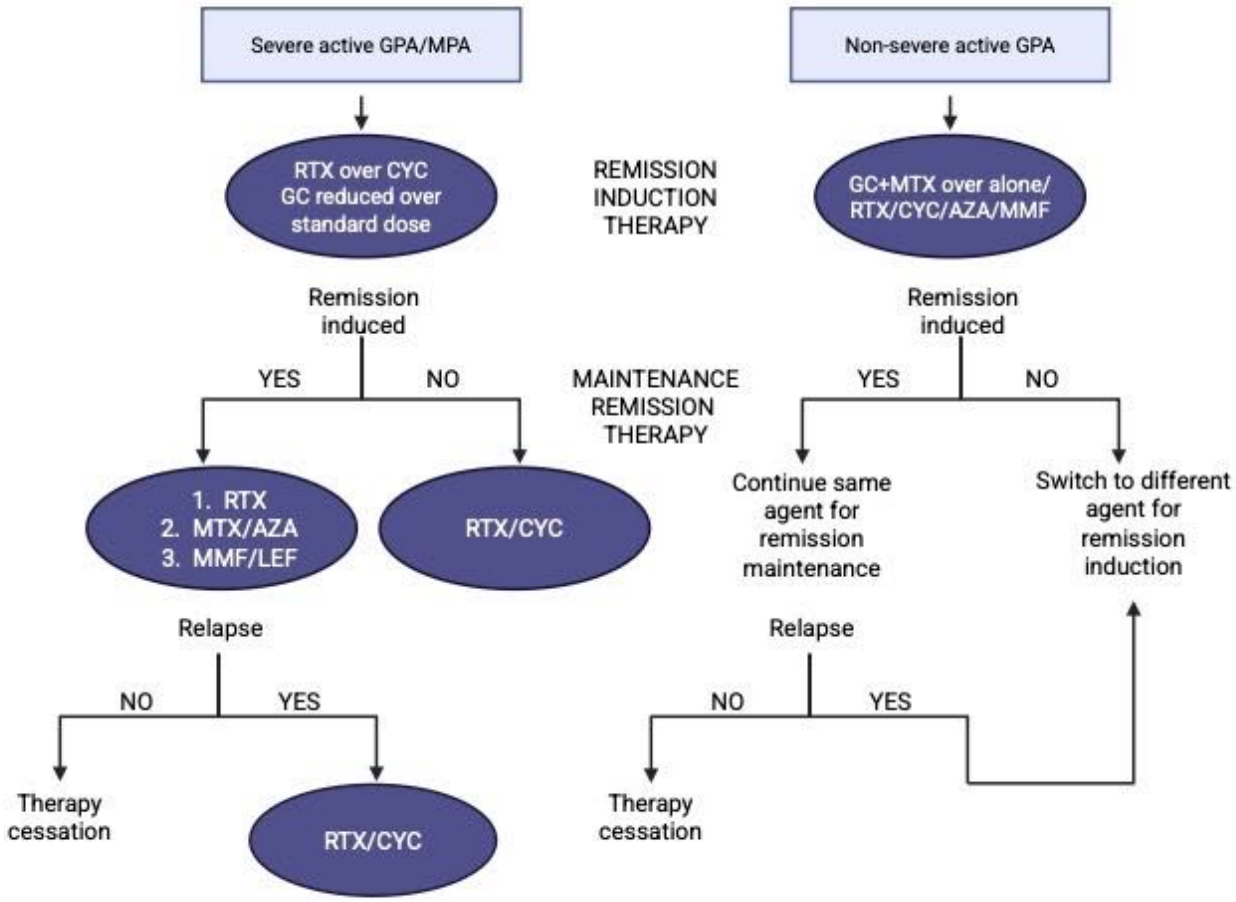


Figure 6. Treatment algorithm for AAV according to ACR/Vasculitis Foundation. [135] Severe disease is managed with rituximab (RTX) or cyclophosphamide (CYC) in combination with glucocorticoids (GC), while non-severe GPA can be treated with GC plus methotrexate (MTX) or alternative immunosuppressants (RTX, CYC, AZA, MMF). After induction, remission maintenance includes RTX, MTX, azathioprine (AZA), mycophenolate mofetil (MMF), or leflunomide (LEF). Therapy is adjusted based on remission status and relapse occurrence. Granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA). *Created with BioRender.com.*

The induction therapy

According to the most recent international guidelines, the induction therapy for newly diagnosed ANCA-associated vasculitis (AAV) presenting with organ-threatening or life-threatening manifestations should consist of high-dose ***glucocorticoids*** in combination with a cytotoxic agent. The corticosteroid regimen typically includes oral prednisone or prednisolone at an initial dose of 1 mg/kg/day (up to a maximum of 80 mg/day), followed by a gradual tapering schedule over several weeks to minimize long-term toxicity. This is co-administered with either cyclophosphamide (CYC) or rituximab (RTX), depending on disease phenotype, patient characteristics, and treatment availability. [136,137] ***Cyclophosphamide*** may be given as daily oral doses (2 mg/kg/day, maximum 200 mg/day) or in pulsed intravenous form (10–15 mg/kg every 2–3 weeks), the latter approach being associated with lower cumulative exposure and fewer adverse events. The CYCLOPS trial (Cyclophosphamide Oral versus Pulse Trial) demonstrated that pulsed IV CYC was as effective as oral administration in inducing remission, with a more favourable safety profile and reduced risk of

leukopenia and infections. Typically, the induction phase with CYC lasts between 3 to 6 months. [138] Alternatively, ***rituximab***—administered at a dose of 375 mg/m² weekly for four consecutive weeks—has been shown to be non-inferior to cyclophosphamide for remission induction in the pivotal RAVE trial. This monoclonal anti-CD20 antibody is particularly favoured in patients with relapsing disease, fertility concerns, or contraindications to alkylating agents. The CYCAZAREM trial (Cyclophosphamide versus Azathioprine for Remission) reinforced the use of daily oral cyclophosphamide in the early remission phase, demonstrating its superior efficacy over azathioprine in achieving and sustaining remission in over 90% of patients with generalized AAV. [139]

The maintenance therapy

Following successful induction of remission, maintenance therapy plays a critical role in preventing disease relapse and minimizing long-term organ damage. This phase typically extends for a minimum duration of 24 months, as premature discontinuation of immunosuppression has been consistently associated with higher relapse rates. The standard maintenance regimen consists of low-dose ***glucocorticoids*** in combination with one of the following immunosuppressive agents: azathioprine (2 mg/kg/day), rituximab (administered as fixed-dose infusions every 6 months), methotrexate (in patients with preserved renal function), or mycophenolate mofetil (MMF). [140] Among these agents, ***azathioprine*** has traditionally been considered the first-line option, supported by early trials demonstrating its efficacy in maintaining remission. However, recent data suggest that ***mycophenolate*** may offer advantages in certain immunological profiles. A small comparative study reported that MMF may suppress interleukin-6 (IL-6) production by B cells more effectively than azathioprine, suggesting an additional anti-inflammatory mechanism. [141] ***Rituximab*** has emerged as an equally effective and often preferred agent for relapse prevention, particularly in patients with relapsing disease or intolerance to conventional maintenance agents. ***Methotrexate*** remains a valuable option for non-severe AAV without renal involvement. ***Leflunomide*** (20–30 mg/day) may be considered as a second-line alternative for maintenance therapy in select patients, especially those with contraindications to first-line drugs. Long-term use of cyclophosphamide is generally avoided during the maintenance phase due to its well-documented cumulative toxicity, including a significantly increased risk of malignancy, gonadal dysfunction, and hematologic suppression. [142] Therefore, CYC is now reserved for induction therapy or refractory cases with close monitoring. [143]

In recent years, **rituximab**—a chimeric murine-human monoclonal antibody targeting the CD20 antigen on B lymphocytes—has gained prominence as a highly effective agent for both remission induction and maintenance in ANCA-associated vasculitis. Rituximab acts by selectively depleting circulating B cells, thereby limiting autoantibody production and modulating the autoimmune response. Its efficacy in inducing remission has been demonstrated through two pivotal

randomized controlled trials: RITUXVAS and RAVE. The RITUXVAS trial (an open-label, international multicentre study) compared a rituximab-based regimen with standard therapy comprising cyclophosphamide followed by azathioprine in patients with newly diagnosed, severe AAV. [144] Simultaneously, the RAVE trial evaluated rituximab versus oral cyclophosphamide for remission induction in patients with either granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA), including relapsing cases. [145] In both studies, rituximab was administered at a dose of 375 mg/m² once weekly for four consecutive weeks. The findings of both trials established rituximab as a non-inferior, and in certain patient subsets—particularly those with relapsing disease—a potentially superior alternative to cyclophosphamide (administered at 2 mg/kg/day). [146] As a result, rituximab is now considered a first-line option in patients for whom alkylating agents are contraindicated, or where fertility preservation and long-term toxicity are of concern.

A series of randomized controlled trials has firmly established rituximab as a highly effective agent for remission maintenance in AAV. The landmark MAINRITSAN trial (ClinicalTrials.gov Identifier: NCT00748644) enrolled 115 patients with newly diagnosed or relapsing AAV who had already achieved remission through cyclophosphamide and glucocorticoid-based induction. [147] Participants were then randomized to receive either fixed-dose rituximab or oral azathioprine for maintenance. After 28 months of follow-up, rituximab demonstrated superior efficacy in sustaining remission, with a comparable incidence of severe adverse events to the azathioprine group. Subsequently, the RITAZAREM trial (ClinicalTrials.gov, Identifier: NCT01697267), which focused on relapsing AAV, randomized 170 patients post-induction therapy to receive either rituximab infusions or oral azathioprine for relapse prevention. [148] Results confirmed that rituximab was more effective in re-inducing and maintaining remission in this high-risk group, particularly among PR3-ANCA–positive individuals. To further refine optimal dosing strategies, two follow-up trials—MAINRITSAN-2 (ClinicalTrials.gov, Identifier: NCT02433522) and MAINRITSAN-3 (ClinicalTrials.gov, Identifier: NCT02433522)—were conducted. MAINRITSAN-2 [149] compared fixed-schedule versus individualized, relapse-driven rituximab administration, while MAINRITSAN-3 [150] explored the long-term benefits and safety of continued rituximab maintenance beyond 18 months. Collectively, these studies demonstrated that prolonged maintenance therapy with low-dose rituximab significantly reduces relapse risk, especially in patients with PR3-ANCA serotype, without increasing serious infection rates.

Despite its proven efficacy, rituximab therapy—particularly in the context of repeated or long-term administration—has been associated with certain adverse effects, most notably hypogammaglobulinemia. [151] This condition, reflecting reduced serum immunoglobulin levels (especially IgG), is most commonly observed following induction treatment and may predispose patients to serious infections. Accordingly, current guidelines developed by expert panels in the

United Kingdom recommend regular monitoring of immunoglobulin levels in patients receiving rituximab-based regimens, particularly before each subsequent treatment cycle. [152] Nevertheless, based on the favourable balance between efficacy and safety observed across multiple clinical trials, rituximab remains a cornerstone of AAV management. It is now endorsed not only as an effective alternative to cyclophosphamide for induction therapy, but also as the preferred agent for remission maintenance in many patients. The long-term data from the MAINRITSAN and RITAZAREM trials, among others, continue to support its use across a broad spectrum of disease phenotypes and ANCA serotypes.

Plasma exchange (also known as **plasmapheresis**), typically administered as seven sessions over two weeks, has historically been considered as an adjunctive therapeutic option for patients with ANCA-associated vasculitis presenting with life-threatening manifestations, particularly severe renal involvement - defined as a serum creatinine concentration ≥ 500 μmol/l i.e. 5,7 mg/dl - due to rapidly progressive glomerulonephritis. [153] In addition, some guidelines have proposed its use in cases of severe diffuse alveolar haemorrhage (DAH), although evidence supporting its benefit in this subset remains limited and inconclusive. [154] The MEPEX trial (Methylprednisolone versus Plasma Exchange) was a multicentre, randomized controlled study that compared plasma exchange with intravenous methylprednisolone as adjunctive therapy in patients with AAV and severe renal impairment. Findings demonstrated that plasma exchange could significantly reduce the incidence of end-stage renal disease at 12 months, likely via the mechanical removal of circulating ANCAs and other inflammatory mediators, whereas corticosteroids primarily attenuate immune activation and autoantibody production. [153] To further explore this approach, the PEXIVAS (Plasma Exchange and Glucocorticoids in Severe AAV) trial—a large, international, randomized, open-label study—investigated whether adding plasma exchange to standard immunosuppressive therapy, including glucocorticoids, would improve clinical outcomes. [155] Despite the biologically plausible rationale, the study found no significant reduction in mortality or progression to end-stage kidney disease. As a result, the routine use of plasma exchange in AAV patients with severe disease is no longer widely recommended, and its application is now limited to selected high-risk cases based on individual clinical judgment.

The PEXIVAS trial (Plasma Exchange and Glucocorticoids for Treatment of ANCA-Associated Vasculitis), registered under ClinicalTrials.gov Identifier: NCT00987389, represented a pivotal multicentre, randomized study that enrolled 704 patients with severe AAV, including those with renal dysfunction and/or pulmonary haemorrhage. Participants were randomized to receive either adjunctive plasma exchange or no plasma exchange, and additionally, to follow either a standard- or reduced-dose oral glucocorticoid regimen, with a follow-up period extending up to seven years. The trial results indicated that the addition of plasma exchange did not significantly lower the

composite outcome of death or progression to end-stage kidney disease (28.4% in the plasma exchange group vs. 31.0% in the control group). Consequently, the findings did not support the routine use of plasma exchange in severe AAV. Furthermore, the study provided compelling evidence that a reduced-dose glucocorticoid protocol was non-inferior to the standard regimen in preventing death or kidney failure, while significantly lowering the risk of treatment-related toxicity. [155] Prior studies [156] have already identified high-dose glucocorticoids as a key contributor to serious infections, which represent one of the leading causes of morbidity and mortality in AAV patients. These results strongly support the implementation of reduced-dose glucocorticoid regimens as the new standard of care, particularly in patients at higher risk of infectious complications.

In cases of newly diagnosed AAV without organ- or life-threatening manifestations, remission induction can be effectively achieved with a less intensive regimen consisting of oral glucocorticoids in combination with either methotrexate (administered orally or parenterally at a dose of 20–25 mg/week) or mycophenolate mofetil. These agents are particularly suitable for patients with limited disease or lower inflammatory burden, offering a favourable safety profile compared to cyclophosphamide- or rituximab-based protocols. [157]

Avacopan, a first-in-class orally administered selective antagonist of the complement 5a receptor (C5aR, also known as CD88), has emerged as a novel therapeutic option in the management of ANCA-associated vasculitis (AAV). C5aR is a G protein-coupled receptor expressed on both myeloid cells—such as neutrophils, macrophages, mast cells, and dendritic cells—and certain non-myeloid tissue types. Avacopan acts by blocking the binding of the potent inflammatory peptide C5a to its receptor, thereby interrupting complement-mediated neutrophil activation, chemotaxis, degranulation, and subsequent endothelial damage. [158] This therapeutic approach aims to replace or reduce the use of glucocorticoids, which have long been associated with significant treatment-related toxicity in AAV. In early phase I trials, Avacopan was well tolerated in healthy volunteers. The phase II CLEAR study (ClinicalTrials.gov Identifier: NCT01363388), conducted by EUVAS, evaluated its safety and efficacy in combination with reduced-dose prednisone or as a steroid-sparing agent in combination with either rituximab or cyclophosphamide. Results demonstrated promising disease control with lower glucocorticoid exposure. The subsequent CLASSIC trial (ClinicalTrials.gov, Identifier: NCT02222155), a phase II randomized, placebo-controlled study, assessed the tolerability of Avacopan (10 mg or 30 mg twice daily) as an adjunct to standard induction therapy. [159] Patients in the 30 mg group experienced improved disease control with a favourable safety profile, supporting further investigation in larger cohorts. The pivotal phase III ADVOCATE trial (ClinicalTrials.gov, Identifier: NCT02994927) included 331 patients with newly diagnosed or relapsing AAV and compared oral Avacopan (30 mg twice daily) to a conventional tapering regimen of oral prednisone, both in combination with standard induction therapy (rituximab or

cyclophosphamide). While remission rates at 26 weeks showed non-inferiority of Avacopan, by week 52, Avacopan demonstrated superiority in achieving sustained remission. Additionally, patients receiving Avacopan experienced fewer glucocorticoid-related adverse effects. [124] Based on these findings, both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved Avacopan for use in AAV as part of a glucocorticoid-sparing regimen in combination with standard induction therapy.

Although current treatment strategies for AAV are largely evidence-based and clinically effective, the use of immunosuppressive agents carries considerable risk for both short- and long-term adverse outcomes. Among the most notable complications are serious infections resulting from treatment-induced hypogammaglobulinemia [160]—commonly observed following rituximab or cyclophosphamide therapy—as well as osteoporosis, infertility, and an elevated lifetime risk for malignancies such as hematologic cancers and bladder carcinoma, particularly linked to historical long-term cyclophosphamide exposure. [161] Given these concerns, a major therapeutic objective in AAV has shifted toward optimizing individualized treatment approaches that preserve efficacy while minimizing toxicity. In this regard, rituximab remains the only biologic agent currently approved for both induction and maintenance of remission in AAV. Nevertheless, ongoing research is actively exploring novel therapeutic targets aimed at reducing or eliminating glucocorticoid exposure, enhancing immune modulation, and tailoring treatment protocols based on disease phenotype, serotype, and relapse risk. Advances in understanding the pathogenesis of AAV—particularly in relation to B-cell biology, complement pathways, and neutrophil-mediated injury—are central to these evolving strategies. [162]

Beyond rituximab and avacopan, several novel biologic agents targeting distinct immunological pathways are currently being investigated for AAV. One such agent is **belimumab**, a monoclonal antibody directed against B-cell activating factor (BAFF), which may be particularly effective in MPO-ANCA–positive patients who tend to show elevated BAFF levels. Although primarily used in systemic lupus erythematosus, belimumab has shown promise as a maintenance therapy in AAV when combined with rituximab, as explored in the ongoing COMBIVAS trial (NCT03967925). [163] Another candidate is **tocilizumab**, an interleukin-6 (IL-6) receptor antagonist, which has demonstrated clinical benefit in case reports and small open-label trials in patients with refractory AAV. [164] Of particular interest is the use of chimeric antigen receptor T (CAR-T) cells targeting CD19+ B cells, offering a highly selective and long-lasting B-cell depletion approach. A recent preclinical study by Lodka et al. [131] showed that CD19-targeted CAR-T cells conferred protection against ANCA-induced glomerulonephritis in murine models, suggesting potential for translation into clinical settings.

1.2.8. Complications in AAV

It is now well established that patients with AAV are at an increased risk of developing thromboembolic events, with a notably hypercoagulable state observed both during active disease and in clinical remission. [165,166] This was first observed in paediatric populations [167] and has since been validated in multiple adult cohorts. One of the most recent multicentre cohort studies, comprising 417 patients, highlighted a strong correlation between disease activity and the incidence of venous thromboembolism (VTE). [168] The overall incidence of VTE among AAV patients is significantly higher compared to the general population, with rates reported at 6.7 per 100 person-years during active disease and 1.8 per 100 person-years during remission [169], as opposed to 0.3 per 100 person-years in the general population.[170] The risk appears to be particularly pronounced in patients with renal involvement, who exhibit enhanced thrombin generation even beyond the acute disease phase.

Although the precise mechanisms remain incompletely understood, several pathophysiological explanations have been proposed. Among them is the observation of increased coagulation activity and impaired fibrinolysis, particularly elevated factor VIII levels, which tend to persist even in remission. [171] Additionally, autoantibodies against components of the fibrinolytic system—such as plasminogen and tissue plasminogen activator—have been detected in a significant proportion of PR3-ANCA-positive patients who developed VTE. [172] Similar findings have been reported in MPO-ANCA-positive individuals, indicating that antiplasminogen antibodies may be broadly implicated in AAV-related hypercoagulability, even during remission. [173] Their presence has been correlated not only with thrombotic events but also with the severity of renal vasculitis. [174]

Another compelling mechanism involves the role of neutrophil extracellular traps (NETs), which are increasingly recognized as key mediators of immunothrombosis in AAV. NETs have been identified in glomerular crescents of patients with microscopic polyangiitis and within thrombotic lesions themselves. [175] In one illustrative case, a markedly higher concentration of NETs was observed in the thrombus of an MPA patient than in patients with pulmonary embolism or bacterial sepsis, supporting their contributory role. [176] NETs appear to promote thrombosis through their histone content, which can activate platelets and bind various coagulation factors, while also directly injuring the endothelium. [177] This evidence supports the hypothesis that NET-driven immunothrombosis may be a central mechanism underlying the elevated thromboembolic risk in AAV and represents a potential therapeutic target.

