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Diagnostic and prognostic aspects of anti-neutrophil cytoplasmic antibodies in systemic vasculitis

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Abstract

Primary systemic vasculitis is associated with a high mortality if left untreated and it is essential to make the diagnosis before permanent organ damage has occurred. In this thesis the diagnostic and prognostic significance of anti-neutrophil cytoplasmic antibodies (ANCA) in systemic vasculitis is investigated.

The clinical significance of a capture enzyme-linked immunosorbent assay (ELISA) based on the monoclonal antibody 4A3 for detection of antibodies against proteinase 3 (PR3-ANCA) was evaluated and the method was found to have a high sensitivity and specificity. It was shown that patients with high levels of PR3-ANCA as measured by the capture ELISA have a poorer survival rate and worse renal outcome and are more prone to relapse. In comparison the symptoms, signs and clinical course of a cohort of patients with vasculitis but without ANCA, with the diagnosis of polyarteritis nodosa, were described.

Recombinant chimeric molecules were produced in order to map epitopes of monoclonal antibodies to PR3. The clinical utility of these chimeric proteins in the diagnosis and follow-up of patients with PR3-ANCA positive vasculitis was studied and these chimeric molecules were not found to be better than capture ELISA using native PR3. It was observed that samples from the same patient during one exacerbation episode always exhibited a similar pattern of reactivity and that some patients exhibited the same pattern at diagnosis and subsequent flares, while in many patients the pattern changed considerably over time indicating epitope shift during the course of the disease.

Key words: ANCA, vasculitis, proteinase 3, epitope, capture ELISA, outcome, Wegener's granulomatosis, microscopic polyangiitis, polyarteritis nodosa

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Original papers

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I. Westman K, Selga D, Bygren P, Segelmark M, Baslund B, Wiik A, Wieslander J: Clinical evaluation of a capture ELISA for detection of proteinase-3 antineutrophil cytoplasmic antibody. Kidney Int 1998;53:1230-1236.

II. Westman K, Selga D, Isberg PE, Bladström A, Olsson H: High proteinase 3-anti-neutrophil cytoplasmic antibody (ANCA) level measured by the capture enzyme-linked immunosorbent assay method is associated with decreased patient survival in ANCA-associated vasculitis with renal involvement. J Am Soc Nephrol 2003;14:2926-2933.

III. Selga D, Mohammad A, Sturfelt G, Segelmark M: Polyarteritis nodosa when applying the Chapel Hill nomenclature – a descriptive study on ten patients. Rheumatology 2006;45:1276-1281.

IV. Selga D, Segelmark M, Wieslander J, Gunnarsson L, Hellmark T: Epitope mapping of anti-PR3 antibodies using chimeric human/mouse PR3 recombinant proteins. Clin Exp Immunol 2004;135:164-172.

V. Selga D, Hellmark T, Gunnarsson L, Westman K, Segelmark M: Capture ELISA for PR3-ANCA is superior to detect and predict relapses of vasculitis compared to direct ELISA based on native PR3, recombinant PR3 or chimeric human/mouse PR3 constructs. Manuscript.

Contents

Abbreviations

AAV	anti-neutrophil cytoplasmic antibody associated vasculitis
ACR	American college of rheumatology
ANCA	anti-neutrophil cytoplasmic antibody
BVAS	Birmingham vasculitis activity score
CHCC	Chapel Hill consensus conference
ELISA	enzyme-linked immunosorbent assay
EMEA	European medicines agency
ENT	ear, nose and throat
FFS	five factor score
HBV	hepatitis B virus
HLE	human leukocyte elastase
hPR3	human proteinase 3
IgG	immunoglobulin G
IIF	indirect immunofluorescence
mab	monoclonal antibody
MPA	microscopic polyangiitis
MPO	myeloperoxidase
mPR3	murine proteinase 3
PAN	polyarteritis nodosa
PCR	polymerase chain reaction
PR3	proteinase 3
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
VDI	vasculitis damage index
WG	Wegener's granulomatosis

Introduction

Vasculitis means inflammation of blood vessels and the term can be applied to blood vessel inflammation of differing aetiologies. The inflammation can be secondary to for example infections, drugs or chemicals, or part of a systemic disease such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). Primary vasculitis is, as the term implies, of unknown aetiology and can affect blood vessels of various sizes and locations. Systemic vasculitides can affect any blood vessel in the body and are associated with a high mortality if left untreated. Immunosuppressive treatment is required to bring the diseases into remission and the nature of these diseases is remitting-relapsing in many patients. Systemic vasculitis often takes a rapid course and it is essential to make the diagnosis and start treatment before any permanent organ damage has occurred. When treating patients with potentially dangerous drugs it is of utmost importance that the diagnosis is correct and that there is no other cause of the symptoms that would require a different treatment. Patients with systemic small vessel vasculitis frequently have anti-neutrophil cytoplasmic antibodies (ANCA) in their blood at the time of diagnosis. These antibodies can be detected with various different detection methods and the presence of certain types of ANCA is strongly suggestive of systemic vasculitis, which is of significant help in making the diagnosis.

Classification of systemic vasculitis

In 1866, Kussmaul and Maier [1] described a patient dying from a previously unknown disease to which they gave the descriptive name of periarteritis nodosa. This and other similar designations such as polyarteritis nodosa (PAN) were subsequently used as diagnostic terms for an increasing number of different vasculitic conditions. The separation into specific subgroups of vasculitides was started by Wegener [2] in 1939. In 1948, a microscopic form of polyarteritis was described by Davson *et al* [3] who suggested that segmental necrotising glomerulonephritis was a feature of this form of the disease. In 1952, Zeek [4] pointed out that the term PAN had gradually come to encompass a wide variety of vascular conditions and proposed a classification for necrotising angiitides in which PAN was separated from other forms. Although many authors adopted this terminology, most continued to include glomerulonephritis as a manifestation of PAN. The relationship between ANCA and small vessel vasculitis was first described by van der Woude *et al* [5] in 1985, but ANCA have not been included in any classification criteria until recently in the EMEA classification [6].