In addition to the well-documented thromboembolic risk, patients with AAV are also at elevated risk for cardiovascular complications, particularly within the first five years following diagnosis. This increased cardiovascular morbidity is believed to result from a combination of

traditional risk factors and disease-specific mechanisms, including chronic systemic inflammation and endothelial dysfunction. Persistent immune activation and elevated proinflammatory cytokine levels may accelerate the development of atherosclerosis, thereby contributing to premature cardiovascular events such as myocardial infarction, stroke, and peripheral artery disease. [178] Furthermore, the use of high-dose glucocorticoids and cytotoxic immunosuppressive therapies may exacerbate underlying metabolic conditions, including hypertension, dyslipidaemia, and insulin resistance, further enhancing the overall cardiovascular risk profile in these patients. Early identification of high-risk individuals and implementation of cardioprotective strategies—including lifestyle modification, blood pressure and lipid control, and close monitoring—are therefore essential components of long-term AAV management.

Infectious complications represent a major cause of morbidity and mortality in AAV patients, particularly during the early phase of treatment when high-dose immunosuppression is administered. Glucocorticoids, cyclophosphamide, and rituximab significantly impair both innate and adaptive immune responses, predisposing patients to opportunistic and community-acquired infections. The risk is highest within the first six months of therapy and is notably associated with severe infections such as pneumonia, sepsis, and reactivation of latent pathogens (e.g., tuberculosis, herpesviruses). [179] Prophylactic measures, including trimethoprim-sulfamethoxazole for *Pneumocystis jirovecii* pneumonia and vaccination against influenza and pneumococcus, are widely recommended in clinical practice to mitigate this risk. [60] Long-term B-cell depletion with rituximab may also lead to hypogammaglobulinemia, necessitating immunoglobulin monitoring and, in selected cases, replacement therapy. [180]

Beyond thromboembolic and infectious complications, AAV patients are susceptible to a spectrum of long-term sequelae that impact prognosis and quality of life. [181] Chronic kidney disease (CKD) is one of the most frequent complications, especially among those with renal involvement at onset. Even with successful induction of remission, irreversible glomerular damage often results in persistent renal dysfunction or progression to end-stage kidney disease (ESKD), requiring dialysis or transplantation. [182] Pulmonary complications, such as pulmonary fibrosis, may develop following episodes of alveolar hemorrhage, while chronic sinus disease and subglottic stenosis are common in granulomatosis with polyangiitis (GPA). Long-term glucocorticoid use further contributes to secondary complications including osteoporosis, diabetes mellitus, cataracts, and muscle wasting. [183] Neuropsychiatric manifestations such as cognitive impairment, fatigue, and depression have also been reported, often underrecognized but significantly affecting daily functioning. These findings underscore the need for multidisciplinary care, vigilant follow-up, and early identification of organ damage in the long-term management of AAV.

1.3. Extracellular vesicles in AAV

In contrast to the well-documented involvement of extracellular vesicles (EVs) in the pathogenesis of various inflammatory, autoimmune, and cardiovascular diseases, their role in vasculitis has been relatively underexplored. This is likely due to the rarity of ANCA-associated vasculitis in the general population and the consequent limited availability of study cohorts. Research into EVs— back then known as Microparticles—in the context of vasculitis began nearly two decades ago, when elevated levels of circulating EVs, primarily of endothelial and platelet origin, were observed in children with active systemic vasculitis. [184] These levels showed a strong correlation with disease activity, independent of immunosuppressive treatment. [184] Then, back in 2006 Daniel *et al.* [185] expanded this line of research by reporting increased levels of EVs expressing neutrophil marker CD66b and platelet marker CD41a in patients with vasculitis, as well as in those undergoing haemodialysis-associated inflammation. Subsequent studies confirmed similar findings in adult populations, reinforcing the relationship between endothelial-derived vesicles and disease activity. [186] At that time, extracellular vesicles were interpreted as *in vivo* markers of neutrophil and platelet activation. However, their precise contribution to disease pathogenesis remained unclear, and their functional role was not yet systematically investigated.

In the years that followed, understanding of extracellular vesicles in the context of vasculitis gradually progressed. (Figure 10.) While early studies confirmed that circulating EVs are elevated in various forms of autoimmune vasculitis, particularly those associated with antineutrophil cytoplasmic antibodies (ANCAs), their precise pathophysiological role remained elusive. The functional significance of these vesicles in ANCA-associated vasculitis (AAV) had not yet been fully delineated.

A notable advancement was made by Hong *et al*. [187], who were among the first to demonstrate that neutrophil-derived microparticles (NMPs) carrying surface adhesion molecules and ANCA autoantigens—namely, myeloperoxidase (MPO) and proteinase 3 (PR3)—are significantly elevated in the plasma of children with active AAV. Importantly, their levels were shown to fluctuate in accordance with disease activity. Furthermore, the study highlighted that NMPs released from neutrophils primed with tumour necrosis factor-alpha (TNF-α) and subsequently activated by ANCA are capable of inducing endothelial activation through a reactive oxygen species (ROS)-dependent pathway. These particles were shown to be highly prothrombotic, with potent thrombin-generating capacity, implicating them as possible contributors to vascular injury and microthrombotic complications in AAV.

Previous investigations have provided compelling evidence that neutrophil-derived microparticles (NMPs) possess the capacity to activate endothelial cells and function as proinflammatory mediators within the vascular microenvironment. [188] These particles may thereby contribute to the propagation of vascular injury in inflammatory disorders such as AAV. In a key study, Pitanga *et al*. [38] demonstrated that extracellular vesicles released from activated human neutrophils contain active myeloperoxidase (MPO). The presence of functional MPO within EVs supports the hypothesis that the MPO–hydrogen peroxide–chloride system, when delivered via EVs, may serve as a localized source of oxidative stress. This mechanism is capable of inducing direct endothelial damage, especially under conditions of heightened neutrophil activation characteristic of vasculitic syndromes. These findings underscore the potential of neutrophil-derived EVs not only as biomarkers of disease activity but also as effectors contributing to vascular pathology.

The role of extracellular vesicles in AAV pathogenesis is recognized, supported by increasing evidence from previous studies. As presented on the timeline below (Figure 7), collectively, this progression highlights the shift from viewing EVs as by-products of cellular activation to recognizing them as active mediators of immunopathogenesis in AAV.

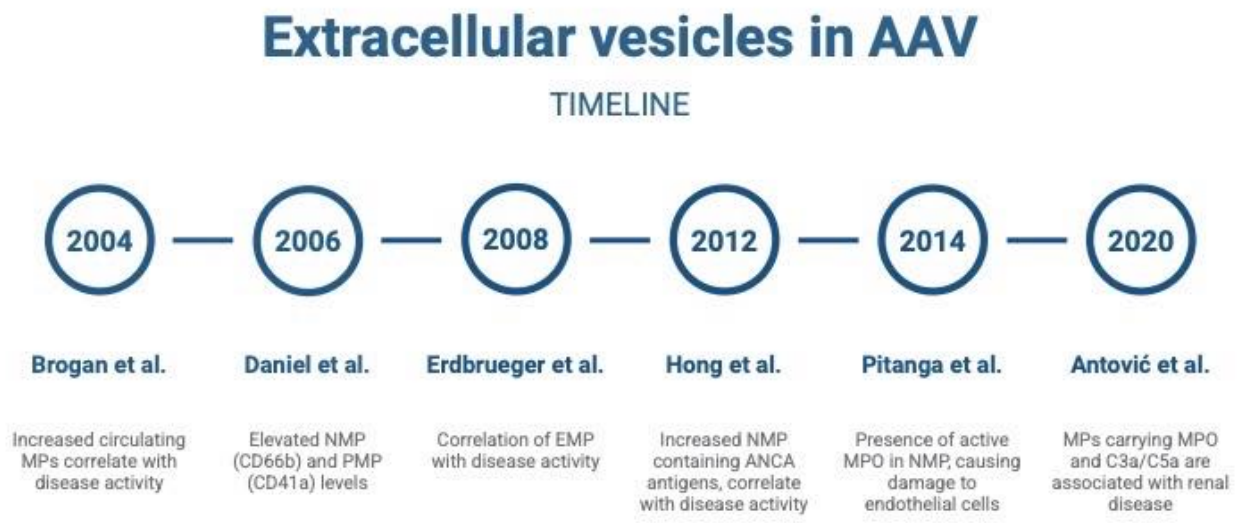


Figure 7. The key milestones in the discovery and characterization of extracellular vesicles (EVs) in ANCA-associated vasculitis (AAV). In earlier studies, EVs were commonly referred to as microparticles (MPs), mainly derived from neutrophils, platelets, and endothelial cells. Over time, multiple studies demonstrated their association with disease activity, their content of ANCA autoantigens such as MPO and PR3, and their role in endothelial activation and injury. More recent work established their involvement in complement activation (C3a, C5a) and renal pathology.

1.3.1. Biomarkers on extracellular vesicles

In 2015, the U.S. Food and Drug Administration (FDA) and the National Institutes of Health (NIH) jointly established the Biomarkers, EndpointS, and other Tools (BEST) Resource as a collaborative initiative to provide standardized definitions and guidance regarding the use of biomarkers in medical research. According to this working group, a biomarker is defined as a measurable indicator of normal biological processes, pathogenic processes, or responses to an exposure or therapeutic intervention. Biomarkers may aid in the identification of disease presence or severity, assist in the classification of subtypes, and guide diagnostic or therapeutic decisions relevant to a specific condition. [189]

Over the years, considerable research effort has been directed toward identifying reliable biomarkers capable of accurately diagnosing and monitoring disease activity in ANCA-associated vasculitis, with the aim of distinguishing active disease from remission. [190] Despite numerous investigations, an ideal biomarker has yet to be identified. The need persists for tools that can reliably predict relapse, confirm remission, monitor disease progression, and guide individualized therapeutic strategies—particularly given the highly variable clinical course observed in patients following initial treatment. Current serological markers, including ANCA titers and conventional inflammatory indicators such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), are widely used in clinical practice. However, their diagnostic and prognostic value is limited due to low specificity and their inability to distinguish between infectious complications and vasculitis flares—one of the most common and critical challenges in the clinical management of AAV. As a result, these conventional tools often fail to offer sufficient accuracy for guiding treatment decisions or staging disease activity. In the absence of reliable predictors of disease relapse, clinicians are frequently faced with the difficult task of balancing the risks associated with prolonged immunosuppressive therapy against the potential for uncontrolled disease activity. In light of these limitations, recent research efforts have turned toward the exploration of novel biomarkers, including extracellular vesicles (EVs), which may offer improved specificity and functional relevance in the context of AAV. The following section will explore these emerging biomarkers in greater detail.

1.3.1.1. Tissue factor (TF)

Tissue factor (TF) represents the primary initiator of the coagulation cascade and is recognized as a central trigger of venous thromboembolism (VTE). Its expression on neutrophil extracellular traps (NETs) and neutrophil-derived microparticles (NMPs) has been extensively investigated in the

context of ANCA-associated vasculitis (AAV). In a pivotal study by Kambas *et al*. [191], TF-bearing neutrophil-derived extracellular vesicles were shown to correlate strongly with active disease states, implicating them as potential mediators of thrombogenicity in AAV and suggesting their utility as biomarkers for identifying patients at heightened risk of VTE.

Previous research has pointed toward a mechanistic link between inflammation and hypercoagulability in AAV. *In vitro* experiments demonstrated that C5a-primed neutrophils, when subsequently stimulated with ANCA, released TF-positive extracellular vesicles and NETs, resulting in increased thrombin generation and activation of the coagulation pathway. [192] This cascade not only supports the concept of neutrophil-driven thrombosis but also emphasizes the procoagulant role of extracellular vesicles in AAV pathogenesis.

Supporting this concept, more recent studies have confirmed elevated levels of microparticle-associated tissue factor activity in AAV patients—even during clinical remission. These findings, particularly when accompanied by increased titers of anti-plasminogen antibodies, appear to serve as strong and independent indicators of thromboembolic risk, regardless of underlying renal function. [173] Altogether, TF-bearing EVs may represent a promising biomarker candidate for identifying subclinical disease activity and guiding thromboprophylactic strategies in AAV.

1.3.1.2. Neutrophil extracellular traps (NETs)

Given their strong thrombogenic potential, tissue factor-bearing extracellular vesicles are now recognized not only as biomarkers but also as active participants in the hypercoagulable state observed in AAV. Notably, these vesicles often originate from activated neutrophils, which play a central role in the pathophysiology of vasculitis through multiple mechanisms. One such mechanism is the formation of neutrophil extracellular traps (NETs)—a process increasingly recognized for its dual function in antimicrobial defence and promotion of vascular injury in autoimmune diseases.

Neutrophil extracellular traps (NETs) are web-like structures composed of chromatin fibers and granule-derived antimicrobial proteins, actively released into the extracellular space by neutrophils through a specialized form of cell death termed NETosis. These DNA-based networks are heavily decorated with histones and proinflammatory enzymes—including the primary ANCA autoantigens, myeloperoxidase (MPO) and proteinase 3 (PR3)—which not only help trap and neutralize pathogens, but also inadvertently promote autoimmune responses and tissue damage. [193] In AAV, NETs are thought to amplify inflammation and perpetuate vasculitic injury through multiple pathways: by exposing ANCA antigens to the immune system, by directly damaging endothelial cells, and by providing a scaffold for thrombosis formation. [194] Furthermore, posttranslational modifications of proteins within NETs may enhance their immunogenicity, thereby fostering a break in self-tolerance and perpetuating autoimmunity. [195]

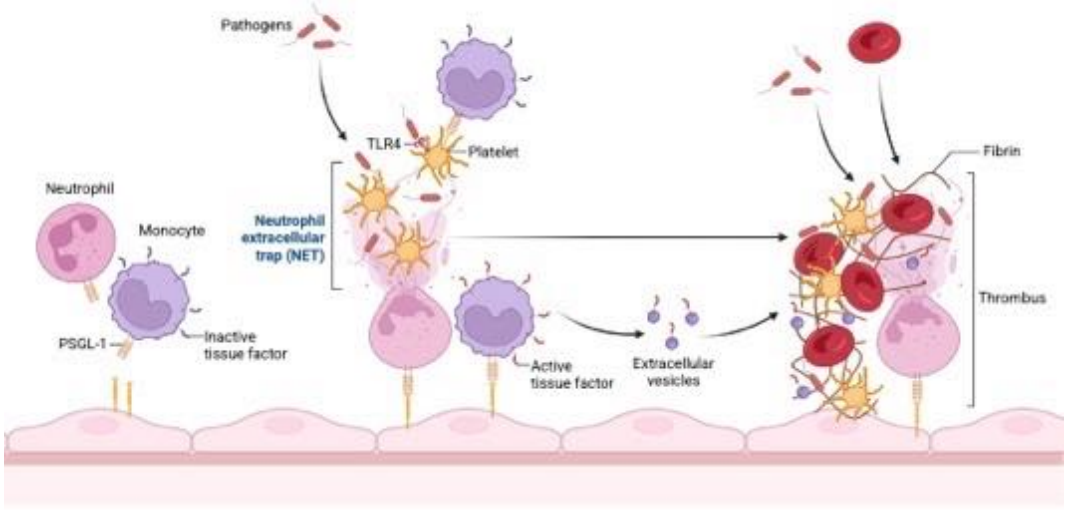


Figure 8. Prothrombotic role of extracellular vesicles and neutrophil extracellular traps (NETs). Activation of neutrophils and monocytes leads to the release of NETs and EVs enriched in tissue factor (TF), which interact with platelets and the coagulation cascade. These mechanisms contribute to thrombin generation, fibrin formation, and ultimately thrombus development and vascular inflammation. *Created with BioRender.com (2025). Retrieved from https://app.biorender.com/biorender-templates.*

A fundamental step in the formation of neutrophil extracellular traps (NETs) involves histone H3 citrullination, catalysed by the enzyme peptidylarginine deiminase 4 (PAD4). This posttranslational modification leads to chromatin decondensation, facilitating the extrusion of DNA and granule proteins into the extracellular space. [196] Citrullinated histone 3 (H3Cit), released during this process, is considered a specific biomarker of NET formation, and its circulating levels can be quantified in plasma samples. [197] Moreover, the presence of H3Cit on extracellular vesicles (EVs) was demonstrated in human models of endotoxemia [198], suggesting a potential role of vesicle-associated citrullinated histones in inflammation. However, this association remains largely unexplored in the context of systemic autoimmune diseases, including ANCA-associated vasculitis.

The pathogenic role of NETs in AAV was first proposed by Kessenbrock *et al*. in 2009. [175] Their seminal study showed that activation of neutrophils by ANCA leads to excessive degranulation and respiratory burst, resulting in the formation of NETs enriched with MPO and PR3—the main autoantigens targeted in AAV. Simultaneously, the formation of extracellular vesicles was observed, with their levels correlating with disease activity assessed by the Birmingham Vasculitis Activity Score (BVAS). [199] Subsequent *in vitro* and *in vivo* [200] studies confirmed that ANCAs directly induce NETs formation, supporting the idea that NETs are not merely byproducts, but active contributors to tissue injury in AAV.

Elevated levels of circulating NET remnants have been consistently detected in the sera of AAV patients, particularly during the active phase of disease. [201,202] Furthermore, NETs have been visualized in affected tissues - glomerular crescents in the kidneys [175,203], skin lesions [204],

and even in intravascular thrombi [176], linking them to both vasculitis and thrombosis. However, findings across studies have not always been consistent. While some authors found that citrullinated H3-positive NETs correlate with disease activity only in MPO-ANCA - positive patients [200], others failed to establish a clear link between NET levels and BVAS scores. Instead, they observed significantly elevated NET biomarkers in AAV patients compared to healthy controls, without clear discrimination between active and inactive disease. [205]

The multifaceted role of NETs in the immunopathogenesis of AAV – spanning autoantigen exposure, endothelial injury, and thrombosis – has been comprehensively reviewed by O`Sullivan *et al.* [206] Their work also introduces the concept of targeting NETs therapeutically, and proposes novel experimental NET-inhibiting strategies, including PAD4 inhibitors and DNase-based approaches, as potential future directions in AAV treatment.

The interplay between the complement system and NETs formation has become increasingly relevant in understanding AAV pathogenesis. *In vitro* studies have demonstrated that activation of the alternative complement pathway can trigger NET release, linking complement activation to neutrophil-driven inflammation. [204] This has led to the hypothesis that both NETs and complement components act synergistically as amplifiers of acute inflammatory responses in AAV, thereby perpetuating vascular damage and immune dysregulation. [207]

Interestingly, Kraaij *et al.* [208] provided a nuanced perspective, suggesting that excessive NETs formation in AAV patients may represent a hallmark of autoimmunity rather than infection. Their study showed that excessive NETs formation *in vitro* was independent of C5a receptor (C5aR) inhibition, indicating that complement activation, although essential in other steps of disease pathogenesis, may not directly regulate NET release in all circumstances. Moreover, they observed increased NETs formation in patients with active disease, even in the absence of detectable ANCA, highlighting alternative pathways contributing to neutrophil activation and NETosis.

More recently, an expanded model of AAV immunopathogenesis has been proposed, positioning NETs not only as effectors of inflammation, but also as initiators of autoimmunity. [209] NET-associated autoantigens such as MPO and PR3 are believed to drive the development of ANCAs, thereby reinforcing a self-perpetuating inflammatory loop. This reciprocal relationship between NET formation and ANCA production contributes to chronic autoimmunity, localized tissue damage, and disease amplification. Therefore, NETs are increasingly viewed not only as bystanders but as central elements in AAV progression—serving as potential biomarkers of disease activity and attractive therapeutic targets in precision-based treatment strategies.

1.3.1.3. Pentraxin 3 (PTX3)

Among the various candidates proposed as biomarkers in AAV, Pentraxin 3 (PTX3) has emerged as a promising molecule. PTX3 is an acute-phase protein that belongs to the long pentraxin family and differs structurally and functionally from the short pentraxins such as C-reactive protein (CRP). [210] It is produced locally at the site of inflammation by a variety of cells including neutrophils, endothelial cells, and macrophages in response to pro-inflammatory stimuli, such as tumour necrosis factor alpha (TNFα) and interleukin 1 (IL-1) among others, and its expression is not mediated by IL-6. Unlike CRP, which is synthesized primarily in the liver, PTX3 is rapidly released by activated neutrophils, often stored in specific granules and co-localized with MPO. Importantly, PTX3 levels remain stable under corticosteroid treatment, making it a potentially more reliable marker of reflecting inflammation in certain contexts. [210]

Many biologic effects of PTX3 are discovered in inflammation: detection of pathogens and further activation of opsonization and phagocytosis, as well as activation of complement system. [211] Since this protein, together with MPO and PR3, is stored in neutrophil granules and released upon neutrophil activation and respiratory burst, it can be found in NETs. [212] Ultimately, it is involved in regulation of neutrophil recruitment. [213]

For some time, it has been known that levels of PTX3 may reflect reactivity of neutrophils in systemic immune-mediated diseases (including AAV), [214] likewise correlation with disease activity, acute phase reactants and prednisone dose was found. [215] Biological role of anti-Pentraxin-3 antibodies still remains unclear, though they could be found in sera from AAV patients as demonstrated in one French cohort, [216] and recently suggested as biomarker of AAV, particularly EGPA. [217]

1.3.1.4. High mobility group box 1 (HMGB1)

High Mobility Group Box 1 (HMGB1) represent a group of nuclear factors that serve as transcription and growth regulators, playing a key role in chromatin remodelling and gene expression. Under conditions of cellular stress, activation, or damage, HMGB1 can be actively secreted into the extracellular space or passively released by necrotic and apoptotic cells. In this extracellular context, HMGB1 acquires proinflammatory properties, acting as a damage-associated molecular pattern (DAMP) molecule and contributing to the initiation and amplification of innate immune responses. [218] Due to these immunomodulatory features, HMGB1 has been implicated in the pathogenesis of various autoimmune and inflammatory disorders, including systemic lupus erythematosus, rheumatoid arthritis, idiopathic inflammatory myopathies, and sepsis.

For some time now, serum HMGB1 is widely analysed in AAV. Growing attention has been directed toward its potential role in disease pathophysiology and its utility as a biomarker. Notably, HMGB1 has been shown to promote neutrophil activation by enhancing the translocation of ANCA antigens (such as MPO and PR3) to the cell surface, thereby facilitating respiratory burst and degranulation. (Figure 9) These effects are mediated through interaction with receptors for glycation and products – RAGE and Toll-like receptors 2 and 4. [219] However, data on circulating HMGB1 levels in AAV remain somewhat inconsistent. While some studies [220] have reported reduced serum HMGB1 concentrations in patients with renal involvement and no significant differences between clinical subtypes of AAV, others have suggested that elevated HMGB1 levels may reflect disease activity [221,222] and renal involvement. [223] This variability may, in part, be attributed to the influence of immunomodulatory therapies, including corticosteroids and statins, on HMGB1 expression and release. [224] Interestingly, given that HMGB1 can be excreted in the urine following glomerular damage, urinary HMGB1 has emerged as a potential non-invasive marker of renal involvement in AAV. Preliminary studies have shown significantly higher urinary HMGB1 levels in patients with active nephritis compared to those in remission and healthy individuals, supporting its utility in disease monitoring and renal assessment. [221,225]

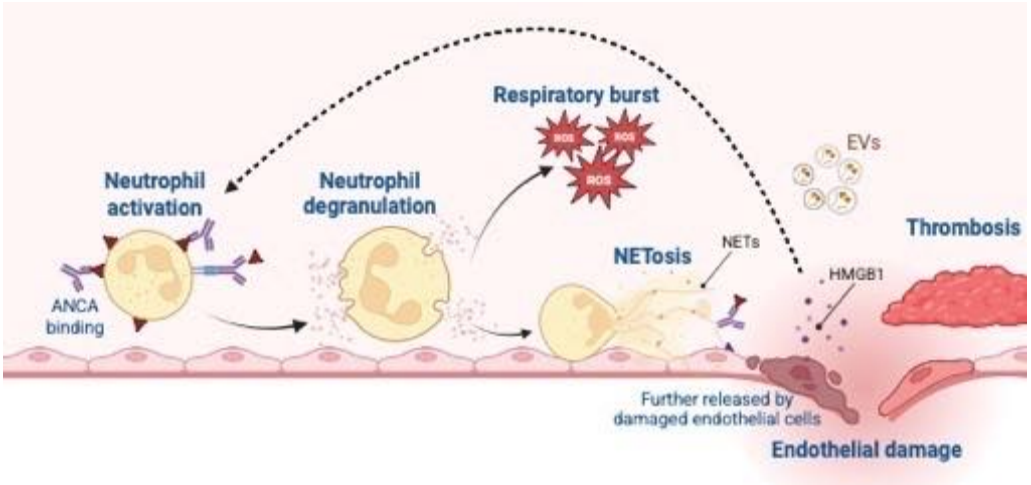


Figure 9. Schematic illustration of the role of HMGB1 in the pathogenesis of ANCA-associated vasculitis (AAV). Activated neutrophils undergo NETosis, releasing neutrophil extracellular traps (NETs) and HMGB1. HMGB1 released by neutrophils and damaged endothelial cells propagates inflammation and promotes thrombosis through interaction with extracellular vesicles (EVs). The dotted arrow indicates the ability of HMGB1 to enhance neutrophil activation by increasing the translocation of ANCA antigens, thereby stimulating degranulation and respiratory burst. Damaged endothelial cells further release HMGB1, contributing to vascular injury. *Created with BioRender.com*

1.3.1.5. Soluble tumour necrosis factor-like weak inducer of apoptosis (sTWEAK)

sTWEAK (soluble Tumor necrosis factor-like weak inducer of apoptosis) is a circulating, type II transmembrane glycoprotein belonging to the TNF ligand superfamily, which exerts its biological functions through binding to the Fn14 receptor (fibroblast growth factor-inducible 14). This

interaction regulates a broad spectrum of physiological and pathological processes, including inflammation, tissue regeneration, and fibrosis. [226] In addition to Fn14, TWEAK can also bind to the hemoglobin-haptoglobin scavenger receptor CD163, a soluble form of which (usCD163) has recently emerged as a promising urinary biomarker for detecting active renal vasculitis. [227]

TWEAK signalling has been implicated in the pathogenesis of both cardiovascular and renal damage, with studies in lupus nephritis providing the basis for exploring anti-TWEAK targeted therapies in inflammatory kidney diseases. [228] In fact, therapeutic modulation of TWEAK activity has shown potential in attenuating renal injury, leading to the proposal of TWEAK as a therapeutic target. [229] Furthermore, serum TWEAK levels have been evaluated as a possible guide for optimizing corticosteroid therapy in 131 systemic lupus erythematosus (SLE) patients, particularly to assist in tapering glucocorticoid doses more effectively. [230]

Elevated circulating concentrations of TWEAK have been documented in various autoimmune inflammatory disorders and appear to reflect disease activity. [231,232,233] In parallel, the urinary form—uTWEAK—has been proposed as a specific indicator of renal involvement, reflecting local tissue inflammation. [234] Despite growing interest in its clinical utility, and its established role in other systemic autoimmune diseases, to date, no studies have systematically examined TWEAK levels or function in patients with ANCA-associated vasculitis. This underscores the need for further investigation into its potential diagnostic and prognostic value in this setting.

1.3.1.5. Plasminogen (Plg)

Another molecule drawing increasing scientific attention in the context of ANCA-associated vasculitis (AAV) is plasminogen, an inactive zymogen and essential member of the fibrinolytic system. Once converted into its active form, plasmin, it contributes to the degradation of fibrin and resolution of thrombi. [235] (Figure 10)

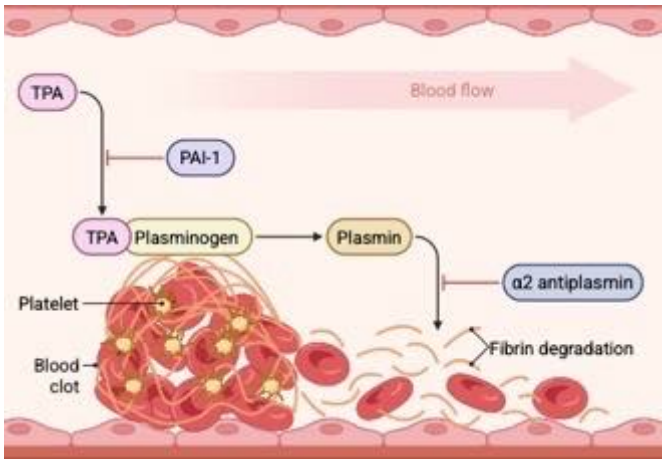


Figure 10. Fibrinolytic system and plasmin-mediated clot degradation**.** Illustration of the fibrinolytic pathway initiated by tissue plasminogen activator (tPA), which converts plasminogen to plasmin. Plasmin cleaves fibrin within the thrombus, leading to its degradation and dissolution, thereby enabling restoration of normal blood flow. *Created with BioRender.com (2025). Retrieved from https://app.biorender.com/biorender-templates.*

Approximately a decade ago, plasminogen was identified as a potential autoantigen, particularly in patients with PR3-ANCA-associated vasculitis, prompting exploration of its immunological significance in disease pathophysiology. [172] The presence of anti-plasminogen autoantibodies has since been associated with a heightened risk of venous thromboembolism (VTE). Mechanistically, this association is likely explained by interference with the normal conversion of plasminogen to plasmin, thereby impairing fibrinolysis and prolonging fibrin clot stability, which supports a prothrombotic milieu. [172] Beyond their role in thrombogenesis, these autoantibodies have also been detected in both PR3- and MPO-ANCA–positive patients and have shown correlation with more severe renal impairment, particularly in those with histological evidence of glomerular fibrinoid necrosis and cellular crescents—features indicative of active renal vasculitis. [174]

In a focused study on 104 patients with biopsy-proven AAV and renal involvement, anti-plasminogen antibodies were significantly more prevalent in those with active disease, compared to patients in remission or healthy individuals. [236] Importantly, the presence of these antibodies correlated with higher Birmingham Vasculitis Activity Score (BVAS), elevated erythrocyte sedimentation rate (ESR), serum creatinine, and D-dimer levels, while showing an inverse correlation with estimated glomerular filtration rate (eGFR). These associations suggest a dual clinical utility of anti-plasminogen antibodies: both as biomarkers of disease activity and as indicators of renal involvement and thromboembolic risk.

In summary, accumulating evidence points to anti-plasminogen antibodies as meaningful markers in AAV. Their presence reflects active systemic inflammation, particularly within the renal vasculature, and is linked to compromised fibrinolysis and an increased propensity for thrombosis, thus underscoring their potential diagnostic and prognostic value.

1.3.1.6. Complement C3a and C5a

The complement system comprises three distinct activation pathways—classical, lectin, and alternative—each of which converges on a common terminal cascade leading to the generation of key effector molecules. Despite differing in their mode of initiation, all pathways result in the cleavage of complement proteins, notably C3 and C5, producing the active fragments C3a and C5a. These anaphylatoxins act as potent chemoattractants, mediating the recruitment and activation of a range of immune cells, including those of the adaptive immune system, through binding to specific receptors such as C5aR (CD88), which is broadly expressed on leukocytes. (Figure 11)

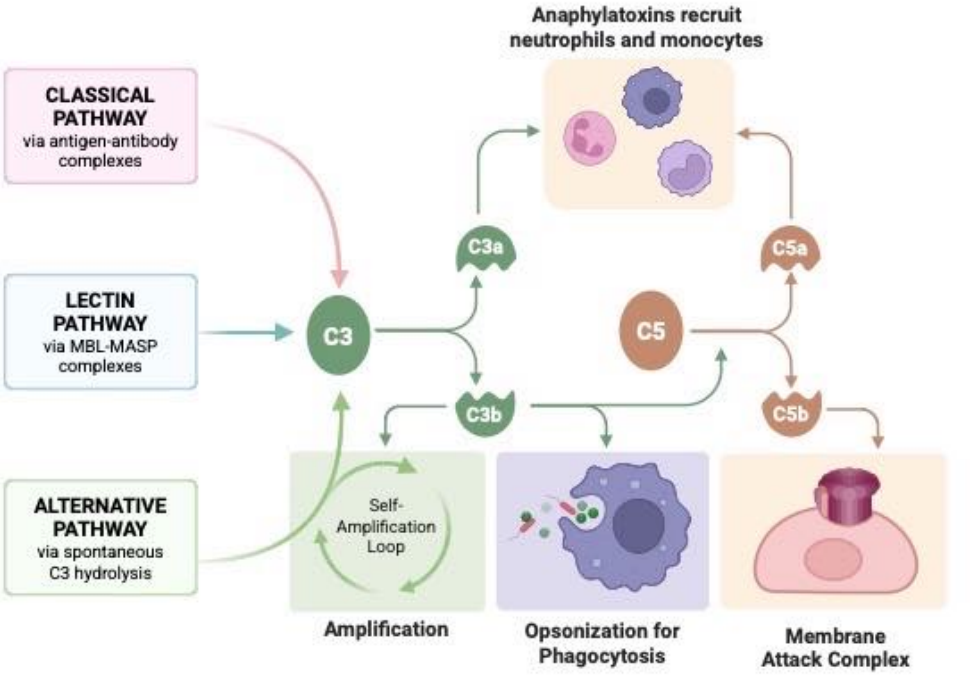


Figure 11. Overview of complement system activation pathways. The complement system can be activated through the classical, lectin, or alternative pathway, all of which converge at the central components C3 and C5. Cleavage of C3 and subsequently C5 generates the effector molecules C3a and C5a, which act as anaphylatoxins and recruit immune cells, while C3b mediates opsonization and C5b initiates formation of the membrane attack complex (MAC). *Created with BioRender.com.*

Recent experimental and clinical investigations have elucidated the pivotal role of complement activation in the pathogenesis of ANCA-associated vasculitis. [237] In particular, the alternative pathway has emerged as a key contributor to disease progression. Upon activation, this pathway amplifies neutrophil activation, establishing a positive feedback loop that intensifies local inflammation, fosters necrotizing tissue injury, and further stimulates the complement cascade, thereby perpetuating disease activity. [58,122]

Specifically, C5a has been shown to upregulate the membrane expression of PR3 on neutrophils, enhancing their binding to circulating ANCA, which subsequently triggers oxidative burst and degranulation. [238,239] Moreover, C5a plays a central role in the formation of neutrophil extracellular traps (NETs) [240] and promotes a prothrombotic state by inducing the release of tissue factor (TF)-expressing extracellular vesicles (EVs) and NETs from primed neutrophils [192]—mechanisms that establish a molecular link between complement activation, inflammation, and hypercoagulability in AAV. (Figure 12)

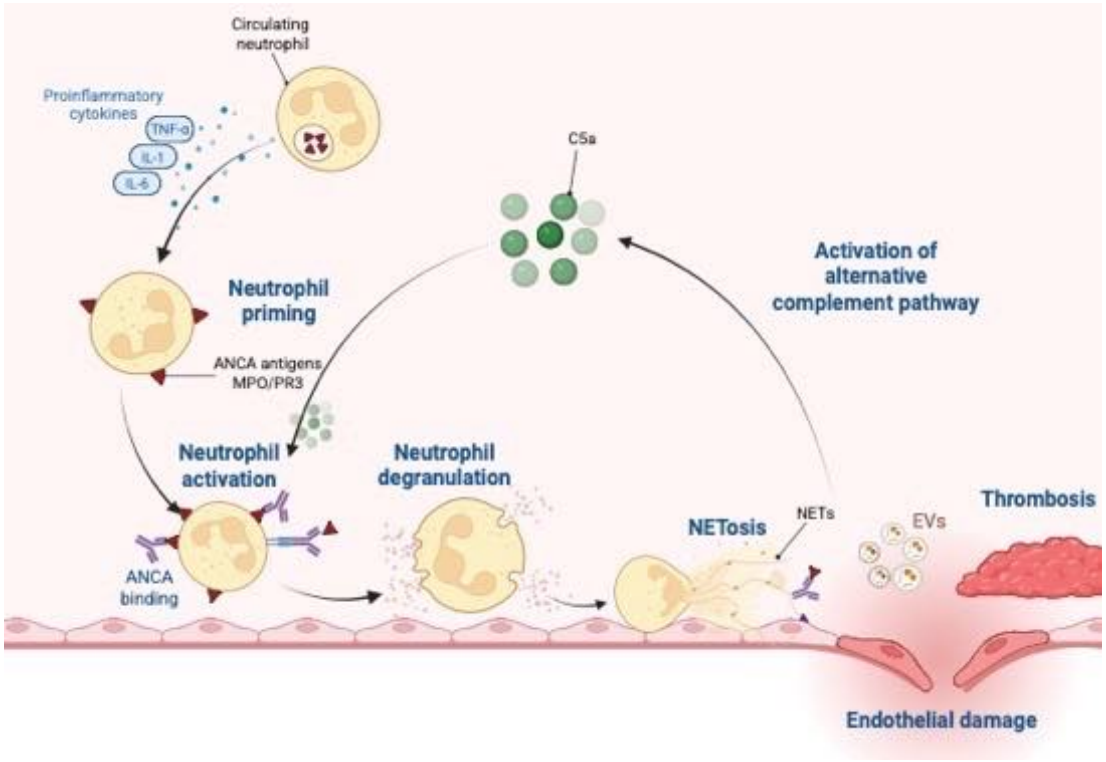


Figure 12. The central role of the complement system in the pathogenesis of ANCA-associated vasculitis (AAV). Proinflammatory cytokines (TNF-α, IL-1, IL-6) prime circulating neutrophils by increasing surface expression of ANCA antigens (MPO/PR3). Binding of ANCAs leads to neutrophil activation, followed by degranulation and the release of cytotoxic mediators. C5a, generated through activation of the alternative complement pathway, amplifies neutrophil activation, thereby establishing a feed-forward loop. Activated neutrophils undergo NETosis, releasing neutrophil extracellular traps (NETs) and extracellular vesicles (EVs), which further propagate endothelial injury and promote thrombosis. This positions complement, and particularly C5a, as a central driver of vascular damage in AAV. *Created with BioRender.com.*

Emerging evidence further supports the clinical relevance of complement components as biomarkers and therapeutic targets. For instance, reduced C3a serum levels at diagnosis have been associated with severe renal involvement and unfavourable prognosis, likely reflecting complement consumption and deposition within glomerular and small vessel lesions. [241,242] Additionally, elevated systemic levels of complement factors, including C5a and C3a, have been reported in AAV patients compared to healthy controls, with their concentrations positively correlating with disease activity assessed by the Birmingham Vasculitis Activity Score (BVAS). [124,243]

Building on these findings, our research group previously published data demonstrating increased levels of MPO-positive extracellular vesicles expressing C3a and C5a in patients with AAV—particularly in those with renal involvement. [244] Importantly, these EV-associated complement components were found to correlate with BVAS, underscoring their potential as biomarkers of disease activity and suggesting a broader role of complement-carrying vesicles in AAV immunopathology.

2. THE OBJECTIVES

The overarching aim of this doctoral thesis is to investigate the degree of systemic inflammation and disease activity in patients diagnosed with ANCA-associated vasculitis in comparison to healthy individuals.

The specific and individual objectives of our scientific investigation are:

1. To compare the expression of surface markers on extracellular vesicles between AAV patients and healthy controls.
2. To analyse the correlation of potential biomarkers expressed on the surface of extracellular vesicles with disease activity as measured by the BVAS score.
3. To assess whether specific extracellular vesicle biomarkers represent potential risk factors for increased disease activity and progression in AAV.
4. To investigate correlation between serum levels of biomarkers and disease activity, measured by the BVAS.
5. To determine the impact of therapy on the levels of selected monoclonal antibodies expressed on extracellular vesicles.
6. To evaluate the activity and procoagulant properties of extracellular vesicles by measuring their thrombin generation potential across different study groups.

3. MATERIALS AND METHODS

3.1. Study design

This study was designed as an observational, cross-sectional cohort investigation aimed at examining extracellular vesicles (EVs) in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) in comparison with healthy controls. The research was conducted within the framework of an ERASMUS+ mobility project for PhD students and academic staff, established through a bilateral agreement between the University of Niš and Karolinska Institutet, Stockholm, Sweden, formally signed in 2016 by the legal representatives of both institutions. The experimental component of the study was carried out at the Karolinska University Hospital, Solna, Sweden.

3.1.1. Cohort details

Since 2008, patients with AAV diagnosed, treated, and followed up at both Rheumatology and Nephrology Departments of Karolinska University Hospital have been enrolled in a cross-sectional study known as VASKA. For the purposes of the present work, clinical characteristics of patients were retrieved from the VASKA database as well as from the electronic medical record system *TakeCare*®.

Inclusion criteria for the study were:

- Confirmed diagnosis of AAV, with subtypes GPA or MPA according to ACR/EULAR recommendation based on Chapel Hill Consensus Conference criteria;
- Positive ANCA serology for either MPO and/or PR3 at any time point;
- Signed written informed consent.

Exclusion criteria from initial study:

- Diagnosis of EGPA, due to the small number of available cases;
- ANCA-negativity.

Assessment of disease activity was performed using the Birmingham Vasculitis Activity Score (Figure 10), version 3 (BVAS v3), in accordance with the European League Against Rheumatism (EULAR) recommendations [60,69]. A total BVAS score of 0 was defined as complete remission, whereas a score ≥ 1 (in the absence of infection) indicated active disease. The BVAS v3 is a standardized and validated tool that quantifies vasculitic involvement across multiple organ systems, including renal, pulmonary, dermatological, neurological, and gastrointestinal domains.

Each manifestation is assigned a weighted score reflecting its clinical severity and relevance; for instance, life-threatening features such as pulmonary hemorrhage or severe renal dysfunction are given higher weight compared to milder symptoms such as arthralgia or cutaneous purpura. The cumulative score thus reflects the overall burden of vasculitic activity at the time of assessment.