ACR classification criteria

In 1990 the American College of Rheumatology (ACR) published classification criteria for Wegener's granulomatosis, polyarteritis nodosa, Churg-Strauss syndrome, hypersensitivity vasculitis, Henoch-Schönlein purpura, giant cell arteritis and Takayasu arteritis [7]. These classification criteria were developed and validated on the basis of data from 1020 consecutive patients from 48 centres in the United

Table 1. (A) The 1990 ACR criteria for the classification of polyarteritis nodosa [148]. For classification purposes, a patient shall be said to have polyarteritis nodosa if at least 3 of these 10 criteria are present.

	Criterion	Definition
1	Weight loss ≥4 kg	Loss of 4 kg or more of body weight since illness began, not due to dieting or other factors
2	Livedo reticularis	Mottled reticular pattern over the skin of portions of the extremities or torso
3	Testicular pain or tenderness	Pain or tenderness of the testicles, not due to infection, trauma or other causes
4	Myalgias, weakness, or leg tenderness	Diffuse myalgias (excluding shoulder and hip girdle) or weakness of muscles or tenderness of leg muscles
5	Mononeuropathy or polyneuropathy	Development of mononeuropathy, multiple mononeuropathies, or polyneuropathy
6	Diastolic BP >90 mm Hg	Development of hypertension with the diastolic BP higher than 90 mm Hg
7	Elevated BUN or creatinine	Elevation of BUN >40mg/dl or creatinine >1.5mg/dl, not due to dehydration or obstruction
8	Hepatitis B virus	Presence of hepatitis B surface antigen or antibody in serum
9	Arteriographic abnormality	Arteriogram showing aneurysms or occlusions of the visceral arteries, not due to arteriosclerosis, fibromuscular dysplasia, or other inflammatory causes
10	Biopsy of small or medium-sized artery containing PMN	Histological changes showing the presence of granulocytes or granulocytes and mononuclear leukocytes in the artery wall

BP=blood pressure; BUN=blood urea nitrogen; PMN=polymorph neutrophil

(B) The 1990 ACR criteria for the classification of Wegener's granulomatosis [149]. For classification purposes, a patient shall be said to have Wegener's granulomatosis if at least 2 of these 4 criteria are present.

	Criterion	Definition
1	Nasal or oral inflammation	Development of painful or painless oral ulcers or bloody nasal discharge
2	Abnormal chest radiograph	Chest radiograph showing the presence of nodules, fixed infiltrates, or cavities
3	Urinary sediment	Microhaematuria (>5 red blood cells per high power field) or red cell casts in urine sediment
4	Granulomatous inflammation on biopsy	Histological changes showing granulomatous inflammation within the wall of an artery or in the perivascular or extravascular area (artery or arteriole)

States, Canada and Mexico with a fresh diagnosis of vasculitis. The participating rheumatologists provided each patient's diagnosis, information on symptoms and relevant laboratory, biopsy and angiographic data. Thus, the participating rheumatologists' diagnoses served as a gold standard in developing the ACR criteria. Furthermore, they were developed as classification criteria for research and not for diagnostic purposes and they do not include microscopic polyangiitis (MPA) or ANCA. Table 1 shows the ACR criteria for WG and PAN. For PAN one of the classification criteria is the presence of Hepatitis B virus (HBV) surface antigen or antibody in serum, a fact that should indicate that the diagnosis is secondary, and not primary, vasculitis. Causal therapy of hepatitis-associated PAN with antiviral drugs usually results in a benign clinical course.

CHCC nomenclature

In 1992 a committee of clinicians and pathologists from different countries and multiple medical disciplines convened for the Chapel Hill Consensus Conference on the Nomenclature of Systemic Vasculitis (CHCC) [8]. The goals of the conference were to reach consensus on the names for some of the most common forms of non-infectious systemic vasculitides and to construct root definitions for the vasculitides so named. The vasculitides were grouped according to the size of the affected blood vessels, always referring to the smallest vessel involved, into three groups; large vessel vasculitis, medium-sized vessel vasculitis and small vessel vasculitis (Table 2). Patients with small vessel vasculitis may have involvement of larger vessels, but not the contrary. With this nomenclature PAN is restricted to disease in which there is arteritis in medium-sized and small arteries without the involvement of smaller vessels, and patients with vasculitis affecting smaller vessels, e.g. with glomerulonephritis, are excluded from this diagnostic category. Although the importance of ANCA was recognised, these antibodies were not included in the definitions. The CHCC nomenclature was rapidly and widely accepted and is now applied in most studies on systemic vasculitis. However, the CHCC definitions were not intended as classification criteria.

Table 2. The CHCC nomenclature of systemic vasculitides.