Birmingham Vasculitis Activity Score (version 3)

Patient ID: **Date of birth:**

Total score:

Assessor: **Date of assessment**

Tick an item **only** if attributable to active vasculitis. If there are no abnormalities in a section, please tick 'None' for that organ-system.

If **all** abnormalities are due to persistent disease (active vasculitis which is not new/worse in the prior 4 weeks), tick the **PERSISTENT** box at the bottom right corner

Is this the patient's first assessment? **Yes** ○ **No** ○

	None	Active disease
1. General	○	
Myalgia		○
Arthralgia / arthritis		○
Fever ≥38° C		○
Weight loss ≥2 kg		○
2. Cutaneous	○	
Infarct		○
Purpura		○
Ulcer		○
Gangrene		○
Other skin vasculitis		○
3. Mucous membranes / eyes	○	
Mouth ulcers		○
Genital ulcers		○
Adnexal inflammation		○
Significant proptosis		○
Scleritis / Episcleritis		○
Conjunctivitis / Blepharitis / Keratitis		○
Blurred vision		○
Sudden visual loss		○
Uveitis		○
Retinal changes (vasculitis / thrombosis / exudate / haemorrhage)		○
4. ENT	○	
Bloody nasal discharge / crusts / ulcers / granulomata		○
Paranasal sinus involvement		○
Subglottic stenosis		○
Conductive hearing loss		○
Sensorineural hearing loss		○
5. Chest	○	
Wheeze		○
Nodules or cavities		○
Pleural effusion / pleurisy		○
Infiltrate		○
Endobronchial involvement		○
Massive haemoptysis / alveolar haemorrhage		○
Respiratory failure		○

	None	Active disease
6. Cardiovascular	○	
Loss of pulses		○
Valvular heart disease		○
Pericarditis		○
Ischaemic cardiac pain		○
Cardiomyopathy		○
Congestive cardiac failure		○
7. Abdominal	○	
Peritonitis		○
Bloody diarrhoea		○
Ischaemic abdominal pain		○
8. Renal	○	
Hypertension		○
Proteinuria >1+		○
Haematuria ≥10 RBCs/hpf		○
Serum creatinine 125-249 µmol/L*		○
Serum creatinine 250-499 µmol/L*		○
Serum creatinine ≥500 µmol/L*		○
Rise in serum creatinine >30% or fall in creatinine clearance >25%		○
***Can only be scored on the first assessment**		
9. Nervous system	○	
Headache		○
Meningitis		○
Organic confusion		○
Seizures (not hypertensive)		○
Cerebrovascular accident		○
Spinal cord lesion		○
Cranial nerve palsy		○
Sensory peripheral neuropathy		○
Mononeuritis multiplex		○
10. Other	○	
a.		○
b.		○
c.		○
d.		○
PERSISTENT DISEASE ONLY: (Tick here if **all** the abnormalities are due to persistent disease)		☐

References:

Version 1: Luqmani, RA, et al. (1994). "Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis." QJM **87**(11):671-8.

Version 2: Luqmani, RA, et al. (1997). "Disease assessment and management of the vasculitides." Baillieres Clin Rheumatol **11**(2): 423-46.

Version 3: Mukhtyar C, et al (2008). "Modification and validation of the Birmingham Vasculitis Activity Score (version 3) Ann Rheum Dis. 2008 Dec 3. [Epub ahead of print]

Figure 13. The Birmingham Vasculitis Activity Score (BVAS) version 3 (*available online at EUVAS website http://www.vasculitis.org/disease*)

Demographic and clinical data for the entire cohort, including age, sex, autoantibody status, specific AAV diagnosis, and disease phenotype at the time of diagnosis, were extracted from the VASKA database and corresponding medical records. Data on prior venous thromboembolic events (VTEs)—defined as events occurring more than three months before AAV diagnosis—were also collected through medical history review; however, these earlier VTEs were excluded from the incidence rate calculations. Disease phenotype at diagnosis, classified as either renal or non-renal involvement, was determined for all patients. Renal involvement was primarily based on histopathological findings from renal biopsies. In the absence of biopsy data, assessment was made using serum creatinine levels and urinalysis. If the biopsy demonstrated pathological findings not exclusively attributable to vasculitis, renal involvement was still considered present. Conversely, if biopsy findings were non-specific but renal dysfunction was evident through lab results, the final determination relied on the clinical judgment documented in the medical record. This multifaceted definition was adopted to ensure maximal clinical accuracy in categorizing disease phenotype subgroups.

The control group consisted of 24 individuals from a population-based cohort [245], matched to AAV patients by age and sex. These controls had no self-reported comorbidities, did not use any medications, and had no history of thrombotic events. All participants in the control group provided written informed consent.

3.1.2. Treatment

Data on immunosuppressive treatment regimens were collected in accordance with current clinical guidelines [135], encompassing both induction and maintenance phases of therapy. The cumulative glucocorticoid (GC) exposure was quantified and expressed in terms of prednisolone-equivalent milligrams (mg) to ensure standardized dosage comparisons.

3.1.3. Routine laboratory analysis

Routine laboratory assessments included measurements of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and serum creatinine. These analyses were performed on peripheral venous blood samples using standardized procedures at the Department of Clinical Chemistry, Karolinska University Hospital, Solna. To estimate renal function, the Cockcroft–Gault equation was applied to calculate creatinine clearance, serving as an approximation of glomerular filtration rate (eGFR). The resulting eGFR values were subsequently used for patient classification, with a threshold of 60 mL/min/1.73 m² employed to distinguish between normal and impaired renal function.

3.1.4. Immunology measurements

Serological markers were analysed in accordance with clinical practice at the Department of Immunology, Karolinska University Hospital. Anti-neutrophil cytoplasmic antibodies (ANCAs) were detected using standard Enzyme-Linked Immunosorbent Assay (ELISA) techniques, either through direct ELISA (Euro Diagnostica) or a multiplex platform (BIO-RAD, BioPlex™ 2200). In cases where both PR3-ANCA and MPO-ANCA were present, the antibody specificity with the higher titer was selected for use in inferential statistical analyses. Diagnosis of ANCA-associated vasculitis (AAV) in all patients was based on the validated European Medicines Agency (EMEA) classification algorithm for epidemiological studies [59]. Among the entire patient cohort, two individuals tested positive for both MPO- and PR3-specific ANCA. For consistency, the antibody with the higher titer was used as the reference specificity in statistical modelling.

Serum concentrations of Pentraxin 3 (PTX3) were measured using a commercially available ELISA kit (R&D Systems Europe Ltd., Abingdon, UK).

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) levels were determined with the Human TWEAK ELISA kit (Thermo Scientific, USA), and high-mobility group box 1 (HMGB1) concentrations were quantified using the Tecan HMGB1 ELISA kit (Fisher Scientific, USA).

Serum levels of complement component C3 were analysed using turbidimetric methods, following clinical routine procedures at the Department of Immunology, Karolinska University Hospital. The established reference interval for C3 was 0.77–1.62 g/L.

Measurement of serum C3a concentrations was conducted using the MicroVue C3a Enzyme Immunoassay (Quidel Corp.), as previously described by our research group. [244] This assay follows a three-step protocol comprising:

1. A microplate pre-coated with a murine monoclonal antibody specific to a neoepitope unique to human C3a;
2. A horseradish peroxidase (HRP)-conjugated polyclonal antibody targeting the C3a region of the parent C3 molecule; and
3. A chromogenic substrate that reacts with the HRP complex

The intensity of the resulting colour reaction directly correlates with the C3a concentration in standards, controls, and diluted serum samples. Final concentrations are determined via a four-parameter logistic regression model based on the standard curve and reported in nanograms per milliliter (ng/mL). The inter-assay coefficient of variation (CV) was 5.3%, and the intra-assay CV was 8.3%, indicating acceptable analytical precision.

Serum concentrations of complement component C5a were determined using the MicroVue C5a Enzyme Immunoassay (Quidel Corp.), as previously reported by our research group. [244] This

direct capture immunoassay is designed for the quantification of C5a in human serum, plasma, and other biological or experimental samples. The assay involves a three-step process consisting of:

1. A microplate pre-coated with a murine monoclonal antibody that recognizes a neoepitope specific to human C5a;
2. A horseradish peroxidase (HRP)-conjugated monoclonal antibody targeting the C5a domain of the C5 molecule; and
3. A chromogenic substrate that reacts with the enzyme-antibody complex.

The intensity of the colorimetric reaction is directly proportional to the concentration of C5a in standards, quality controls, and diluted test specimens. Concentrations are calculated from a standard curve using a four-parameter logistic (4PL) regression model and are expressed in nanograms per milliliter (ng/mL). The inter-assay coefficient of variation (CV) was 3.8%, and the intra-assay CV was 7.8%, indicating high assay reproducibility and reliability.

3.1.5. Ethics

Whole study was performed in accordance with the Declaration of Helsinki principles as well as Good Clinical Practice Guidelines. A part of the collection of data involved going through medical records containing patient information that had not been de-identified. The ethical aspects of such procedures had to be considered, since they are to be regarded as an intrusion of the patients' integrity. The sensitive patient information was handled with great confidentiality to avoid harm to the patients. Non-digital patient data were kept at the rheumatology clinic at Karolinska University Hospital, Solna, and digital data transferred to computers outside of the clinic were completely de-identified. The presentation of the results was also completely de-identified. All recruited patients received oral and written consent for inclusion in the VASKA study prior to enrolment. Ethical permit has been acquired and approved by the Regional Ethical Review Board in Stockholm and the Swedish Ethical Review Authority (ethical review number: 2008/1143-31/3). The Ethical committee of the Medical Faculty University in Niš obtained the permit as well (ethical review number: 12-11272/2-9, date 25/9/2023).

3.2. Detection of extracellular vesicles

We accessed the number and phenotype of extracellular vesicles in plasma identified by flow cytometry, during active disease and remission, compared to healthy controls.

3.2.1. Blood sampling and preparation of samples

Following enrolment into the VASKA cohort, blood samples were collected in accordance with standardized protocols established by the haemostasis laboratory. Venous blood was drawn into Vacutainer tubes (Becton Dickinson) containing either a clot activator or 0.129 mol/L trisodium citrate buffer (pH 7.4), using a 1:9 ratio of anticoagulant to blood. To reduce platelet activation and obtain platelet-poor plasma (PPP), all samples were centrifuged within one hour of collection at room temperature for 10 minutes at high speed. The resulting plasma was then aliquoted and stored in an upright position at –80°C for subsequent analysis. (Figure 14)

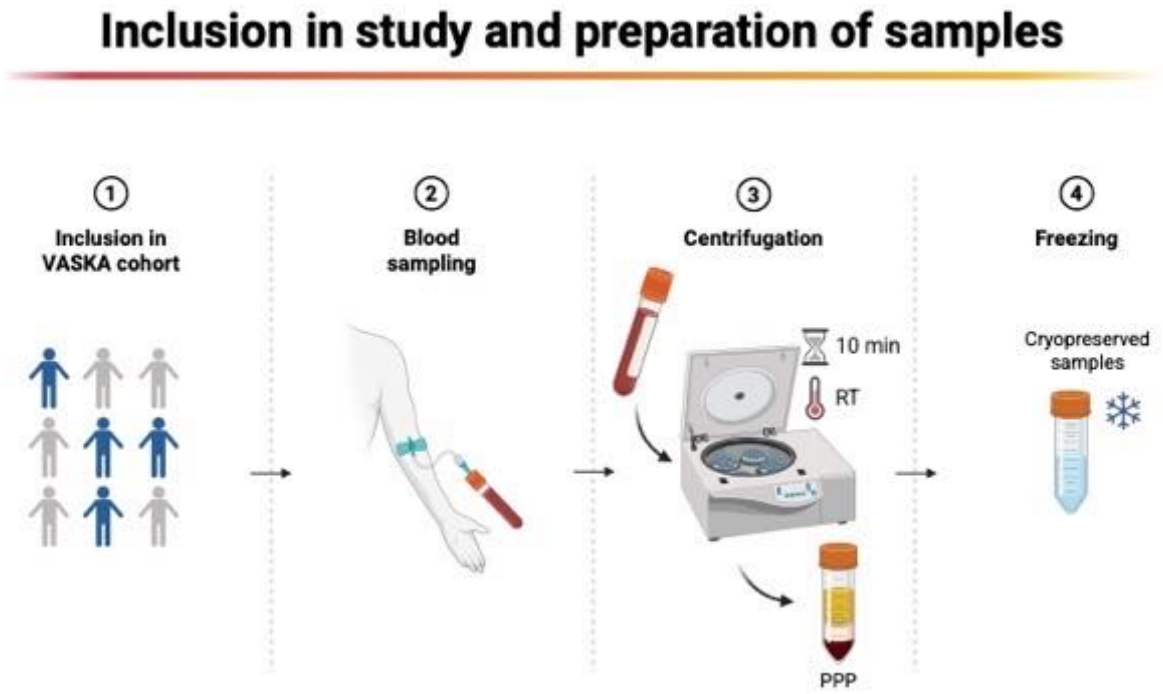


Figure 14. Inclusion of study participants and preparation of plasma samples: Patients were recruited into the VASKA cohort and underwent standard venous blood sampling. Collected blood was processed by centrifugation at room temperature (2000 g for 10 minutes) in order to obtain platelet-poor plasma (PPP). The resulting plasma was then aliquoted, cryopreserved, and stored at –80 °C until further analysis. *Created with BioRender.com.*

3.2.2. Detection of extracellular vesicles using flow cytometry

Cell and particle detection was conducted using flow cytometry, wherein each element is directed through a flow chamber and intersected by one or more laser beams. The resulting optical signals are typically visualized as dot plots or histograms for further interpretation.

Platelet-poor plasma (PPP) samples were initially thawed in a 37°C water bath for approximately 5 minutes. Following thawing, a two-step centrifugation protocol was applied to eliminate residual cells or debris that could interfere with flow cytometric analysis. The first centrifugation was performed at 2,000×g for 20 minutes at room temperature, followed by a second

high-speed centrifugation at 13,000×g for 2 minutes, also at room temperature. Subsequently, 20 µL of the resulting supernatant was incubated in the dark for 20 minutes with 5 µL of fluorescently labelled monoclonal antibodies (details listed in Table 5). The incubation process was terminated by the addition of 500 µL of Cellfix™ (Becton Dickinson, BD, NJ, USA). All samples were processed under strictly standardized conditions, including consistent centrifugation speeds, rotor type, deceleration settings, and temperature control across all measurements. (Figure 15)

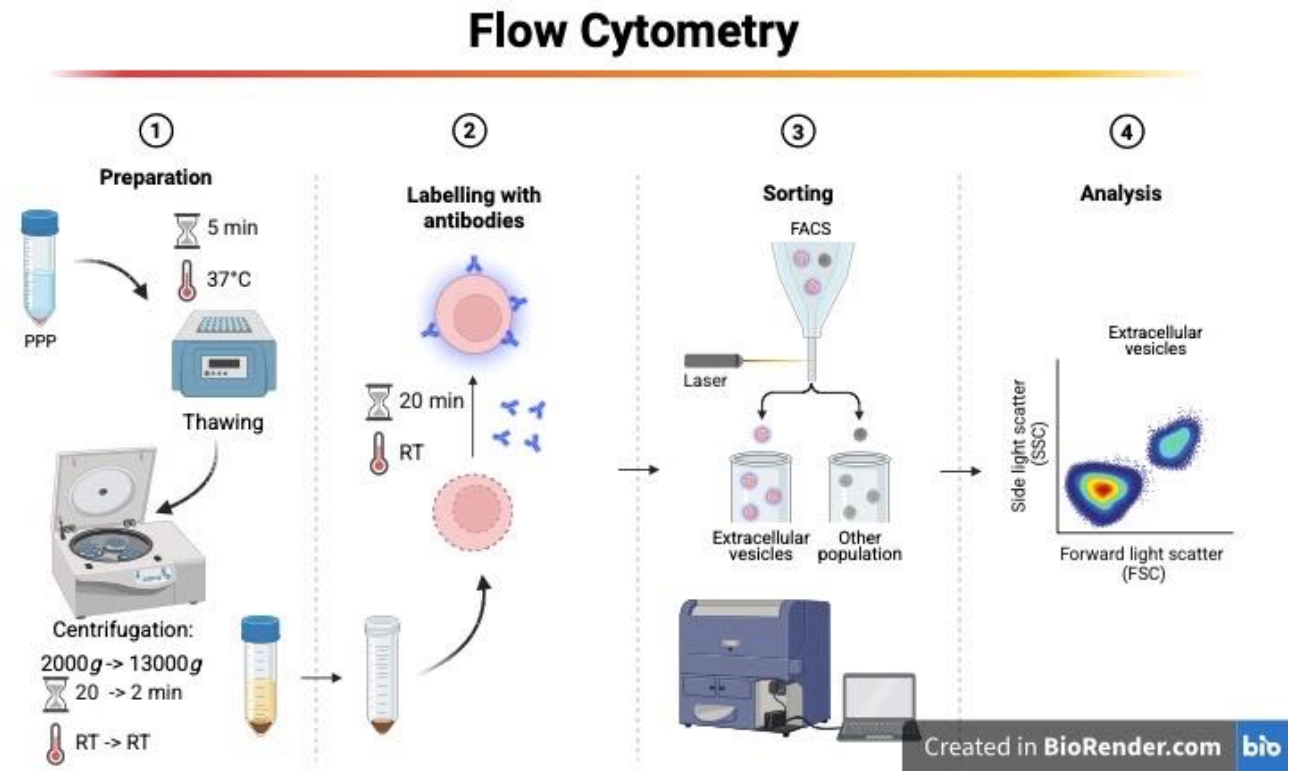


Figure 15. Isolation of extracellular vesicles using flow cytometry: Platelet-poor plasma (PPP) was thawed in a water bath and subjected to sequential centrifugation (2000 g for 20 minutes followed by 13,000 g for 2 minutes) to obtain platelet- and debris-free supernatant. The pellet was incubated in dark with fluorescent monoclonal antibodies for 20 minutes in order to label extracellular vesicles (EVs) with specific surface markers. Thereafter, fluorescence-activated cell sorting (FACS) was performed, where a laser beam enabled discrimination of EVs from other particle populations. Finally, the samples were analysed according to forward scatter (FSC) and side scatter (SSC) parameters, providing both quantitative and phenotypic characterization of extracellular vesicles. *Created with BioRender.com.*

Measurement of extracellular vesicles (EVs) was conducted using flow cytometry on a Beckman Coulter Gallios™ instrument (Beckman Coulter, Brea, CA, USA). Size-based gating for EVs was established using Megamix beads (BioCytex, Marseille, France) ranging from 0.3 to 3.0 µm. In accordance with the MISEV guidelines for the classification of medium/large EVs [246], extracellular vesicles were operationally defined as particles smaller than 1 µm in diameter and further characterized based on surface protein expression.

To control for nonspecific binding and establish background signal, isotype-matched immunoglobulin controls (IgG1-FITC, IgG1-PE), lacking reactivity to human antigens, were used in

all assays. EV counts were expressed as the number of events detected during a fixed acquisition time of 30 seconds. The intra- and inter-assay coefficients of variation (CVs) for EV quantification were both below 9%, indicating high reproducibility of the method.

Table 5. Antibodies and target proteins

Antibody	Company name	Target protein
CD142 (PE)	BD, NJ, USA	TF
Anti-H3cit	Abcam, Cambridge, UK	Cit H3
Anti-MPO	BD, NJ, USA	MPO
Anti-PR3	Abcam, Cambridge, UK	PR3
Anti-Pentraxin 3	Abcam, Cambridge, UK	PTX3
HMGB1	R&D Systems, MN, USA	HMGB1
Anti-MIF	LSBio. Inc., Seattle, WA, USA	MIF
Anti-sTWEAK	LSBio. Inc., Seattle, WA, USA	sTWEAK
Anti-plasminogen	Abcam, Cambridge, UK	Plasminogen
Anti-C3a	Thermo Fisher Scientific Inc., Waltham, MS, USA	C3a
Anti-C5a	Thermo Fisher Scientific Inc., Waltham, MS, USA	C5a

Abbreviations: TF - tissue factor; CitH3 - citrullinated histone H3; MPO - myeloperoxidase; PR3 - proteinase3; PTX3 - pentraxin 3; HMGB1 - high mobility group box 1; MIF - macrophage migration inhibitory factor; sTWEAK - soluble tumour necrosis factor-like weak inducer of apoptosis; C3a and C5a - components of complement system C3a and C5a.

3.2.3. Analysis of thrombin generation using activity assay

The procoagulant activity of extracellular vesicles (EVs) was further evaluated in vitro using a modified version of the Calibrated Automated Thrombogram (CAT) assay, as originally described by Hemker *et al.* [247] The assay was performed on a Fluoroskan Ascent reader (Thermo Scientific) using transparent U-bottom 96-well microplates.

In detail, platelet-poor plasma (PPP) samples were thawed in a 37°C water bath for approximately 5 minutes and subsequently processed through a two-step centrifugation protocol: first at 2,000×g for 20 minutes, followed by centrifugation at 20,000×g for 45 minutes, both at room temperature. The resulting EV-enriched pellet was resuspended by diluting 50 µL of the pellet in 450 µL of phosphate-buffered saline (PBS, pH 7.6), according to the isolation protocol described by Pereira et al, in order to obtain an vesicles-enriched pellet. [32] To initiate thrombin generation, 20 µL of the EV-enriched suspension was added to 80 µL of previously centrifuged pooled normal plasma from healthy donors (20,000×g for 30 minutes at room temperature), without the addition of tissue factor (TF) or exogenous phospholipids, which are typically required in the standard CAT

protocol. [248] Vesicle-poor plasma (i.e., plasma devoid of EVs and any added trigger) was used for control reactions. (Figure 16)

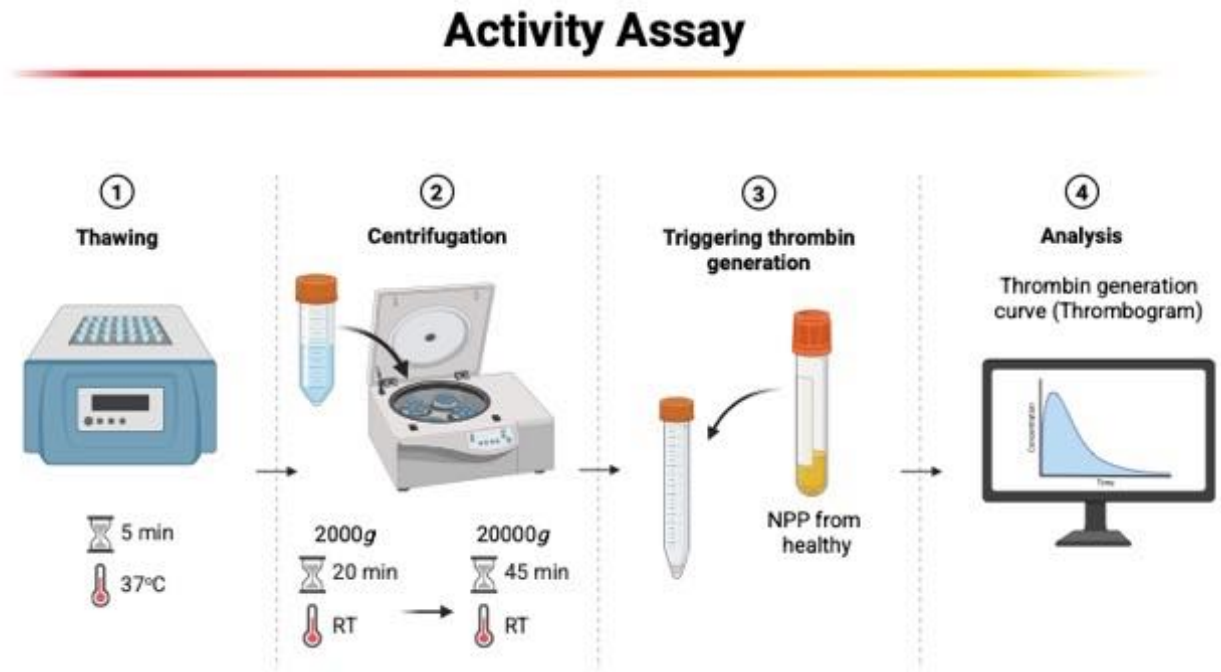


Figure 16. Workflow of the Activity assay for thrombin generation: Plasma samples were first thawed in a water bath at 37 °C for 5 minutes, followed by sequential centrifugation (2000 g for 20 minutes and 20,000 g for 45 minutes at room temperature) to obtain an extracellular vesicle (EV)-enriched pellet. This pellet was resuspended and added to previously centrifuged normal pooled plasma (NPP) from healthy controls in order to trigger thrombin generation without exogenous tissue factor or phospholipids. Thrombin generation was continuously monitored over 60 minutes at 37 °C, and results were expressed as thrombin generation curves (thrombograms). *Created with BioRender.com.*

Thrombin generation was monitored for 60 minutes at 37°C. Fluorescence signals were recorded every 20 seconds, allowing for construction of a thrombin generation curve ("thrombogram"). (Figure 17) Quantitative parameters, including lag time, peak thrombin, endogenous thrombin potential (ETP), and time to peak, were derived from the thrombogram using dedicated analysis software (Thrombinoscope BV, Maastricht, Netherlands) according to the manufacturer's instructions.

The thrombogram, illustrated in Figure 17, represents the graphical profile of thrombin generation over time. The area under the thrombin generation curve (AUC) reflects the total amount of thrombin produced and is referred to as **the endogenous thrombin potential (ETP)**. **The lag time** denotes the duration from the start of the assay to the initiation of thrombin generation, measured in minutes. **The peak thrombin** refers to the highest concentration of thrombin formed during the measurement period, while **the time to peak** corresponds to the time required to reach this maximum thrombin concentration, also expressed in minutes. [249]

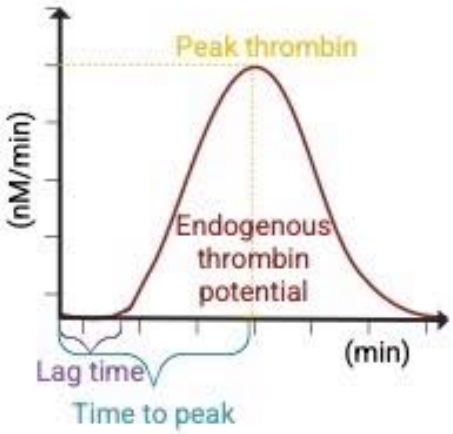


Figure 17. Schematic presentation of thrombin generation curve (Thrombogram): The lag time represents the initiation phase until thrombin generation begins, while the time to peak indicates the time needed to reach maximum thrombin formation. The peak thrombin corresponds to the highest concentration of thrombin generated during the assay. The endogenous thrombin potential (ETP), calculated as the area under the curve, reflects the total amount of thrombin formed over time. *Created with BioRender.com.*

3.4. Statistical analysis

Upon completion of the study database, including all relevant variables, statistical analysis was conducted. Descriptive statistics were used to summarize patient characteristics. Continuous variables were reported as means with standard deviations (SD) or medians with ranges (minimum–maximum), depending on the distribution of the data. Categorical variables were expressed as percentages. The normality of data distribution was assessed using the Shapiro–Wilk test.

Group comparisons for continuous variables were performed using either independent samples t-tests (for normally distributed data) or Mann–Whitney U tests (for non-normally distributed data). Categorical variables were compared using the Chi-square (χ^2) test. Associations between variables were evaluated using Pearson's correlation coefficient for parametric data and Spearman's rank correlation coefficient for non-parametric data, depending on the data type and distribution characteristics.

To assess the prognostic utility of individual variables in predicting disease activity, univariate logistic regression analysis was applied. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA) for Windows.

4. RESULTS

4.1. General demographic characteristic of study cohort

A total of forty-eight patients diagnosed with ANCA-associated vasculitis (AAV) and twenty-three age- and sex-matched healthy control subjects were initially recruited into the study. During screening, one patient was excluded from all analyses due to ongoing plasmapheresis treatment. In addition, two patients were partially excluded: one was receiving anticoagulant therapy with warfarin and therefore could not be included in the thrombin generation assay, while another exhibited markedly elevated serum lipid levels that interfered with the detection of extracellular vesicles (EVs). Following these adjustments, the final study population comprised forty-six patients with AAV who fully met all predefined inclusion criteria. Importantly, none of the patients included in the final analysis had a history of venous thromboembolism (VTE) or major cardiovascular events, and none were receiving anticoagulation therapy or undergoing plasma exchange at the time of blood sampling. (Figure 18)

The majority of participants in both the patient and control groups were of Swedish ancestry, reflecting the demographic structure of the recruitment base. Comprehensive demographic and clinical characteristics of the patient cohort—including age, sex distribution, disease subtype (GPA or MPA), ANCA serotype (MPO- or PR3-ANCA), disease activity measured by the Birmingham Vasculitis Activity Score (BVAS), and renal function—are summarized in Table 2. Comparative analysis revealed no statistically significant differences in these baseline variables between patients with active disease and those in remission. These observations indicate that the active and inactive patient groups were broadly comparable with respect to demographic and major clinical characteristics, thus reducing the risk of confounding factors when interpreting subsequent analyses.

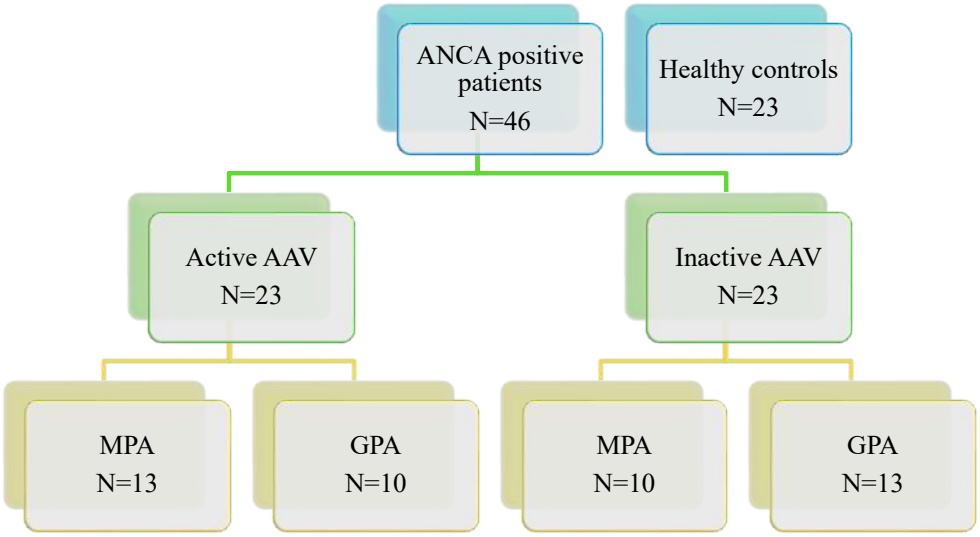


Figure 18. Flowchart of cohort study. **Abbreviations:** ANCA – antineutrophil cytoplasmic antibody; AAV – ANCA associated vasculitis; MPA – microscopic polyangiitis; GPA- granulomatosis with polyangiitis.

Half of the patients (n=23) presented with active small-vessel vasculitis, defined as a BVAS score greater than zero in the absence of intercurrent infection. The mean BVAS among active patients was 13.9 ± 7.8 (maximum possible score 63). Within this subgroup, 13 were male and 11 female. Thirteen patients were diagnosed with microscopic polyangiitis (MPA) and 10 with granulomatosis with polyangiitis (GPA). Regarding ANCA specificity, 12 patients were MPO-ANCA positive, 10 were PR3-ANCA positive, and one patient displayed double positivity for both MPO and PR3.

Patients in remission (n=23) had a mean age of 63.8 ± 12.8 years and the same sex distribution as the active group (13 males, 11 females). Ten patients were diagnosed with MPA and 13 with GPA. ANCA specificity was distributed similarly, with 12 patients MPO-ANCA positive, 10 PR3-ANCA positive, and one double positive. As per definition, all patients in remission had a BVAS of 0.

As shown in Table 6, no statistically significant differences were found between active and remission groups with regard to age, sex, disease subtype, or ANCA specificity. The only difference between groups was BVAS score, by definition.

The control group consisted of 23 healthy individuals matched for age and sex ($p>0.999$, i.e. $p=0.222$).

Table 6. Patients and controls general features

	Total AAV	Active vasculitis	Vasculitis in remission	p[1]	Healthy controls	p[1]
Patient's characteristics						
Subject number	46	23	23		23	
Gender (M/F)	25/21	13/11	13/11	>0.999	12/11	>0.999
Age at sampling (years)†	62.5±13.33	61.3±14.0	63.8±12.8	0.535	66.0±9.54	0.222
Disease characteristics						
BVAS* †	/	13.9±7.8	0		/	
Disease subtype						
Microscopic polyangiitis ‡	23 (50.0)	13 (56.5)	10 (43.5)		/	
Granulomatosis with polyangiitis ‡	23 (50.0)	10 (43.5)	13 (56.5)		/	
ANCA status						
MPO-ANCA positivity ever‡	26 (54.1)	13 (54.2) ◊	13 (54.2) ◊	>0.999	/	
PR3-ANCA positivity ever ‡	22 (45.9)	11 (45.8) ◊	11 (45.8) ◊	>0.999	/	

* Birmingham Vasculitis Activity Score (BVAS) ≥ 1 is defined as active disease; † (mean±SD), ‡ (absolute #, %), 1 Chi-squared test, ◊ Two patients were double positive for both MPO and PR3 ANCA. **Abbreviations:** MPO – myeloperoxidase; PR3 - proteinase 3; ANCA - Antinuclear cytoplasmic antibody; AAV – ANCA associated vasculitis. Statistically significant p values (<0.05) are shown in italics.

4.2. Laboratory analysis and immunology

Serum creatinine levels were significantly higher in patients with AAV compared to healthy controls ($p<0.001$), while estimated glomerular filtration rate (eGFR) values were significantly lower in the AAV group than in controls ($p<0.001$). These findings were consistent irrespective of disease activity. Among inflammatory markers, both ESR and CRP were significantly elevated in patients with active vasculitis compared to those in remission ($p=0.026$ and $p<0.001$, respectively). Table 7 summarizes renal function parameters and acute phase reactants in the study cohort.

Table 7. Basic laboratory analysis and immunology findings in study cohort

	Total AAV	**Active vasculitis**	**Vasculitis in remission**	**p[1]**	**Healthy controls**	**p[1]**
Markers of renal function						
Serum creatinine (mg/dl) †	124.1±73.0	143.3±94.0[a]	104.9±35.9[b]	0.448	75.6±12.4	*<0.001*
Creatinine clearance/eGFR (ml/min/1,73m2) †	62.8±28.4	60.7±32.9[a]	64.8±23.6[b]	0.826	85.3±16.3	*<0.001*
Inflammatory markers						
ESR (mm/h) †	28±23.4	37.6±28.3	19.9±4.4	0.026	/	
CRP (mg/ml) †	9.2±12.5	14.1±15.8	4.6±5.3	*0.001*	/	

† (mean±SD), [1] Mann-Whitney test, [a] vs control group (Creatinine p=0.001, eGFR p=0.006), [b] vs control group (Creatinine p=0.002, eGFR p=0.001). **Abbreviations:** ANCA - Antinuclear cytoplasmic antibody; AAV – ANCA associated vasculitis; eGFR – estimated glomerular filtration rate; ESR – erythrocyte sedimentation rate; CRP – C reactive protein. Statistically significant p values (<0.05) are shown in italics.

Among the investigated serum markers, only pentraxin 3 (s-PTX3) was significantly elevated in active AAV patients compared to those in remission ($p<0.001$) and to healthy controls ($p<0.001$). In contrast, no significant differences were observed in serum levels of TWEAK or HMGB1 between AAV patients and controls.

With respect to complement components, serum C3a levels tended to be lower in active AAV compared to remission or healthy controls ($p=0.06$ and $p=0.07$, respectively). Conversely, serum C5a levels showed the opposite trend, being higher in active disease and lower in remission, though without reaching statistical significance.

Table 8 summarizes serum concentrations of PTX3, TWEAK, HMGB1, and complement components C3a and C5a in AAV patients and controls.

Table 8. Levels of serum markers found in AAV patients and healthy controls

Serum markers †	Total AAV	Active vasculitis	Vasculitis in remission	p[1]	Healthy controls	p[1]
s-PTX3 (ng/ml)	3.12±4.66	5.0±6.0	1.2±0.7	*<0.001*	0.85±0.46	*<0.001*
s-sTWEAK (pg/ml)	1672.16±1906.76	1530.3±537.7	1807.9±2637.4	0.093	1537.87±561.13	0.385
s-HMGB1 (ng/ml)	3.6	4.3	3.3	0.34	4.4	0.49
s-C3a (ng/ml)	1887±744.1	1738±658.8	2036±807.6	0.06	2066±837.9	0.07
s-C5a (ng/ml)	22.6±10.8	23.3±10.5	21.9±11.3	ns	20.7±7.8	ns

† (mean±SD), [1] Mann-Whitney test. **Abbreviations:** ANCA - Antinuclear cytoplasmic antibody; AAV – ANCA associated vasculitis; s-PTX3 – serum pentraxin3; s-sTWEAK – serum soluble tumour necrosis factor-like weak inducer of apoptosis; s-HMGB1 – serum levels of high mobility group box protein 1; s-C3a and s-C5a - serum levels of components of complement system C3a and C5a. Statistically significant p values (<0.05) are shown in italics.

4.3. Treatment

Data regarding treatment regimens in the study cohort are presented in Table 9. At the time of sampling, ongoing glucocorticoid therapy was recorded in 22 of 23 patients with active vasculitis (95.7%) and in 15 of 23 patients in remission (65.2%), with a significantly higher frequency in the active group (p=0.021). The mean daily prednisolone dose was also significantly higher among patients with active vasculitis compared to those in remission (32.3 ± 26.6 mg vs. 5.8 ± 5.8 mg, p=0.0022).

Regarding additional immunosuppressive treatment, methotrexate was administered to 3 active patients (13.0%) and 4 patients in remission (17.4%). Azathioprine was used more frequently in the remission group (26.1% vs. 4.3%), though without statistical significance. Similarly, mycophenolate mofetil was reported in 5 active (21.7%) and 1 remission patient (4.3%). Cyclophosphamide was significantly more often prescribed in the active group (21.7% vs. 0.0%, p=0.049). Previous exposure to cyclophosphamide was also more frequent in patients with active disease compared to those in remission (34.8% vs. 4.3%, p=0.022). No patients in either group were treated with rituximab at the time of sampling.

Table 9. Treatment of patients with active disease and in remission

Drugs	Total AAV	Active vasculitis	Vasculitis in remission	p-value
I. Ongoing treatment				
Glucocorticoids‡	37 (80.4)	22 (95.7)	15 (65.2)	*0.02*[1]
Prednisolone dose (mg/day) †	19.0±23.2	32.3±26.6	5.8±5.8	*0.002*[2]
Methotrexate ‡	7 (15.2)	3 (13.0)	4 (17.4)	>0.999[1]
Azathioprine ‡	7 (15.2)	6 (26.1)	1(4.3)	0.101[1]
Mycophenolate mofetil ‡	6 (13.0)	5 (21.7)	1 (4.3)	0.189[1]
Cyclophosphamide‡	5 (10.9)	5 (21.7)	0 (0.0)	*0.049*[3]
II. Previous treatment				
Cyclophosphamide previous ‡	9 (19.6)	8 (34.8)	1 (4.3)	*0.022*[3]

† (mean±SD), ‡ (absolute #, %), [1] Chi-squared test, [2] Mann-Whitney test, [3] Fisher test. Statistically significant p values (<0.05) are shown in italics.

4.4. Expression of investigated biomarkers on extracellular vesicles

Under physiological conditions, extracellular vesicles (EVs) are released into the extracellular space by activated or dying cells. Thus, in his study, the presence of specific surface antigens on EVs was assessed using fluorescence-labelled antibodies in order to determine their immunophenotype, as described in previous section.

As shown in Table 10, levels of all measured EV subsets were significantly elevated in patients with AAV compared to healthy controls (all $p<0.001$). Both active and inactive AAV subgroups also displayed significantly higher EV levels compared to controls (all $p<0.01$). Within the patient cohort, individuals with active vasculitis showed significantly higher levels of EVs expressing tissue factor (TF), neutrophil extracellular traps (NETs), pentraxin 3 (PTX3), HMGB1, C3a, and C5a compared to those in remission ($p=0.012$, $p=0.023$, $p=0.014$, $p=0.006$, $p<0.001$, and $p<0.01$, respectively). No significant differences were observed for MPO, plasminogen, or sTWEAK between active and inactive groups.