Large vessel vasculitis
Giant cell (temporal) arteritis
Takayasu arteritis
Medium-sized vessel vasculitis
Polyarteritis nodosa
Kawasaki disease
Small vessel vasculitis
Wegener's granulomatosis
Churg-Strauss syndrome
Microscopic polyangiitis
Henoch-Schönlein purpura
Essential cryoglobulinaemic vasculitis
Cutaneous leukocytoclastic vasculitis

EMEA algorithm for classification

Recently a group of clinicians from multiple medical disciplines met at the European Medicines Agency (EMEA) and developed a stepwise algorithm for the classification of the ANCA-associated vasculitides (AAV) and PAN for epidemiological studies [6]. Their aim was to develop a consensus on the application of the CHCC definitions and the ACR criteria for the AAV and PAN, which could facilitate the comparison of epidemiological data. Clear entry criteria are required prior to use of the algorithm, avoiding inclusion of patients with other conditions. The algorithm incorporates ANCA and surrogate markers for vasculitis, e.g. mononeuritis multiplex shown by neurophysiologic tests for PAN or X-ray evidence of fixed pulmonary infiltrates, nodules or cavitations for WG. The stepwise algorithm is shown in Figure 1. For final validation the algorithm was tested using 80 paper cases and there was good agreement between the classification produced by the algorithm and the clinical diagnosis.

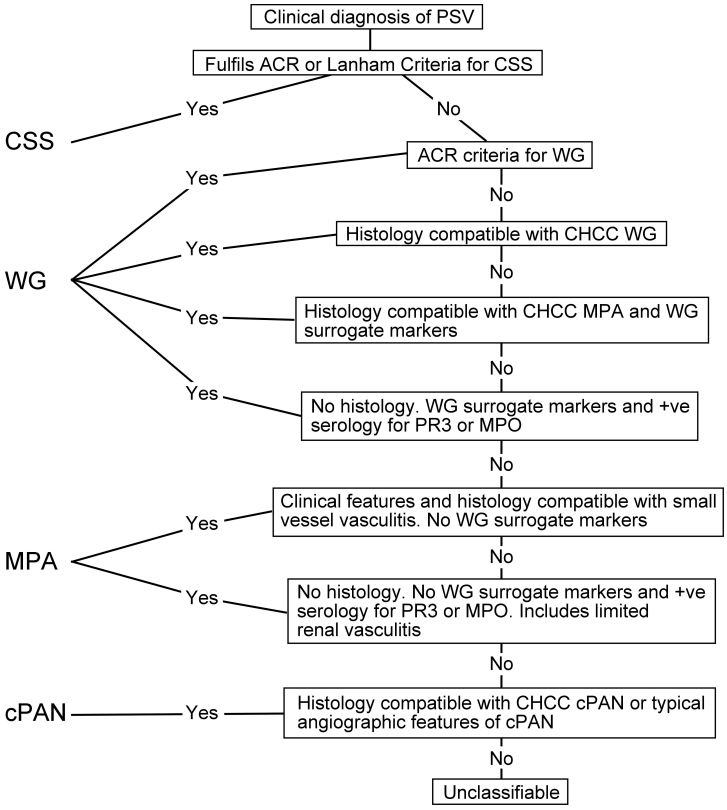

Figure 1. The stepwise EMEA algorithm. Prior to entering the algorithm the patient must fulfil the following entry criteria: (1) symptoms and signs compatible with a diagnosis of ANCA associated vasculitis or PAN, (2) histological, serological or imaging proof of vasculitis or eosinophilia, and (3) no other diagnosis to account for symptoms and signs (with special reference to malignancy and infection). PSV=primary systemic vasculitis; CSS=Churg-Strauss syndrome; cPAN=classical PAN

Diagnosis of systemic vasculitis

Early diagnosis is of paramount importance since the vasculitides often have a progressive course that rapidly results in an increased extent and severity of organ involvement. First the suspicion has to be raised and then the diagnosis must be confirmed. There is no international consensus on how this should be done, but many clinicians already work as it is proposed by the EMEA algorithm [6]. The clinical picture has to be compatible with a diagnosis of vasculitis and other causes of the symptoms, e.g. malignancy or infection, have to be ruled out. It is always desirable to have a histological confirmation of vasculitis. However, usually it takes some time to get the tissue biopsy done and it is not even always possible to obtain a biopsy. When the patient has ANCA and surrogate markers of vasculitis the probability of a diagnosis of small vessel vasculitis is reasonably high. If a patient has a fulminant course of his symptoms and biopsy is not immediately available, but

the symptoms are compatible with vasculitis, if other causes are ruled out and if ANCA are positive, the treatment with immunosuppressive drugs should not be delayed, although one should always strive to attain a histological confirmation.

Clinical characteristics at the time of diagnosis in polyarteritis nodosa, Wegener's granulomatosis and microscopic polyangiitis

Polyarteritis nodosa

Since Kussmaul and Maier described periarteritis nodosa in 1866 [1] many studies have reported on PAN. As stated above PAN is restricted to arteritis in medium-sized and small arteries without involvement of smaller vessels. However, although the CHCC definitions were widely accepted most studies still abide by the ACR criteria, including secondary forms of vasculitis, e.g. HBV associated vasculitis, and patients with small vessel vasculitis. Studies strictly adhering to the CHCC definitions are scarce and therefore the data presented in this section are based on studies applying the old definitions to large extent.

Most information about PAN from recent decades originates from the French Vasculitis Study Group (FVSG). From their reports PAN is a disease without sex preference that most often starts between 40 and 60 years of age [9-11]. Clinical studies with the old definitions report that PAN typically develops subacutely, with the onset of constitutional symptoms over weeks to months, but acute onset is seen in a minority of patients. The peripheral nervous system is the most frequently involved organ system and symptoms from the peripheral nerves are found in 20-75% of the patients [12-15]. Sometimes these symptoms are interpreted as a primarily neurological disease, but mononeuritis multiplex, which is typical of vasculitis, shows a characteristic picture on electromyography. Gastrointestinal involvement is also commonly reported, affecting 31-60% of the patients [12-15], and can be life-threatening. Involvement of the abdominal arteries can be demonstrated by angiography showing multiple aneurysms and irregular constrictions. The frequency of renal involvement is reported to be 20-44%, cutaneous involvement 28-46% and cardiac involvement 17-40% [12-15]. Myalgia is common and may be part of the constitutional symptoms or be due to vasculitis in muscular arteries. In epidemiological studies strictly adhering to the CHCC nomenclature the annual incidence of PAN is only 0-2 per million [16].