Table 10. Expression of extracellular vesicles in plasma of patients with AAV and healthy controls

Antigens expressed on EVs[I]	Total AAV	Healthy controls	p[†]	Active vasculitis	Inactive vasculitis	p[‡]
***Biomarkers expressed on extracellular vesicles (mean*±SD)**						
TF	253.6±151.3	122.4±63.1	*<0.001*	320.6±176.8	186.6±78.4	*0.012*
NETs	107.6±20.1	51.0±26.0	*<0.001*	114.4±20.6	100.8±17.4	*0.023*
MPO	235.5±356.6	46.7±23.0	*<0.001*	225.70±240.5	245.2±449.5	0.717
PTX3 DIM	821.1±757.4	216.2±112.5	*<0.001*	1186.9±850.3	455.3±413.1	*0.014*
HMGB1	639.5±355.0	89.4±46.1	*<0.001*	769.0±372.6	510.0±289.5	*0.006*
Plasminogen	96.1±36.3	48.0±20.7	*<0.001*	93.5±27.4	98.7±43.9	0.956
sTWEAK	145.9±76.5	77.7±41.1	*<0.001*	138.9±78.4	152.9±75.6	0.455
C3a	110.8±49.7	62.2±32.1	*<0.01*	120.2±51.3	101.4±47.3	*<0.001*
C5a	83.7±71.8	29.9±15.0	*<0.01*	92.7±57.6	74.7±44.7	*<0.01*

[†] Total AVV vs healthy controls, Mann-Whitney test, ‡ - Active vasculitis vs vasculitis in remission, Mann-Whitney test, [I] - Mean±SD, Mann-Whiteney test: (p<0,01). **Abbreviations:** EVs – extracellular vesicles; AAV – ANCA associated vasculitis; TF - tissue factor; NETs - neutrophil extracellular traps; MPO - myeloperoxidase; PTX3 - pentraxin 3; HMGB1 - high mobility group box 1; sTWEAK - soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

No correlations were observed between patient age, disease duration, renal function parameters (serum creatinine and eGFR), ANCA serotype (PR3- or MPO-ANCA), and the levels of any of the investigated extracellular vesicle (EV) markers.

The following figures (19-26) are demonstrating levels of extracellular vesicles expressing the investigated biomarkers in AAV patients (both active and in remission) and controls. As well as among vasculitis group when divided in certain subgroups based on ANCA positivity

Figures 16a and 16b illustrate plasma levels of EVs expressing tissue factor (TF). Significantly higher levels of TF-positive EVs were detected in patients with active vasculitis compared to those in remission (p<0.01) and to healthy controls (p<0.001). No significant differences were observed between patients in remission and healthy controls (Figure 19a). When stratified by ANCA serotype, the elevation of TF-positive EVs was preserved only in the MPO-ANCA positive subgroup (p<0.01), while no such difference was found in PR3-ANCA positive patients (Figure 19b).

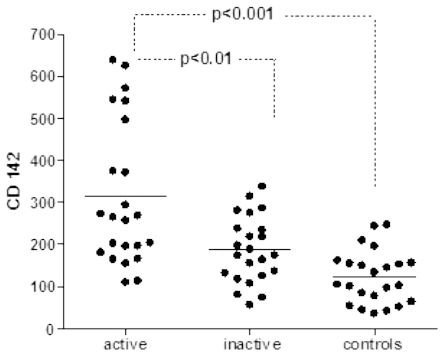


Figure 19a. EVs expressing TF

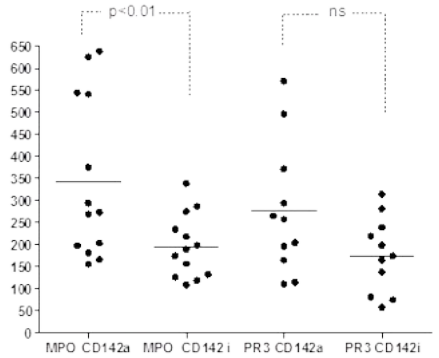


Figure 19b. TF in MPO/PR3 group

Figures 20a and 20b illustrate plasma levels of EVs expressing neutrophil extracellular traps (NETs). Patients with active AAV demonstrated significantly higher levels of NET-positive EVs compared with patients in remission ($p<0.05$) and healthy controls ($p<0.0001$). Moreover, patients in remission also showed significantly elevated NET-positive EV levels compared with controls ($p<0.001$) (Figure 20a).

When stratified according to ANCA specificity, no significant differences in NET-positive EV levels were observed between active and inactive patients within the MPO-ANCA or PR3-ANCA subgroups. However, a marked difference was noted between MPO- and PR3-ANCA positive patients at diagnosis, with higher levels in the MPO group ($p<0.001$) (Figure 20b).

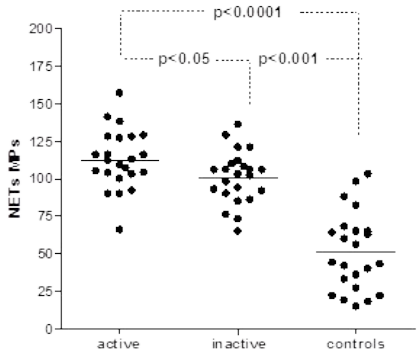


Figure 20a. EVs expressing NETs

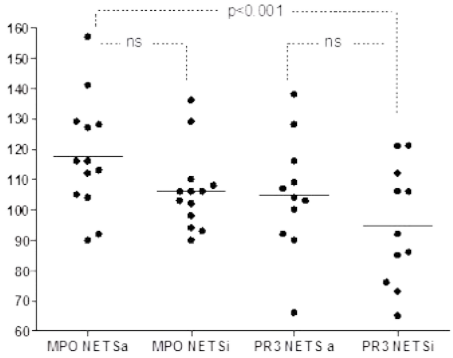
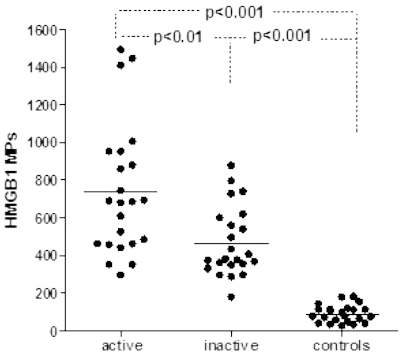


Figure 20b. NETs in MPO/PR3 group

Extracellular vesicles expressing HMGB1 were significantly elevated in patients with active AAV compared to those in remission ($p<0.01$). Healthy controls demonstrated markedly lower levels of HMGB1-positive vesicles than both active and inactive AAV groups ($p<0.001$ for both comparisons) (Figure 21a). When stratified by ANCA specificity, HMGB1-bearing vesicles remained significantly higher in MPO-positive patients with active disease relative to those in remission ($p<0.05$), whereas no significant difference was observed in the PR3-positive subset (Figure 21b).



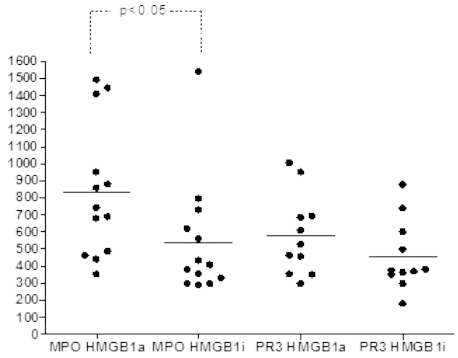


Figure 21a. EVs expressing HMGB1 **Figure 21b.** HMGB1 in MPO/PR3 group

Plasma levels of extracellular vesicles expressing sTWEAK were significantly lower in healthy controls compared with both active AAV patients ($p<0.0001$) and those in remission ($p<0.05$). However, no significant differences were observed between active and inactive patient groups (Figure 22).

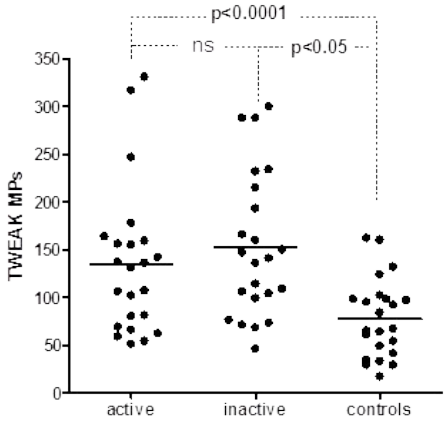


Figure 22. EVs expressing TWEAK

Both patients with active AAV and those in remission exhibited significantly higher plasma levels of extracellular vesicles expressing plasminogen compared with healthy controls ($p<0.001$ and $p<0.01$, respectively). However, no significant difference was observed between the active and inactive patient groups (Figure 23).

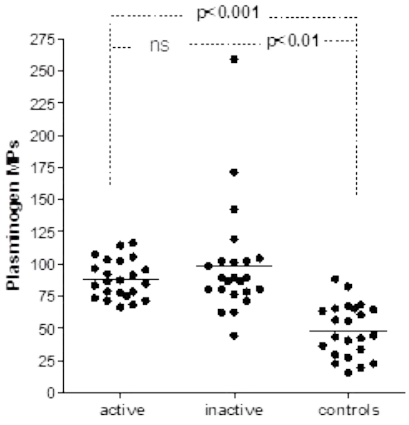
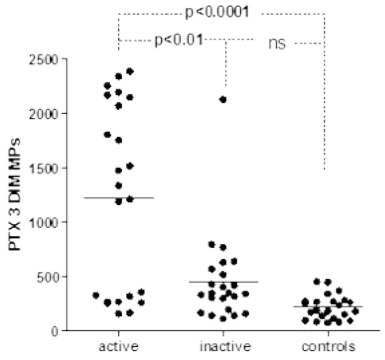


Figure 23. EVs expressing plasminogen

Flow cytometry analyses revealed two distinct extracellular vesicle populations expressing PTX3: a DIM population, which was closer to the levels observed in healthy controls, and a BRIGHT population, which did not show statistically significant differences between active and inactive AAV patients. The DIM PTX3+ EVs were significantly elevated in AAV patients compared with controls ($p<0.001$), whereas no difference was observed for the BRIGHT subset. (Figure 24a–c)



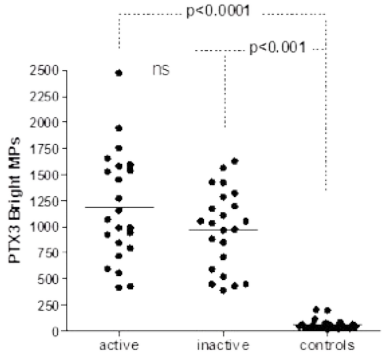


Figure 24a/b. EVs expressing PTX3 dim/bright

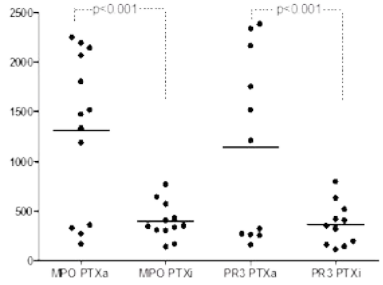


Figure 24c. PTX3 in MPO/PR3 group

The following figures are displaying significant difference of extracellular vesicles expressing C3a (Figure 25) and C5a (Figure 26) between AAV patients and healthy controls. Even thought, statistical significance was not reached between active and inactive groups of AAV patients, we observed significantly higher levels of extracellular vesicles expressing both C3a and C5a in AAV remission than in healthy controls.

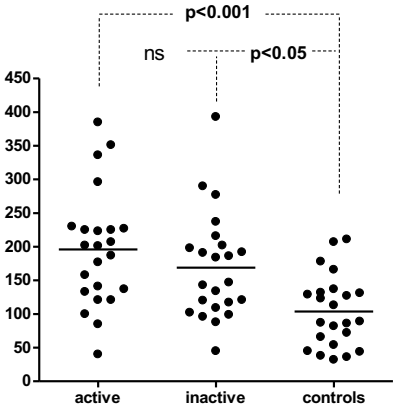


Figure 25. EVs expressing C3a

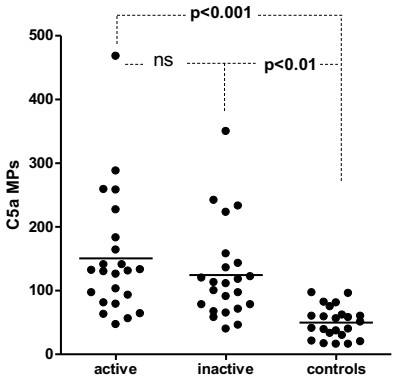


Figure 26. EVs expressing C5a

A significant positive correlation was observed between disease activity, measured by BVAS, and extracellular vesicles expressing complement components C3a and C5a. Specifically, EVs expressing C3a correlated with BVAS ($r=0.37$, $p<0.01$), while EVs expressing C5a showed an even stronger correlation ($r=0.41$, $p<0.01$). (Figure 27)

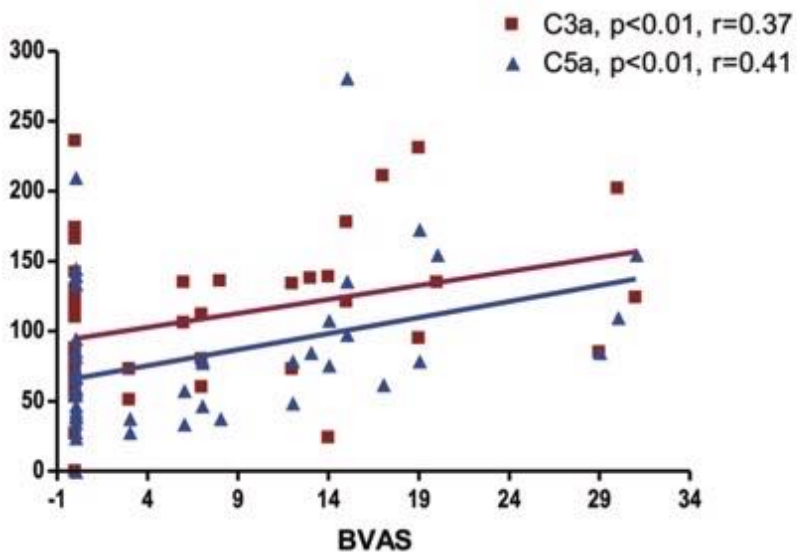


Figure 27. Association of disease activity with C3a and C5a expressed on extracellular vesicles. BVAS – Birmingham Vasculitis Activity Score.

When stratifying patients according to MPO-ANCA positivity, only extracellular vesicles expressing MPO were significantly elevated in the MPO+ active vasculitis group compared with MPO– active patients ($p=0.046$). No other significant differences were observed between MPO+ and MPO– patients in either active or inactive disease for EVs expressing TF, NETs, PTX3, HMGB1, plasminogen, or sTWEAK.

With respect to complement components, EVs expressing C3a showed a trend toward higher levels in MPO+ compared with MPO– patients in active disease, though this did not reach statistical

significance (p=0.116). In remission, however, C3a-expressing EVs were significantly increased in MPO+ patients compared with MPO– (p<0.001). For C5a, no statistically significant differences were observed between MPO+ and MPO– patients, regardless of disease activity (active p=0.198; remission p=0.339). (Table 11)

Table 11. Levels of extracellular vesicles compared to MPO+ in active and inactive disease

EVs expressing markers[I]	Active vasculitis			Inactive vasculitis		
	MPO+	MPO-	p[1]	MPO+	MPO-	p[1]
Extracellular vesicles expressing biomarkers						
TF	356.93±181.52	264.22±162.93	0.159	196.31±71.53	173.9±88.8	0.605
NETs	119.57±19.39	106.33±20.97	0.159	106.23±13.25	93.7±20.22	0.148
MPO	305.93±279.68	100.89±56.74	*0.046*	337.08±581.94	125.8±119.52	0.101
PTX3	1282.64±768.81	1038±993.3	0.516	527.15±510.76	361.8±228.39	0.522
HMGB1	876.43±405.73	602±250.62	0.141	539.54±344.48	471.6±209.3	>0.999
Plasminogen	92.93±33.54	94.44±15.49	0.305	88.85±29.59	111.5±56.68	0.343
sTWEAK	149.29±94.31	122.78±44.54	0.975	141.92±82.58	167.1±66.91	0.313
C3a	209.5 ± 94.5	199.7 ± 4.2	0.116	186.0 ± 0.0	183.3 ± 28.9	*<0.001*
C5a	183.4 ± 109.4	140.4 ± 116.3	0.198	111.2 ± 64.3	136.8 ± 83.6	0.339

[I] - Mean±SD, [1] Mann-Whiteney test: (p<0,01). **Abbreviations:** TF - tissue factor; NETs - neutrophil extracellular traps; MPO - myeloperoxidase; PTX3 - pentraxin 3; HMGB1 - high mobility group box 1; sTWEAK - soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

4.5. Performance of biomarkers in predicting disease activity

Univariate logistic regression analysis (Table 12) identified several statistically significant risk factors for disease activity, as measured by BVAS. Among conventional inflammatory markers, elevation of ESR and CRP were associated with higher odds of active disease (OR 1.045, p=0.030; OR 1.157, p=0.015, respectively). Regarding extracellular vesicle–associated biomarkers, higher levels of EVs expressing TF, NETs, PTX3, and HMGB1 emerged as significant predictors of disease activity (OR 1.008, p=0.011; OR 1.040, p=0.029; OR 1.002, p=0.004; OR 1.003, p=0.023, respectively). In addition, both complement-derived EV markers, C3a (OR 1.014, p=0.009) and C5a (OR 1.012, p=0.012), were also significantly associated with disease activity.

Table 12. Risk factors for disease activity measured by BVAS

Parameter	OR	95%CI	p-value
Age	0.986	0.943-1.031	0.527
Sex	0.839	0.263-2.681	0.767
Creatinine	1.009	0.999-1.019	0.095
eGFR	0.995	0.974-1.016	0.619
ESR	1.045	1.004-1.087	*0.030*
CRP	1.157	1.029-1.301	*0.015*
Extracellular vesicles expressing biomarkers			
MPO+	1.197	0.369-3.875	0.765
TF	1.008	1.002-1.015	*0.011*
NETs	1.040	1.004-1.077	*0.029*
MPO	1.000	0.998-1.001	0.852
PTX3	1.002	1.001-1.0003	*0.004*
HMGB1	1.003	1.000-1.005	*0.023*
Plasminogen	0.996	0.980-1.013	0.628
sTWEAK	0.998	0.990-1.005	0.533
C3a	1.005	1.001-1.010	*0.041*
C5a	1.004	1.001-1.008	*0.038*

OR - odds ratio, 95%CI - 95% confidence interval. **Abbreviations:** eGFR – estimated glomerular filtration rate; ESR – erythrocyte sedimentation rate; CRP – C reactive protein; TF - tissue factor; NETs - neutrophil extracellular traps; MPO - myeloperoxidase; PTX3 - pentraxin 3; HMGB1 - high mobility group box 1; sTWEAK - soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

Table 13 and Table 14 present the correlation analyses of serum biomarkers and extracellular vesicles with disease activity, as assessed by the BVAS score. Among serum markers, a significant positive correlation was found for pentraxin 3 (s-PTX3; r=0.696, p<0.001), whereas no significant associations were observed for s-TWEAK (r=-0.095, p=0.535) (Table 13).

Table 13. Correlation of serum levels of biomarkers with disease activity measured by BVAS

Serum markers		BVAS
s-PTX3	r	0.696**
	p-value	*<0.001*
	N	46
s-sTWEAK	r	-0.095
	p-value	0.535
	N	45

Abbreviations: s-PTX3 - serum pentraxin3; s-sTWEAK - serum soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

For extracellular vesicles, strong correlations were identified for those expressing tissue factor (TF; r=0.623, p<0.001) and PTX3 (r=0.505, p<0.001), while vesicles bearing HMGB1 showed a weaker but statistically significant correlation (r=0.298, p=0.044) with disease activity. Additionally, extracellular vesicles expressing C3a and C5a also demonstrated significant associations with BVAS (r=0.230, p=0.050 and r=0.330, p=0.020, respectively). (Table 14)

Table 14. Correlation of extracellular vesicles expressing biomarkers with disease activity measured by BVAS

Extracellular vesicles expressing biomarkers		BVAS
TF	r	0.623**
	p-value	*<0.001*
	N	46
NETs	r	0.200
	p-value	0.182
	N	46
MPO	r	0.052
	p-value	0.730
	N	46
PTX3	r	0.505**
	p-value	*<0.001*
	N	46
HMGB1	r	0.298*
	p-value	*0.044*
	N	46
Plasminogen	r	-0.113
	p-value	0.455
	N	46
sTWEAK	r	0.046
	p-value	0.764
	N	46
C3a	r	0.230*
	p-value	*0.050*
	N	46
C5a	r	0.330**
	p-value	*0.020*
	N	46

Abbreviations: sPTX3 - serum pentraxin3; s-sTWEAK - serum soluble tumour necrosis factor-like weak inducer of apoptosis; TF - tissue factor; NETs - neutrophil extracellular traps; MPO - myeloperoxidase; PTX3 - pentraxin 3; HMGB1 - high mobility group box 1; sTWEAK - soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

No significant correlation was observed between serum levels of PTX3 and extracellular vesicles expressing PTX3 (r=-0.089, p=0.558), nor between serum levels of TWEAK and extracellular vesicles expressing TWEAK (r=-0.114, p=0.457).

4.6. Correlation of biomarkers with treatment

Prednisolone dose at the time of sampling showed a significant positive correlation with serum pentraxin levels (ρ=0.327, p=0.026) and extracellular vesicles expressing NETs (ρ=0.307, p=0.038). No significant associations were found between prednisolone dose and extracellular vesicles expressing TF, MPO, PTX3, HMGB1, plasminogen, sTWEAK, as well as complement components C3a and C5a (all p>0.05). (Tables 15 and 16).

Table 15. Correlation of prednisolone dose with different markers expressed on extracellular vesicles

Extracellular vesicles expressing biomarkers		**Prednisolone dose**
TF	ρ	0.237
	p-value	0.113
	N	46
NETs	ρ	0.307*
	p-value	*0.038*
	N	46
MPO	ρ	-0.117
	p-value	0.437
	N	46
PTX3	ρ	0.261
	p-value	0.080
	N	46
HMGB1	ρ	0.280
	p-value	0.060
	N	46
Plasminogen	ρ	-0.165
	p-value	0.272
	N	46
sTWEAK	ρ	0.054
	p-value	0.723
	N	46
C3a	ρ	0.047
	p-value	0.755
	N	46
C5a	ρ	0.106
	p-value	0.485
	N	46

ρ - Spearman correlation coefficient. Abbreviations: TF - tissue factor; NETs - neutrophil extracellular traps; MPO - myeloperoxidase; PTX3 - pentraxin 3; HMGB1 - high mobility group box 1; sTWEAK - soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

Table 16. Correlation of prednisolone dose with different serum markers

Serum markers		Prednisolone dose
s-PTX3	ρ	0.327*
	p-value	*0.026*
	N	46
s - sTWEAK	ρ	0.126
	p-value	0.411
	N	45

ρ - Spearman correlation coefficient. Abbreviations: s-PTX3 - serum pentraxin3; s-sTWEAK - serum soluble tumour necrosis factor-like weak inducer of apoptosis. Statistically significant p values (<0.05) are shown in italics.

4.7. Activity assay

To assess the prothrombotic potential of extracellular vesicles (EVs), a modified Calibrated Automated Thrombogram (CAT) assay was performed (Tables 17 and 18). The addition of EVs isolated from plasma of AAV patients resulted in significantly accelerated thrombin generation compared with EVs obtained from healthy controls. This was reflected by shorter lag time and time to peak, as well as significantly increased endogenous thrombin potential (ETP) and peak thrombin (all $p < 0.001$).

When comparing EVs from AAV patients, irrespective of disease activity status, with those from healthy controls, ETP and peak thrombin levels were consistently higher in the patient group (both $p < 0.001$), whereas lag time and time to peak were significantly reduced ($p < 0.001$, $p = 0.002$, $p < 0.001$, and $p = 0.003$, respectively) (Table 17).

Table 17. Thrombin generation assay in patients with AAV and healthy controls

CAT assay[‖]	Total AAV	Healthy controls	p[†]	Active vasculitis	Inactive vasculitis	p[‡]
Lag time (min)	17.2±3.2	21.5±4.2	*<0.001*	17.6±2.9	16.8±3.5	0.407
Lag time SD	1.1±1.1	0.7±0.6	0.231	1.2±1.1	0.9±1.0	0.168
ETP (nM/min)	1314.4±491.1	698.6±251.8	*<0.001*	1350.9±457.3	1276.4±532.3	0.376
ETP SD	87.4±66.1	32.1±26.8	*0.001*	61.2±53.3	114.8±68.1	*0.004*
Peak (nM)	89.1±42.7	46.0±16.2	*<0.001*	96.4±44.6	81.5±40.3	0.276
Peak SD	2.7±4.2	1.9±1.5	0.555	3.2±3.6	2.3±4.8	*0.047*
ttPeak (min)	22.8±3.1	27.1±4.0	*<0.001*	23.0±2.8	22.7±3.4	0.525
ttPeak SD	1.0±1.0	0.7±0.6	0.308	1.2±1.1	0.8±0.9	0.111

[†] Total AVV vs healthy controls, Mann-Whitney test, [‡] - Active vasculitis vs vasculitis in remission, Mann-Whitney test, [‖] - Mean±SD, Mann-Whiteney test: (p<0,01). **Abbreviations:** CAT - calibrated automated thrombogram; Lag time - time to start of thrombin generation; ETP - endogenous thrombin potential, total amount of thrombin generated over time; Peak - the maximal concentration of thrombin generated; ttPeak - time to peak, the time to maximal thrombin generation; SD - standard deviation. Statistically significant p values (<0.05) are shown in italics.

On the other hand, no significant differences were found in any of the thrombin generation parameters when AAV patients were stratified according to MPO-ANCA serostatus, both in active disease and remission (Table 18).

Table 18. Difference of MPO positivity in patients with active vasculitis and in remission in regard to CAT assay parameters

CAT assay ǁ	Active vasculitis			Inactive vasculitis		
	MPO+	MPO-	p[1]	MPO+	MPO-	p[1]
Lag time	17.43±2.11	17.99±3.88	>0.999	16.87±3.33	16.7±3.84	0.974
Lag time SD	1.12±0.85	1.28±1.39	0.975	0.9±0.97	0.99±1.2	0.872
ETP	1378.18±466.05	1308.33±467.72	0.643	1255.66±617.32	1301.19±440.69	0.582
ETP SD	63.51±50.39	57.52±60.55	0.516	109.23±67.28	121.58±72.17	0.456
Peak	103.8±50.83	84.8±31.85	0.557	80.04±47.5	83.28±32.0	0.456
Peak SD	4.09±4.16	1.75±2.16	0.159	1.24±1.07	3.54±7.0	0.429
ttPeak	22.79±1.84	23.38±3.88	0.975	22.88±3.4	22.41±3.63	0.674
ttPeak SD	1.18±0.79	1.21±1.46	0.439	0.74±0.89	0.95±1.08	0.418

[l] - Mean±SD, [1] Mann-Whiteney test: (p<0,01). **Abbreviations:** CAT - calibrated automated thrombogram; Lag time - time to start of thrombin generation; ETP - endogenous thrombin potential, total amount of thrombin generated over time; Peak - the maximal concentration of thrombin generated; ttPeak - time to peak, the time to maximal thrombin generation; SD - standard deviation. Statistically significant p values (<0.05) are shown in italics.

No significant associations were observed between parameters of thrombin generation and disease activity measured by BVAS, as shown in Table 19.

Table 19. Parameters of activity assay as risk factors for disease activity measured by BVAS

Parameters of CAT assay	OR	95%CI	p-value
Lag time	1.093	0.903-1.323	0.363
Lag time SD	1.257	0.703-2.246	0.441
ETP	1.000	0.999-1.002	0.608
ETP SD	0.985	0.974-0.997	0.011
Peak	1.009	0.994-1.023	0.245
Peak SD	1.055	0.908-1.226	0.481
TtPeak	1.039	0.856-1.261	0.699
ttPeak SD	1.445	0.770-2.713	0.252

OR - odds ration, 95%CI - 95% confidence interval. **Abbreviations:** CAT - calibrated automated thrombogram; Lag time - time to start of thrombin generation; ETP - endogenous thrombin potential, total amount of thrombin generated over time; Peak - the maximal concentration of thrombin generated; ttPeak - time to peak, the time to maximal thrombin generation; SD - standard deviation.

To further assess the discriminatory potential of thrombin generation, a receiver operating characteristic (ROC) curve analysis was performed for endogenous thrombin potential (ETP). between ANCA-associated vasculitis (AAV) patients and healthy controls, as well as between disease activity subgroups. ETP showed excellent discriminatory capacity between AAV patients and controls, both in active and inactive disease, but failed to discriminate between active and inactive AAV patients. (Figure 24, Table 20).

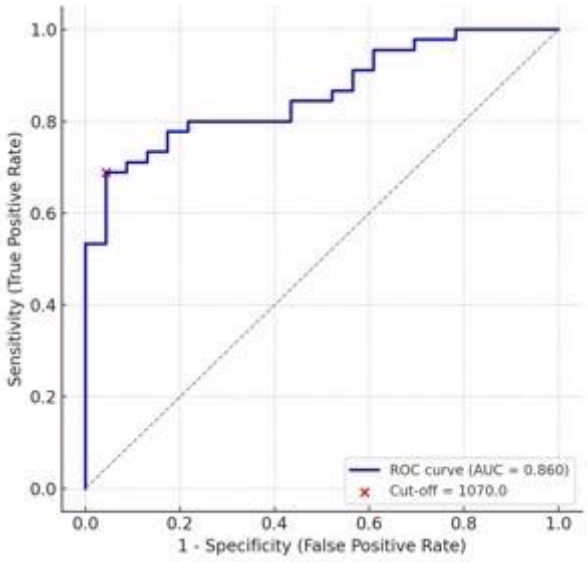


Figure 28. ROC curve for Endogenous thrombin potential (ETP) in AAV patients and healthy controls

Table 20. ROC curve analysis of endogenous thrombin potential (ETP) for discrimination between ANCA-associated vasculitis patients, disease activity subgroups, and healthy controls

	AUC	**95% CI**	**p-value**	**Optimal cut-off (nM/min)**	**Sensitivity (%)**	**Specificity (%)**
AAV vs Controls	0.860	0.757–0.963	*<0.001*	1070.0	76.1	87.0
Active AAV vs Controls	0.872	0.769–0.975	*<0.001*	1134.0	82.6	87.0
Inactive AAV vs Controls	0.849	0.736–0.962	*<0.001*	1070.0	73.9	87.0
Active vs Inactive AAV	0.521	0.354–0.688	0.793	-	-	-

AUC – area under the curve, 95%CI - 95% confidence interval. **Abbreviations:** ETP - endogenous thrombin potential, AAV – ANCA associated vasculitis. Statistically significant p values (<0.05) are shown in italics.

5. DISCUSSION

5.1. Extracellular vesicles in AAV

Extracellular vesicles (EVs) are nano-sized, membrane-bound structures released by eukaryotic cells in response to cellular activation, stress, or apoptosis. They serve as key mediators of intercellular communication, transporting a variety of biomolecules—including proteins, lipids, and nucleic acids—to target cells. EVs are involved in numerous physiological and pathological processes and have garnered increasing attention for their potential role in the pathogenesis of diseases such as ANCA-associated vasculitis (AAV). Given their release into various biological fluids, including blood, urine, and saliva, EVs are easily accessible through non-invasive sampling, making them promising candidates for diagnostic and prognostic biomarkers across a wide range of conditions. [250] However, significant methodological challenges persist in EV research. Detection techniques can substantially influence the measured concentration and composition of EV populations, thereby complicating comparisons across studies. [251,252] Notably, none of the currently available isolation protocols yield a completely pure EV fraction; contaminants such as platelets, plasma protein aggregates, and lipoproteins are frequently co-isolated. [253] Moreover, surface biomarkers on smaller extracellular vesicles may be present at low densities, making detection technically challenging. To address these issues and promote standardization in the field, the International Society for Extracellular Vesicles (ISEV) has published consensus guidelines covering isolation, characterization, and functional studies. [254] In the present study, we employed two complementary techniques in accordance with these recommendations: flow cytometry, to quantify and phenotype of extracellular vesicles, and a modified Calibrated Automated Thrombogram (CAT) assay, to evaluate their procoagulant activity through thrombin generation. Both methods were conducted following established and validated protocols. [251,255]

ANCA-associated vasculitis (AAV) comprises a heterogeneous group of multisystem autoimmune disorders characterized by recurrent disease flares, the need for prolonged immunosuppressive therapy, and frequent complications that collectively exert a profound impact on patients' quality of life. To date, there is a lack of reliable and non-invasive biomarkers that can accurately assess disease activity, predict relapses, or evaluate therapeutic response. As such, the identification of novel biomarkers suitable for integration into routine clinical practice remains a critical unmet need. Although tissue biopsy remains the diagnostic gold standard in AAV, its invasive nature and associated risks limit its use to cases where histological confirmation is essential.

In this thesis, we explored the utility of a novel class of non-invasive biomarkers—extracellular vesicles expressing disease-relevant surface molecules—as indicators of disease activity in AAV. Our principal findings demonstrate significantly elevated levels of EVs bearing tissue factor (TF), neutrophil extracellular traps (NETs), pentraxin 3 (PTX3), high-mobility group box 1 (HMGB1), and the complement components C3a and C5a in patients with active disease compared to both inactive AAV patients and healthy controls. Furthermore, EV expression of TF, PTX3, and HMGB1 showed significant positive correlations with disease activity as measured by the Birmingham Vasculitis Activity Score (BVAS). In contrast, other investigated markers, including plasminogen and TNF-like weak inducer of apoptosis (TWEAK), were found to be elevated in the overall AAV cohort relative to controls, but these did not correlate with disease activity scores, suggesting they may serve more as disease presence markers rather than indicators of current inflammatory burden.

5.2. Biomarkers expressed on extracellular vesicles in AAV

A central feature in the pathogenesis of AAV is injury to the vascular endothelium, a process strongly associated with elevated levels of circulating extracellular vesicles, particularly those originating from endothelial cells and platelets. These subsets of EVs have been shown to correlate with disease activity, underscoring their potential as biomarkers. [185,256] Neutrophil-derived EVs, in particular, are implicated in vascular damage through interactions with ANCA autoantibodies, resulting in enhanced production of reactive oxygen species (ROS) and pro-inflammatory cytokines. [187]

Previous studies have reported increased levels of extracellular vesicles in AAV patients, especially during active disease. [185] Likewise, these particles derived from neutrophil granules seems to be even more accurate in predicting disease activity, since they are enriched with active myeloperoxidase (MPO) [38] or proteinase 3 (PR3), and carry pro-inflammatory mediators such as pentraxin 3 (PTX3), TWEAK, HMGB1, and complement components C3a and C5a. [244,257] Notably, increased levels of platelet-derived extracellular vesicles—often MPO-positive—have been observed in active AAV and found to correlate with systemic inflammation, renal dysfunction (serum creatinine), and glomerular histopathologic lesions. [258]

Despite these promising findings, none of the aforementioned studies have established clinically applicable threshold (cut-off) values for clinical use. As such, their integration into diagnostic or monitoring frameworks remains limited, emphasizing the need for further standardization and validation in larger, prospective studies.

As outlined previously, this thesis investigated several potential biomarkers expressed on the surface of extracellular vesicles in patients with ANCA-associated vasculitis, with the goal of identifying markers that correlate with disease activity and may hold diagnostic or prognostic value. The following subsections present key findings and interpretations related to each individual biomarker evaluated:

5.2.1. Tissue factor

Tissue Factor (TF) plays a critical role in the initiation of the coagulation cascade, ultimately leading to thrombin generation and the development of venous thrombosis. [259] A growing body of evidence supports the involvement of TF in promoting a procoagulant state and elevating the risk of venous thromboembolism (VTE) in patients with AAV. Notably, Kambas *et al.* demonstrated that TF serves as a molecular bridge between inflammation and thrombogenicity in AAV, highlighting its expression within neutrophil extracellular traps (NETs) and on extracellular vesicles derived from neutrophils. Their study reported significantly elevated levels of TF-bearing neutrophil derived EVs in AAV patients compared to both healthy controls and patients with rheumatoid arthritis, along with a correlation between TF-expressing NETs and disease activity. [191]

Building on this, Mendoza *et al.* identified TF activity on circulating extracellular vesicles as a strong predictor of VTE risk in AAV, independent of renal function. [173] More recently, our own research group demonstrated that increased thrombin generation in AAV patients is linked to higher surface expression of both TF and citrullinated histone H3 (H3Cit) on MPO-positive EVs. [260]

Consistent with these previous findings, the current thesis confirms significantly elevated levels of TF-expressing EVs in AAV patients compared to healthy controls. These levels were not only higher during active disease relative to remission, but also showed a positive correlation with the Birmingham Vasculitis Activity Score (BVAS), suggesting their relevance in reflecting disease activity. Taken together, our results further support the hypothesis that TF-bearing EVs contribute to the hypercoagulable state observed in AAV and may serve as both biomarkers of disease activity and potential mediators of thrombotic risk.

5.2.2. Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) contribute significantly to endothelial cell injury in AAV through two primary mechanisms. First, NETs can promote the generation of ANCA autoantibodies by facilitating dendritic cell-mediated cross-presentation to T and B lymphocytes. Second, ANCAs themselves can induce NETosis, which in turn activates the alternative pathway of the complement system. [202] NETs are considered central to AAV pathogenesis, given that patients exhibit both excessive formation and impaired degradation of these web-like chromatin fibres. These

structures also display AAV-associated autoantigens and have been shown to activate complement, contributing to tissue injury, particularly in pauci-immune glomerulonephritis, as seen in this disease.

In the present study, we observed significantly elevated plasma levels of extracellular vesicles expressing NETs components in AAV patients compared to healthy controls, and between patients with active versus inactive disease. However, no statistically significant correlation was found between EV expressing NETs and disease activity as measured by the Birmingham Vasculitis Activity Score (BVAS). Despite their elevation, these EVs did not perform reliably as markers of current inflammatory burden.

Several factors may explain this discrepancy. First, methodological differences in NET visualization—whether via immunofluorescence, flow cytometry, or ELISA-based detection—can yield inconsistent results. Second, variability across *in vivo* and *in vitro* studies may reflect heterogeneity in biological responses that are not fully captured under standardized conditions. Third, our findings revealed a correlation between EVs expressing NETs and the daily dose of prednisolone, suggesting that immunosuppressive therapy may influence NET formation or clearance, thus confounding their interpretive value.

Taken together, while EVs bearing NETs may still hold mechanistic relevance in AAV, their utility as a biomarker for assessing disease activity appears limited based on current evidence.

5.2.3. Pentraxin 3

Pentraxin 3 (PTX3), an acute-phase protein, exhibits proinflammatory functions, including neutrophil recruitment, opsonization, but also is involved in the activation of the complement cascade. In the context of AAV, our findings revealed significantly elevated serum PTX3 levels in patients with active disease compared to those in remission. These serum concentrations also showed a positive correlation with disease activity, as assessed by the Birmingham Vasculitis Activity Score (BVAS). Similarly, extracellular vesicles expressing PTX3 were markedly increased in patients with active AAV relative to both healthy controls and patients in remission, again correlating with disease activity. Furthermore, elevated levels of EVs expressing PTX3 are reported as risk factor for disease activity.

Interestingly, there was no correlation between serum levels of PTX3 and levels of extracellular vesicles expressing this marker, suggesting distinct regulatory mechanisms or compartmentalization of PTX3 production and release. A similar pattern was reported in a multicentre Italian study that examined PTX3 in patients with AAV, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and CREST syndrome. [214] The authors concluded that PTX3 may serve as a tissue-specific marker, reflecting local inflammatory activity at sites of vasculitis rather than systemic inflammatory burden.

From a therapeutic point of view, in particular corticosteroid influence on PTX3, correlation of prednisolone daily dose with serum levels of PTX3 was found. Since both steroids and immunosuppressive therapy influence production of several cytokines (such as IL1 and TNFα) and those further trigger creation of PTX3, obviously prednisolone can modulate PTX3 expression in AAV patients. Positive correlation was found between PTX3 concentrations and activity in systemic immune-mediated diseases. [261] However, an alternative explanation is that patients with more severe disease (and therefore higher PTX3 levels) tend to require higher doses of corticosteroids. Thus, whether PTX3 expression is directly influenced by corticosteroids or merely reflects disease severity remains an open question.

Recently, a longitudinal study from our extended patient cohort provided further insight. It demonstrated that both plasma and urinary PTX3 levels were significantly higher at baseline than at 6-month follow-up, correlating with disease activity, particularly in patients with renal involvement. Notably, no correlation was observed between plasma and urinary PTX3 concentrations. Thus, the authors are implying PTX3 as more reliable biomarker for monitoring disease activity in AAV, and may serve as a reliable marker of renal vasculitic activity. [262]

Although some literature suggests that the only serum marker that remains unaffected by corticosteroid therapy is PTX3, and thus could serve as a robust biomarker for monitoring disease activity regardless of treatment status [214], our findings and others indicate that its expression may indeed be modulated—directly or indirectly—by therapeutic interventions. Therefore, the utility of PTX3 as a treatment-independent marker of disease activity remains promising but warrants further validation in larger and therapeutically stratified cohorts.

5.2.4. HMGB1

High-mobility group box 1 (HMGB1) is a nuclear protein with potent proinflammatory activity once released into the extracellular space, typically during necrosis or other forms of immunogenic cell death. It has been widely studied as a biomarker in systemic autoimmune diseases, particularly systemic lupus erythematosus (SLE), where both serum and urinary HMGB1 levels have been proposed as indicators of disease activity, especially in the context of lupus nephritis. [263,264]

In contrast, the role of HMGB1 as a biomarker in ANCA-associated vasculitis remains less clearly defined and is marked by conflicting evidence. A cross-sectional study reported elevated serum HMGB1 levels in patients with active granulomatosis with polyangiitis (GPA) compared to those with microscopic polyangiitis (MPA). [265] Similarly, Bruchfeld *et al.* [223] observed significantly increased serum HMGB1 levels in AAV patients with active renal involvement (within the same VASKA cohort), regardless of vasculitis subtype, compared to patients in remission. In addition, their study reported higher HMGB1 concentrations in AAV patients versus healthy controls.