Wegener's granulomatosis

Wegener's granulomatosis was initially reported by Klinger in 1931 and later described in more detail as "rhinogenic granulomatosis" by Friedrich Wegener [2] in 1939. In a generalised stage it was clinically and anatomically characterised by a triad: a granulomatous-necrotising and ulcerative inflammatory process of the respiratory tract and the internal organs; a necrotising-granulomatous general vasculitis and a usually focal, necrotising glomerulonephritis.

The clinical picture can vary from a slow and mild course, with nasal congestion and crusting, to a more fulminant picture with respiratory or renal insufficiency.

Typically the upper or lower airways and the kidneys are affected. The frequency of different organ involvement at the time of diagnosis varies depending on the department at which the study is carried out, e.g. higher frequencies of renal involvement are reported from renal departments. Some patients have a history extending over many years of symptoms from the upper airways before the diagnosis is confirmed, but subsequently many of them develop renal or lung involvement [17]. The frequency of ear, nose and throat (ENT) involvement at the time of diagnosis is reported to be 55-85%, renal involvement 18-100%, lung involvement 31-70% and eye involvement 15-53% [17-21]. The signs and symptoms from these organ systems can vary. Renal involvement is usually not perceived by the patient until it is severe enough to produce uraemic symptoms, but it can be found early on a urinary dipstick test for haematuria and proteinuria. Microscopic analysis of urinary sediment can reveal erythrocytes, granular casts and erythrocyte casts as signs of glomerulonephritis. ENT involvement can give rise to a wide range of symptoms, e.g. rhinorrhoea, nasal congestion, purulent or bloody nasal discharge, sinus pain, hoarseness or hearing loss. Patients with these symptoms are often treated repeatedly with antibiotics before the suspicion of WG is raised. Lung involvement, which presents with cough, haemoptysis or pleuritis, can often be misjudged as pneumonia or even malignancy. On X-ray examination granulomas or infiltrates can be seen also in many asymptomatic patients [17]. Ocular manifestations often produce significant symptoms, sometimes with proptosis and pain.

Musculoskeletal involvement is frequent [17,18] with most patients experiencing arthralgia or myalgia, but deformity of the joints is not a feature of systemic vasculitis. Neurological involvement is rare at initial presentation [17-19], but a few patients develop mononeuritis multiplex or central nervous system abnormalities. Other organ systems rarely involved are the gastrointestinal system, the heart, the skin and the mucous membranes [17-19]. It is uncommon to find involvement of other organ systems, but occasionally granulomas can be found in the parotid gland, pulmonary artery, breast, urethra, cervix or vagina [17].

The annual incidence is 4.9-12 per million [16,22]. The median age at diagnosis is reported to be between 50 and 62 years of age in different studies [18-21,23], and older patients have been shown to present with more severe symptoms [24]. There is a 58-73% male predominance [18,19,21,23].

Microscopic polyangiitis

Davson *et al* described a microscopic form of polyarteritis in 1948 and to distinguish it from PAN suggested that segmental necrotising glomerulonephritis was a feature of this disease [3]. According to the CHCC nomenclature microscopic polyangiitis is a necrotising vasculitis affecting small vessels, e.g. capillaries, venules or arterioles, commonly involving the kidneys and lungs. Similarly to Wegener's granulomatosis, MPA affects small vessels, and these diseases can be difficult to discriminate from each other if no granulomas are found on histological examination. In WG a prominent feature is chronic nasal inflammation, but studies on MPA report frequencies of ear, nose and throat (ENT) symptoms ranging from 0% to 30% [18-20,25].

This is probably due to disparate ways of differentiating these two diseases from each other. Glomerulonephritis is found in 79-92% of the patients at the time of diagnosis [19,25,26], and renal insufficiency can develop rapidly. The percentage of patients with pulmonary involvement varies considerably between different studies, e.g. in Guillevin's study only 25% had pulmonary involvement [25], while Pavone *et al* could show that 63% of the patients in their study had pulmonary haemorrhage or infiltrates [26]. Respiratory insufficiency at the time of diagnosis is rare but can be fatal. Eye involvement is uncommon, only 4-12% of the patients presenting with this feature [18,19,25]. Neurological, gastrointestinal, cardiac and skin involvement are also rare [18-20,26]. Myalgia can be part of an influenza-like illness with malaise, fever and weight loss that frequently precede other symptoms.

The annual incidence is 3-7.9 per million [16,22]. The median age at diagnosis is the same as in WG, 53-68 years [18-21,26], and MPA also has a male preponderance varying between 55 and 68% [18,19,21,25].

Histological features of polyarteritis nodosa, Wegener's granulomatosis and microscopic polyangiitis

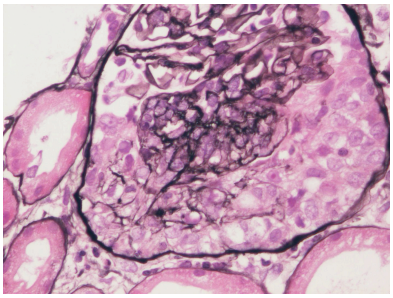

Figure 2. Renal biopsy with crescent formation (Photo Ingela Skogvall)

Histological examinations show focal segmental vasculitis with fibrinoid necrosis of the vessel walls accompanied by mural and perivascular infiltration of leukocytes [27]. Neutrophils predominate in the early lesions and are replaced by mononuclear leukocytes in older lesions. With disease progression fibrosis and sclerosis develop. In small vessel vasculitis a typical glomerulonephritis with segmental fibrinoid necrosis and crescent formation is frequent (Figure 2). Glomerular basement membranes and Bowman's capsule are often disrupted in areas of necrosis. By immunohistology, the glomeruli typically have no or only scanty deposits of immunoglobulin [28]. In WG the most characteristic respiratory tract lesion is irregular focal necrotising granulomatous inflammation with admixed neutrophils [27]. These lesions contain scattered multinucleated giant cells but most of the injured tissue contains non-specific infiltrates of neutrophils, eosinophils and mononuclear leukocytes. It is important to get a tissue biopsy from the site of active disease since these diseases appear focally and a "blind" biopsy is not likely to yield diagnostic material.

Anti-neutrophil cytoplasmic antibodies

Definitions

Anti-neutrophil cytoplasmic antibodies (ANCA) are a family of autoantibodies against neutrophil antigens. ANCA can be detected by different detection methods.

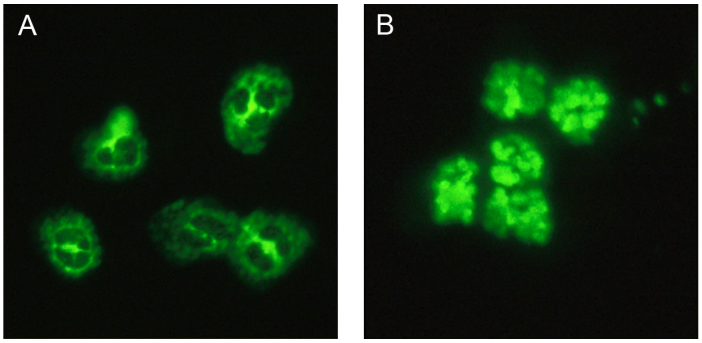

Figure 3. Indirect immunofluorescence on ethanol fixed neutrophils: (A) c-ANCA pattern and (B) p-ANCA pattern. (Photo Jörgen Wieslander)

In indirect immunofluorescence (IIF) using ethanol fixed human neutrophils the patterns significant for systemic vasculitis are the cytoplasmic pattern (c-ANCA) and the perinuclear pattern (p-ANCA) (Figure 3). These patterns are mainly caused by antibodies against proteinase 3 (PR3) and myeloperoxidase (MPO), but since whole neutrophils are used, antibodies against other neutrophil antigens can also yield these patterns, as shown in Figure 4. This is why the designation of the patterns is used: c-ANCA and p-ANCA. When detecting ANCA by enzyme-linked immunosorbent assay (ELISA) one antigen at a time is used, i.e. when testing for PR3-ANCA only PR3 is bound to the ELISA microtitre plates, hence the name of the antigen is used together with ANCA, e.g. PR3-ANCA.

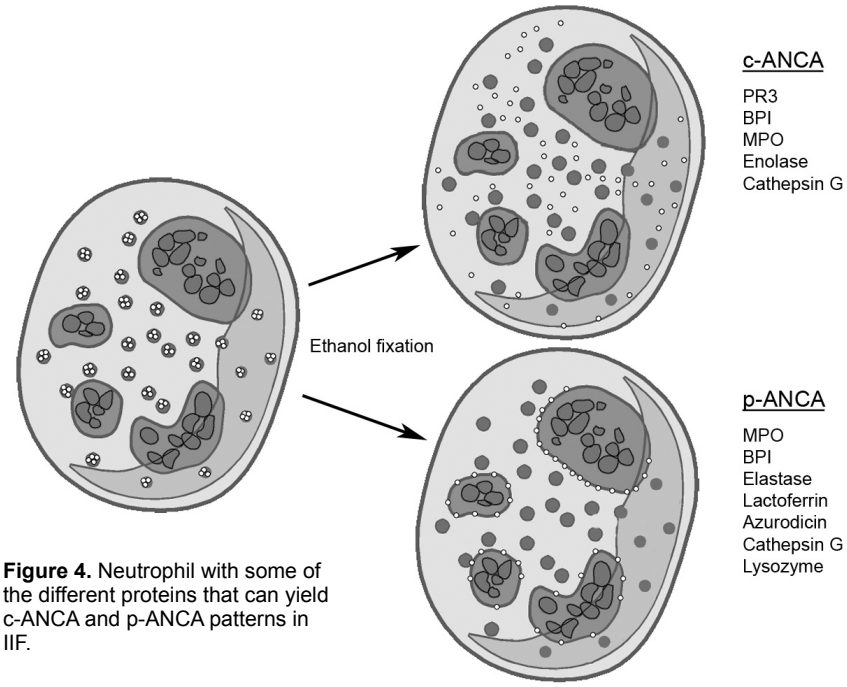

Figure 4. Neutrophil with some of the different proteins that can yield c-ANCA and p-ANCA patterns in IIF.

The history of PR3-ANCA

The first step towards ANCA testing can be traced back to 1959 when Calabresi *et al* first used fluorescein conjugated rabbit anti-human IgG to detect anti-neutrophil cytoplasmic antibodies in ethanol fixed human neutrophils [29]. In 1964 Faber *et al* demonstrated an antinuclear factor specific for leukocytes [30] and in 1972 Wiik *et al* described a technique for detecting granulocyte-specific antinuclear antibodies (GS-ANA) with ethanol fixed granulocytes [31]. These antibodies were specific for neutrophil granulocytes and were not found to react with lymphocytes or other cells. At that time interest was focused on RA and SLE and these autoantibodies were found mainly in patients with Felty´s syndrome. In 1985 van der Woude *et al* linked these antibodies to Wegener's granulomatosis and stated that the titres are related to disease activity in these patients [5]. Three years earlier it had been shown that antineutrophil antibodies occur in patients with segmental necrotising glomerulonephritis, but in that report it was suggested that the causative agent was an arbovirus [32]. After van der Woude's report the interest in these autoantibodies rose markedly and the search for the antigen began.