However, other findings complicate the picture. De Souza *et al.* [220], in a longitudinal study, noted that serum HMGB1 levels were elevated at presentation in patients without renal involvement, while patients with renal manifestations showed levels similar to controls. They proposed that HMGB1 levels may fluctuate depending on renal damage severity and, as such, may not reliably reflect disease activity across all AAV phenotypes. Another potential explanation for these discrepancies lies in the technical variability of HMGB1 measurement; Western blotting has been shown to detect significantly higher levels compared to ELISA—by as much as fivefold. However, it is worth noting that ELISA was used consistently across most studies, including ours, suggesting that measurement technique alone may not fully explain the observed variability. Urinary HMGB1 has also been explored as a potentially more reliable biomarker, particularly in kidney-involved AAV. For instance, Ma *et al.* [221] demonstrated an association between urinary HMGB1 levels and disease activity in AAV, further supporting this approach.

In the present study, serum HMGB1 concentrations did not differ significantly between AAV patients and healthy controls, nor did they correlate with disease activity as assessed by BVAS. In contrast, plasma levels of extracellular vesicles bearing HMGB1 were significantly higher in AAV patients relative to controls and also showed marked elevation in patients with active disease compared to those in remission. Notably, levels of extracellular vesicles expressing HMGB1 correlated with BVAS and emerged as an independent risk factor for disease activity.

Our findings are in alignment with previous work by a Chinese research group that reported circulating HMGB1 levels to reflect both disease activity and renal involvement in a cohort of 74 AAV patients, primarily with MPA. [266] However, the mechanistic role of HMGB1 in promoting hypercoagulability in AAV remains an open question and warrants further mechanistic investigation.

5.2.5. Plasminogen

Plasminogen is a central component of the fibrinolytic system, not only contributing to clot breakdown but also playing a role in modulating inflammation through neutrophil recruitment and activation. [267] In ANCA-associated vasculitis, the presence of anti-plasminogen antibodies has been associated with active disease and is particularly linked to renal pathology, including glomerular lesions. [174,268] These findings underscore the involvement of plasminogen pathways in AAV pathogenesis and immune-mediated tissue injury.

Despite the known significance of anti-plasminogen antibodies, to our knowledge, extracellular vesicles expressing plasminogen have not been previously studied in AAV. In the present study, we identified significantly elevated levels of plasminogen-bearing EVs in AAV patients compared to healthy controls. However, these levels did not correlate with disease activity,

as no significant differences were observed between patients with active versus inactive disease, nor was any correlation found with BVAS scores.

These findings suggest that while EVs expressing plasminogen may be increased in AAV, their levels do not reflect ongoing disease activity and therefore may be more indicative of general disease presence or underlying chronic inflammation, rather than acute flare status.

5.2.6. sTWEAK

TNF-like weak inducer of apoptosis (TWEAK) is a soluble proinflammatory cytokine belonging to the TNF superfamily, primarily secreted by activated macrophages and monocytes and detectable in the circulation. [269] It has been proposed as a potential biomarker of disease activity in several autoimmune and inflammatory conditions, particularly systemic lupus erythematosus (SLE) . [270]

In our cohort, serum TWEAK levels did not show any correlation with disease activity in AAV, as measured by BVAS. These findings are consistent with a previous study by our group, conducted on an expanded cohort of AAV patients followed over a 6-month period. That longitudinal analysis revealed significantly higher serum TWEAK levels at baseline compared to 6-month follow-up; however, no significant difference was observed between AAV patients and healthy controls. Interestingly, in the same study, elevated urinary TWEAK levels were observed at baseline, and these showed significant correlations with both BVAS and albuminuria. [271] This suggests that urinary TWEAK may be a more sensitive marker of disease activity, particularly in the context of renal involvement.

These results align with findings from studies in other immune-mediated kidney diseases, such as lupus nephritis and IgA nephropathy, where urinary TWEAK has shown potential as a non-invasive biomarker reflecting glomerular inflammation. [272,273] In contrast, serum TWEAK levels have been reported to be elevated in various autoimmune diseases including SLE, rheumatoid arthritis, Behçet's disease, systemic sclerosis, and type 1 diabetes mellitus. [274, 275, 276] Interestingly, to the best of our knowledge, neither one study was focused on TWEAK expression in AAV. Notably, Mirioglu *et al.* [277] included a small group of 14 AAV patients with renal involvement, but as disease controls, in their investigation of TWEAK in active and inactive SLE. While serum TWEAK levels were higher in active SLE compared to inactive and healthy controls, no significant difference was observed between patients with active renal SLE and active renal AAV.

Contrary to some of these earlier findings, the present study did not observe any significant differences in serum TWEAK levels between active and inactive AAV patients, nor did serum levels correlate with BVAS. However, we did find elevated levels of extracellular vesicles bearing TWEAK in the plasma of AAV patients compared to controls. These elevations were present regardless of

disease activity status and showed no correlation with BVAS, suggesting that while extracellular vesicles expressing TWEAK may reflect general disease presence or chronic inflammation, it is not a reliable indicator of disease activity.

5.2.7. Components of complement system: C3a and C5a

Although AAV has traditionally been classified as a pauci-immune disease—characterized by the absence or minimal presence of immunoglobulin and complement deposits—accumulating evidence has highlighted the significant involvement of the complement system, particularly the alternative pathway, in AAV pathogenesis. [158,238,278] This obvious influence of components of complement system in disease development, relates mainly relevant at the renal level, as demonstrated initially in murine models [93,114,279], and subsequently confirmed in human studies. Immunohistochemical analyses have shown deposition of complement components at sites of vasculitic injury, and their plasma concentrations have been found to correlate with disease activity. [280] Furthermore, some studies have reported associations between hypocomplementemia and more severe renal involvement, including greater proteinuria and higher disease activity scores. [241,281]

The terminal product of the alternative complement pathway, C5a, is a potent anaphylatoxin with strong proinflammatory and prothrombotic properties. It plays a key role in bridging the inflammatory and coagulation cascades in AAV. [192,244] C5a promotes ANCA antigen (e.g., PR3) expression on neutrophil surfaces, facilitating their priming and subsequent activation by ANCA. This generates a positive feedback loop wherein C5a induces neutrophil and endothelial cell activation, chemotaxis, and NET formation. [282] In turn, activated neutrophils release microparticles that further stimulate alternative pathway activation. [121,281] Additionally, C5a promotes a hypercoagulable state by inducing tissue factor (TF) expression on EVs and enhancing NETosis. [192] Its receptor, C5aR (CD88), has been shown to be essential in the development of MPO-ANCA-induced necrotizing crescentic glomerulonephritis in murine models—an effect that could be prevented by pharmacologic blockade. [93] This has paved the way for targeted therapies such as Avacopan (CCX168), a selective C5aR inhibitor, which has shown promising results in reducing renal inflammation [128] and allowing for steroid-sparing regimens. [283]

In our study, we observed increased serum levels of C5a in patients with active disease compared to those in remission, although this difference did not reach statistical significance. Nonetheless, C5a's biological activity—as both a proinflammatory and prothrombotic mediator—remains compelling in the AAV disease model.

In contrast to C5a, C3a is thought to exert a more regulatory or protective role in AAV. It has been proposed to counteract neutrophil mobilization and dampen local inflammation, functioning in opposition to C5a. [284] Consistent with this, we observed lower serum levels of C3a in patients with

active disease compared to those in remission, although the difference was not statistically significant. No correlation was observed between serum concentrations of C3 or C3a and extracellular vesicles expressing C3a either. However, a novel and important observation from our study was the significant increase in C3a-expressing EVs in AAV patients compared to controls, with even higher levels observed in patients with active disease, even correlating with disease activity. These findings are in line with our group's previous publication [244], suggesting that extracellular vesicles expressing complement components may reflect local or cell-specific activation events not captured by bulk serum measurements.

Taken together, these results reinforce the increasingly recognized role of complement dysregulation—especially involving C5a and C3a—as a key contributor to AAV pathogenesis. Our data on EV-associated complement expression provide additional support for the involvement of both inflammatory and regulatory complement arms, expanding the current understanding of their role in disease activity and progression.

Interestingly, these findings challenge earlier assumptions, which held that circulating complement levels (e.g., C3, C4) remain within normal ranges in most AAV patients. [285] As such, our study supports a growing shift toward examining functional and localized complement activation, including that mediated by EVs, rather than relying solely on systemic complement measurements.

5.3. Procoagulant property of extracellular vesicles in AAV

To address the second objective of this study, we employed a functional activity assay—specifically, a modified version of the Calibrated Automated Thrombogram (CAT) method—to investigate the procoagulant potential of extracellular vesicles (EVs) by assessing their capacity to promote thrombin generation. Commonly referred to as a thrombin generation assay (TGA), this technique provides a dynamic assessment of haemostatic balance by simultaneously measuring both the formation and inhibition of thrombin over time. The assay was originally adapted for EV-based analysis by Bidot *et al.* nearly two decades ago. [248]

In our study, EV-enriched pellets were isolated from the plasma of patients with AAV (including both active and inactive disease states) as well as from age- and sex-matched healthy controls. These vesicles were then used as procoagulant stimuli in the CAT assay. The principal finding was a significantly increased thrombin generation in AAV patients compared to controls, as indicated by higher endogenous thrombin potential (ETP) and peak thrombin, along with shorter lag time and time to peak. Notably, this procoagulant enhancement was observed regardless of disease activity, as no significant differences were found between patients with active versus inactive vasculitis. These results suggest that EVs derived from AAV patients carry intrinsic procoagulant

properties, potentially contributing to the hypercoagulable state commonly observed in this disease. The lack of correlation with disease activity may indicate that EV-mediated coagulation imbalance persists even during clinical remission, reflecting an underlying predisposition to thrombosis.

Comparable findings were reported in a smaller study involving children with systemic vasculitis, where a similar assay demonstrated elevated thrombotic potential in patients compared to healthy controls. [286] Interestingly, that study also found significantly higher thrombin generation during active disease relative to remission. The authors attributed this effect to an increase in neutrophil-derived EVs during active inflammation.

Our findings support the hypothesis that EVs contribute to the hypercoagulable state observed in AAV, independently of overt disease activity. The presence of enhanced thrombin generation even in patients in remission suggests a persistent subclinical prothrombotic milieu, possibly contributing to the increased risk of venous thromboembolic events (VTE) described in AAV cohorts. Given that EVs serve as carriers of tissue factor (TF), neutrophil components, and complement fragments—all of which were elevated in our study—their functional role in coagulation appears not merely associative but mechanistically significant.

This aspect of EV biology underscores their potential as both biomarkers and active contributors to the pathophysiology of AAV-related thrombosis. Importantly, our findings highlight the value of thrombin generation assays (TGAs) as a functional readout of EV-induced coagulation, going beyond simple enumeration or marker expression. The application of such functional assays may prove useful in risk stratification, especially in identifying patients who may benefit from preventive antithrombotic strategies—even in the absence of clinically active disease.

Further longitudinal studies are needed to determine whether thrombin-generating EVs predict thrombotic events or disease flares, and to evaluate their responsiveness to immunosuppressive or anticoagulant therapies. Additionally, integration of EV analysis with other biomarkers of endothelial injury and coagulation could provide a more comprehensive profile of vascular risk in AAV.

5.4. Contribution

Through this investigation, we have expanded current understanding of the immunopathogenesis of ANCA-associated vasculitis (AAV), particularly by exploring the functional and phenotypic role of extracellular vesicles (EVs) in disease activity and hypercoagulability. Our findings highlight several candidate biomarkers—such as extracellular vesicles expressing TF, PTX3, HMGB1, and C5a—that may serve as indicators of disease activity or contribute to underlying prothrombotic risk. Importantly, we also demonstrate the utility of functional assays, such as modified

thrombin generation testing, in evaluating the pathogenic role of EVs beyond surface marker expression.

Although the investigated biomarkers are not intended for use in primary diagnosis—mainly due to the complexity and cost of their detection methods—they hold promise as tools for disease monitoring. Pending validation in larger, multicentre cohorts, these markers could aid in differentiating patients with active disease from those in remission, thereby informing clinical decision-making regarding therapy intensity and duration.

A particularly relevant observation from our study is the differential effect of immunosuppressive therapy on biomarker distribution. While serum levels of certain molecules (e.g., PTX3, HMGB1) appeared to correlate with corticosteroid dose, this was not observed for their EV-associated counterparts. This suggests that extracellular vesicle profiling may offer a therapy-independent insight into ongoing pathophysiological activity—an advantage over conventional serum biomarkers whose levels may be masked or suppressed by treatment.

More broadly, the exploration of EVs provides a new lens through which to study the complex interplay between inflammation, immunity, and coagulation in AAV. By identifying functional markers associated with EVs, our study contributes to a growing body of evidence that supports the development of immune-targeted, precision therapies, such as complement inhibitors (e.g., Avacopan), which may offer safer and more effective alternatives to traditional cytotoxic immunosuppressants. Importantly, we further demonstrate that assessing EV-mediated thrombin generation and their procoagulant activity provides unique mechanistic insight into how inflammation and coagulation intersect in AAV, thereby offering not only novel biomarkers of thrombotic risk but also potential targets for therapeutic intervention. Ultimately, deciphering the cellular and molecular mechanisms behind AAV not only enhances disease stratification but also paves the way toward individualized treatment strategies, focused on minimizing toxicity while maintaining disease control.

5.5. Study limitations

Several limitations of this study must be acknowledged.

First, the process of freezing and thawing plasma samples containing extracellular vesicles (EVs) may have influenced the integrity and quantification of the vesicles. As previously demonstrated in detail in the doctoral thesis by one of our co-authors [287], freeze-thaw cycles can induce morphological changes in EVs and potentially elevate EV counts, particularly due to platelet activation and subsequent vesicle release. This phenomenon may lead to artificially increased EV levels when compared to fresh platelet-poor plasma (PPP) samples. However, some studies have argued that plasma EVs remain relatively stable through freeze-thaw cycles, especially when

standardized protocols are followed [288], suggesting this impact may be minimized with careful handling.

Second, the relatively small sample size represents a limitation of statistical power and generalizability. AAV is a rare disease, and as such, achieving a larger inclusion rate is inherently challenging. Despite this, our cohort size is comparable to similar studies in the field and includes carefully phenotyped patients with both active and inactive disease.

Third, the cross-sectional study design limits the ability to infer temporal or causal relationships. A prospective longitudinal design, ideally beginning at the time of diagnosis and including serial sampling during therapy, remission, and potential flares, would provide more robust data on the dynamics of biomarker expression. Even a structured follow-up design with biomarker analysis at multiple time points would have allowed a more accurate assessment of disease activity correlations and treatment response. We are hoping to address this limitation in future work by implementing longitudinal monitoring of extracellular vesicle profiles, which may reveal their utility as dynamic biomarkers throughout different stages of disease and treatment.

Despite these limitations, the current study provides meaningful insight into the role of EVs in AAV pathogenesis and their potential utility as functional biomarkers. The findings offer a strong foundation for future prospective investigations.

6. CONCLUSIONS

All examined proteins expressed on extracellular vesicles (EVs) were evaluated for their potential utility as biomarkers in patients with ANCA-associated vasculitis (AAV). Based on the findings presented throughout this thesis, we conclude the following:

1. Extracellular vesicles expressing all investigated markers (TF, NETs, PTX3, HMGB1, C3a, C5a, plasminogen, and TWEAK) were significantly elevated in AAV patients compared to healthy controls. This increase was observed both in patients with active disease and those in remission, indicating a persistent biological signal.
2. Active AAV patients demonstrated significantly higher levels of EVs bearing tissue factor (TF), neutrophil extracellular traps (NETs), pentraxin 3 (PTX3), high mobility group box 1 (HMGB1), and complement components C3a and C5a, compared to patients in remission and controls. However, not all markers showed a direct correlation with disease activity measured by the Birmingham Vasculitis Activity Score (BVAS).
3. Among the evaluated markers, higher levels of EVs expressing TF, NETs, and PTX3 emerged as significant independent risk factors for active disease, suggesting their potential utility in clinical stratification.
4. A statistically significant correlation was found between serum PTX3 concentrations and disease activity (BVAS). Additionally, EVs expressing TF, PTX3, and HMGB1 correlated strongly with disease activity, further underscoring their role in the underlying immunopathogenesis of AAV.
5. Prednisolone dosage at the time of sampling was positively correlated with serum levels of PTX3 and EVs expressing NETs, indicating that treatment may partially influence the levels of some circulating biomarkers—especially in soluble form—while EV-associated expression appears more stable and therapy-independent.
6. Finally, the procoagulant properties of extracellular vesicles in AAV were confirmed via a modified thrombin generation assay. Compared to healthy controls, EVs from AAV patients were associated with significantly shorter Lag time and Time to peak, indicating faster thrombin generation. While ETP and Peak of thrombin were significantly higher in AAV patients compared to controls. In terms of EVs derived from plasma of AAV patients (irrespective of disease activity), compared to EVs extracted from controls, higher ETP and Peak levels were found, but shorter Lag time and Time to peak, suggesting a persistent prothrombotic state independent of clinical disease activity.

In summary, this research confirms that extracellular vesicles not only serve as carriers of inflammatory and thrombogenic mediators but also exhibit biologically active properties that may directly contribute to disease pathology. Their expression patterns and functional characteristics offer valuable insight into immune activation, endothelial injury, and coagulation imbalance in ANCA associated vasculitis. Although further prospective, multicentre validation studies are required, EV profiling may become a valuable adjunct in the assessment of disease activity, prognosis, and individualized therapy monitoring.

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8. BIOGRAPHY

Milena Manojlović was born on November 28, 1987, and currently resides in Niš, Serbia. She is married, mother of three children. Primary education was completed at "Učitelj Tasa" Elementary School and her secondary education at "Bora Stanković" Gymnasium in Niš, both with excellent academic performance. She obtained MD degree at Faculty of Medicine, University of Niš, with an average grade of 9.64 (out of 10). Over the course of her studies, she received multiple awards and scholarships, including being a three-time recipient of the City of Niš Scholarship for Talented Students. Additionally, she was awarded the Dositeja Scholarship by the Fund for Young Talents of the Ministry of Youth and Sports of the Republic of Serbia, as part of the competition for the 1,000 best final-year undergraduate students. In the 2012/2013 academic year, she enrolled in doctoral academic studies at the Faculty of Medicine, University of Niš.

She started her residency in pediatrics as a volunteer in 2015 and began her permanent employment at the Pediatrics Clinic of the University Clinical Center in Niš in 2018. On July 2, 2020, she successfully passed the pediatrics specialization exam, with an excellent grade, earning the title of Pediatrics Specialist.

MD Manojlović spent 6 months from December 2016 to June 2017, as a visiting PhD researcher at the Karolinska Institute in Stockholm, Sweden, as part of the ERASMUS+ PhD student exchange program, under the mentorship of Associated Professor Aleksandra Antović.

Throughout her research career, she has contributed to the publication of more than 20 scientific papers, including 9 indexed in SCI-listed journals. She has also actively participated in numerous scientific conferences, both nationally and internationally.

ИЗЈАВА О АУТОРСТВУ

Изјављујем да је докторска дисертација, под насловом

“ЕКСТРАЋЕЛИЈСКЕ ВЕЗИКУЛЕ КАО БИОМАРКЕРИ АКТИВНОСТИ БОЛЕСТИ КОД ВАСКУЛИТИСА УДРУЖЕНИМ СА АНТИТЕЛИМА ПРОТИВ ЦИТОПЛАЗМЕ НЕУТРОФИЛА”

“EXTRACELLULAR VESICLES AS BIOMARKERS OF DISEASE ACTIVITY IN ANTINEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS”

која је одбрањена на Медицинском факултету Универзитета у Нишу:

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Потпис аутора дисертације:

Др Милена Г. Манојловић

ИЗЈАВА О ИСТОВЕТНОСТИ ШТАМПАНОГ И ЕЛЕКТРОНСКОГ ОБЛИКА ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

Наслов дисертације:

“ЕКСТРАЋЕЛИЈСКЕ ВЕЗИКУЛЕ КАО БИОМАРКЕРИ АКТИВНОСТИ БОЛЕСТИ КОД ВАСКУЛИТИСА УДРУЖЕНИМ СА АНТИТЕЛИМА ПРОТИВ ЦИТОПЛАЗМЕ НЕУТРОФИЛА”

“EXTRACELLULAR VESICLES AS BIOMARKERS OF DISEASE ACTIVITY IN ANTINEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS”

Изјављујем да је електронски облик моје докторске дисертације, коју сам предала за уношење у **Дигитални репозиторијум Универзитета у Нишу**, истоветан штампаном облику.

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Потпис аутора дисертације:

Др Милена Г. Манојловић

ИЗЈАВА О КОРИШЋЕЊУ

Овлашћујем Универзитетску библиотеку „Никола Тесла“ да у Дигитални репозиторијум Универзитета у Нишу унесе моју докторску дисертацију, под насловом:

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Дисертацију са свим прилозима предала сам у електронском облику, погодном за трајно архивирање.

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