In 1987 Lockwood *et al* proposed that the antigen was alkaline phosphatase [33], but this was questioned by other groups in the same year [34-36]. Savage *et al* used crude acid extract of neutrophils as a source of antigen in a radioimmunoassay and this was recognised by sera from patients with WG and MPA but it also gave a substantial number of false positive results [37]. By first stimulating neutrophils with phorbol ester, to induce degranulation, and to affinity-purify the antigen from the supernatants, in an immunoabsorbent column with ANCA positive serum from a patient with WG, a more specific ELISA was created [38].

During the "1st International workshop on ANCA" in January 1988 ANCA was defined by the recognition of an uneven, granular staining of the cytoplasm of neutrophils and some monocytes, but not lymphocytes, in standardised IIF using smeared or cytospun healthy, human leukocytes fixed in alcohol [39]. These antibodies were distinguished from a perinuclear staining pattern (p-ANCA) which was caused by antibodies against MPO [40]. A standardised IIF technique was delineated by Wiik [41] and adapted as the reference in studies of ANCA. Soon after this, at the "2nd International ANCA Workshop" in 1989, the nomenclature adopted was to use c-ANCA for the antibodies giving a cytoplasmic pattern and p-ANCA for the antibodies giving a perinuclear staining pattern by IIF [42].

In 1989 it was shown that c-ANCA from patients with WG bound to a 29 kDa serine protease, different from elastase and cathepsin G, present in the azurophilic α-granules of human neutrophils [43-45]. It was speculated that this antigen might be PR3, and Niles *et al* presented a preliminary amino-terminal sequence of mature PR3 [44]. This was confirmed by Jennette *et al* who used purified PR3 and monoclonal antibodies specific for PR3 to show that the neutrophil proteinase with which c-ANCA reacted was indeed PR3 [46].

Proteinase 3

Proteinase 3 (also called p29, NP4, myeloblastin and AGP-7) is a serine protease with a molecular weight of 29 kDa [47]. PR3 is contained in the azurophilic granules of human neutrophils together with other serine proteases: e.g. human leukocyte elastase (HLE) and cathepsin G. These granules are acquired during the promyelocyte stage of myeloid differentiation. PR3 can also be found in secretory vesicles and specific granules [48], as well as in the granules of monocytes [49].

PR3 is synthesised as a pre-proenzyme with a signal sequence directing the translated protein into the endoplasmic reticulum, and an amino-terminal dipropeptide, consisting of alanine and glutamic acid, which is cleaved off by dipeptidyl peptidase-1 to generate an active enzyme [50]. Following removal of the activation dipeptide the amino-terminal folds into the hydrophobic interior of the molecule generating the mature enzyme [51]. A carboxy-terminal septapeptide is also cleaved off before PR3 becomes a fully matured protein of 222 amino acids, but the physiological significance and effects of carboxy-terminal processing remain unknown.

PR3 has four intramolecular disulfide bonds and is a highly folded protein [47]. It has been shown that both potential aspargine-linked glycosylation sites on PR3 are glycosylated [52,53]. The catalytic site is made up of three amino acids: histidine at position 44, aspartic acid at position 91 and serine at position 176 [47]. PR3 can degrade a variety of matrix proteins including elastin, fibronectin, laminin, vitronectin and collagen type IV, and the most important physiological inhibitor is α1-antitrypsine [54].

ELISA for ANCA detection

IIF is a detection method which requires skilled technicians for interpretation of the immunofluorescence findings. Furthermore, c-ANCA and p-ANCA can be caused by other antigens than PR3 and MPO, which are the major autoantigens in small vessel vasculitis. In order to circumvent the problems involved in different interpretations more automatised assays for the detection of these autoantigens were developed. In direct ELISA the antigen is coated on a plastic microtitre plate and the

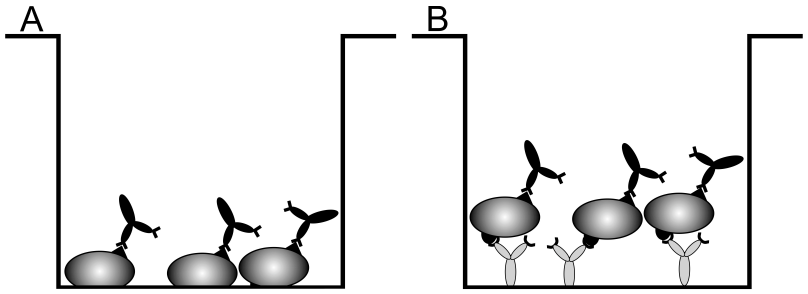

Figure 5. (A) In direct ELISA the antigen is coated directly on a plastic microtitre plate. (B) In capture ELISA the microtitre plate is first coated with a monoclonal antibody that captures the antigen and presents it to the patients' antibodies.

patient's serum or plasma added to allow reaction with the antigen (Figure 5). After washing, ANCA are detected by adding an enzyme-conjugated anti-human-IgG antibody and then adding a chromogen and measuring the colour development, which represents the amount of bound antibody, by optical density scanning of the plate.

In capture ELISA the plastic microtitre plate is first coated with a monoclonal antibody that captures the antigen and presents it to the patient's antibodies (Figure 5). The capturing antibody is thought to present the antigen in native conformation, rendering the epitopes on PR3 more readily accessible for ANCA. Detection of ANCA is performed as in direct ELISA.

In addition to the mentioned conventional assays a few other techniques have been developed for the detection of PR3-ANCA. Image analysis, which is based on the IIF technique, has been shown to be more sensitive than direct ELISA, but less sensitive than IIF and capture ELISA in the detection of relapses in patients with PR3-ANCA positive WG [55]. An ANCA / anti-GBM (anti-glomerular basement membrane antibodies) dot-blot was able to detect 92-95% of the PR3-ANCA, 80-86% of the MPO-ANCA and 100% of the anti-GBM antibodies previously detected by direct ELISA, and had a good specificity for PR3-ANCA [56]. It was concluded that it could be useful as a screening tool in situations where conventional ANCA testing is not readily available. A fully automated fluorescent-enzyme immunoassay (FEIA) was almost comparable to direct ELISA, but less sensitive than capture ELISA, in the detection of PR3-ANCA in patients with WG [57]. None of these assays were found to be superior to the conventional assays and they have not become established for a wider use.

PR3 for ELISA

Prior to the identification of PR3 as the major c-ANCA antigen, ELISA for c-ANCA was performed using a crude α-granule extract as the antigen [58]. This extract contained many different antigens, including substantial amounts of MPO, but for some reason most MPO-ANCA did not react in ELISA with this extract. For the isolation of PR3 large amounts of neutrophils are needed and an alternative source of neutrophils was examined by Ballieux *et al* who used purulent sputum from patients with cystic fibrosis and chronic bronchitis [59]. From these neutrophils a PR3-α1antitrypsin complex as well as noncomplexed PR3 was isolated that was recognised by c-ANCA positive WG sera but not by sera from healthy controls. However, this technique did not come into clinical use, which presumably was due to problems of obtaining sputum in sufficient amounts.

The importance of the form of antigen used was highlighted early by an international cooperative study group which compared ANCA assays in different laboratories [60]. The IIF test, although performed with different methods in 7 laboratories, showed comparable results, but the solid phase assays for PR3-ANCA detection were not well standardised. The best results were obtained with highly purified PR3. The purification process of PR3 from neutrophils is laborious and the yield of PR3 is

low. Therefore, several attempts have been made to express recombinant PR3 that is recognised by PR3-ANCA and can be produced in large amounts. This has proved to be difficult since the epitopes for PR3-ANCA are easily destroyed by denaturation [61]. Thus, an assay for PR3-ANCA detection requires the recombinant PR3 to have been produced with the correct three-dimensional structure. Recombinant PR3 synthesised by wheat germ ribosomes in vitro, and not posttranslationally modified, is not recognised by c-ANCA [61]. Recombinant PR3 expressed in the prokaryotic E. coli [61,62] or in the eukaryotic yeast Pichia pastoris [62] is only recognised by a small percentage of PR3-ANCA positive sera. For recombinant PR3 expressed in insect cells using the baculovirus expression system conflicting results have been obtained [63-65]. Eukaryotic cell systems have proved to be better for this purpose. PR3-ANCA from patients with WG react with recombinant PR3 expressed in the rat basophilic mast cell line RBL-1 and the murine myeloblast-like cell line 32D cl3 [52] as well as PR3 expressed in the human mast cell line HMC-1 [66] and the human epithelial cell line 293 (HEK 293) [67].

Modifications of the N-terminal residues have consequences for protein folding, antigenic properties, zymogen activation and subcellular routing and PR3-ANCA from some patients only react with PR3 after cleavage of its N-terminal activation dipeptide [64,67]. The C-terminal extension appears to be unimportant for activity, intracellular sorting and interaction with target substrates. Recombinant PR3 with a C-terminal c-myc extension used in a capture ELISA is recognised by a majority of PR3-ANCA positive sera [68].

Epitope mapping of PR3-ANCA

Several studies have been carried out to map the epitopes recognised by the PR3-ANCA. Synthetic peptides produced in an overlapping fashion spanning the whole PR3 molecule have been produced by several groups in order to find linear epitopes for PR3-ANCA [69-72]. Sera from patients with WG and MPA have been shown to react more strongly with some of these peptides in ELISA, but in three of the studies the same peptides were also recognised by control sera [70-72]. The failure of these attempts is probably due to the fact that the majority of PR3-ANCA recognise conformational epitopes on PR3 [61]. It has been shown that antibody binding is inhibited by exposure of PR3 to low pH, by reducing disulphide bonds or by boiling PR3 in SDS [61,72]. All these procedures change the conformation of PR3 and thereby destroy epitopes recognised by PR3-ANCA.

In order to investigate epitope specificities of PR3-ANCA monoclonal antibodies (mabs) against PR3 have been generated by several groups [73-75]. The principal mabs used in this thesis were generated by Sommarin *et al* [73]. Mice of the BALB/c strain were immunised with an extract of human neutrophil α-granules, spleen cells from a good responder were fused with Sp2/O cells and hybridomas were generated. Clones were screened using PR3 ELISA and three IgG class clones, designated 4A3, 4A5 and 6A6, were selected and subcloned. These mabs were tested in pairs in competition experiments and found to recognise different epitopes on PR3. It was also shown that the mabs could partially inhibit PR3-ANCA binding to PR3, but a

third of the patient sera were not inhibited by any of the mabs. This showed that the autoimmune response to PR3 is directed against several epitopes.

Competition studies indicate that PR3-ANCA from patients with WG are directed against a restricted number of epitopes on PR3 [69,76]. It has also been implied that epitope shift occurs in some patients with WG, but the study was small and the results have yet to be confirmed [76].

Monoclonal antibodies for capture ELISA

The mabs generated by Sommarin *et al* [73] were used in a study by Baslund *et al* to develop a capture ELISA for the detection of PR3-ANCA [77]. This assay was found to be more sensitive than direct ELISA when the capture antibodies were chosen carefully to avoid competition with antigenic epitopes of primary importance for human autoantibody recognition. Several other groups have also developed capture assays for the detection of PR3-ANCA and Csernok *et al* have evaluated different capture ELISAs in a multicentre study [78]. They found that most capture ELISAs successfully detected PR3-ANCA, but there were significant differences in the IIF and direct ELISA results between laboratories.

ANCA assays for diagnosis of vasculitis

IIF and ELISA are the most common assays adopted for ANCA detection clinically. Many studies have been performed to analyse the sensitivity and specificity of different detection methods. One large study was performed by the European Commission/Measurement and Testing (EC/BCR) project for ANCA assay standardisation [79]. In this study 14 centres participated with 358 patients with small vessel vasculitis (WG, MPA, Churg-Strauss syndrome and idiopathic rapidly progressive glomerulonephritis), 184 disease controls and 740 healthy controls. Patients were included on clinical grounds, not on the basis of ANCA results, and all patients were subsequently classified centrally, using the names and definitions adopted from the CHCC nomenclature. The results from different assays are summarised in Table 3. Combined sensitivity for IIF in newly diagnosed WG was 85% and in MPA 81%, and specificity for this combination was 76% towards disease controls and 94% towards healthy controls. The sensitivity in the ELISAs was somewhat better, but the conclusion from this study was that by combining IIF and ELISA results, either positive c-ANCA plus positive PR3-ANCA or positive p-ANCA plus positive MPO-ANCA, a higher specificity was achieved than by either assay in isolation.

Other studies have shown similar results. Rao *et al* performed a literature review and meta-analysis of 15 studies and found the sensitivities of c-ANCA testing for overall WG to range from 34% to 92% and the specificities ranged from 88% to 100% [80]. They found the sensitivity of c-ANCA to be higher in active disease (91%) than in inactive disease (63%). Unfortunately many other studies do not differentiate between active and inactive disease, which makes it difficult to interpret the results. In a large study from Baltimore 856 consecutive patients tested prospectively for ANCA by IIF were included [81]. Each patient's underlying diagnosis was based

without regard to their ANCA status. The positive predictive value (PPV) of ELISA for AAV was superior to that of IIF, 83% versus 45%. When combining both tests the PPV increased to 88%.

Table 3. Results from the EC/BCR project for ANCA assay standardisation. Comparison between IIF and ELISA results in patients with newly diagnosed small vessel vasculitis (97 WG, 44 MPA), 184 disease controls and 740 healthy controls. Results are shown as percent of patients positive in each assay.

	IIF		ELISA	
	c-ANCA	p-ANCA	PR3-ANCA*	MPO-ANCA
WG	64%	21%	65-67%	24%
MPA	23%	58%	25-27%	58%
Disease controls	4%	19%	11-14%	9%
Healthy controls	2%	4%	1-2%	4%

*3 different PR3-ANCA ELISAs were compared

To determine the prevalence of ANCA in patients with connective tissue disease a large study was performed in Boston, Massachusetts [82]. Serum from 463 patients with connective tissue disease (RA, SLE, scleroderma, inflammatory myositis, Sjögren syndrome, antiphospholipid syndrome and early undifferentiated connective tissue disease) drawn within a year of the onset of symptoms and control sera from 200 blood donors were analysed in both IIF and ELISA. c-ANCA was not detected in any study or control patient, while p-ANCA was commonly detected among patients with SLE (31%), and p-ANCA was associated with antinuclear antibodies in all cases. PR3-ANCA was detected in 9 patients and MPO-ANCA in 2 patients. When a combination of IIF and ELISA was used the test specificity for vasculitis was 99.5% among patients with connective tissue disease.

An international consensus statement on testing and reporting of ANCA was formulated at a symposium on ANCA and vasculitis [83]. This is based on the opinions of the participants and recommends that for ANCA testing in all “new” patients IIF must be performed and serum samples containing ANCA should then be tested in ELISAs for PR3-ANCA and MPO-ANCA.

The performance of assays varies significantly. In one study 4 test sera were tested in 11 different laboratories according to individual laboratory protocols [84]. All test sera were positive for ANCA by IIF in each of the 11 laboratories, but the observed IIF patterns were inconsistent with the consensus patterns in 8 of 43 assays (each from a different laboratory). The variations in ANCA IIF patterns did not necessarily result from the misinterpretation of patterns, but may have resulted from other, probably technical, factors. In another study seven PR3-ANCA ELISA kits were evaluated and in 25 c-ANCA positive sera from patients with WG the PR3-ANCA positivity ranged from 44% to 84% [85]. An absolute concordance among the 7 kits

was noted only in 56% of the c-ANCA positive samples. These studies demonstrate the importance of development and standardisation of detection methods.

Russell *et al* compared the performance of IIF, direct ELISAs for PR3- and MPO-ANCA and capture PR3-ANCA ELISA using 615 consecutive serum samples from patients undergoing evaluation for suspected vasculitis or autoimmune disease [86]. 86 patients had WG or MPA, 118 had other autoimmune diseases and the remaining 411 carried other diagnoses. Capture PR3-ANCA ELISA using recombinant PR3 and the monoclonal antibody MCPR3-2 was positive in 55% of the 23 patients with active WG compared to 58% in direct PR3-ANCA ELISA and 61% in c-ANCA IIF. The highest specificity for WG/MPA was achieved when a positive capture PR3-ANCA ELISA result coincided with a positive c-ANCA by IIF (99.8%). In another study serum samples drawn at the time of diagnosis from 92 patients with WG or MPA, 30 disease controls (SLE, RA) and 30 healthy controls where tested in IIF and direct and capture ELISAs for PR3- and MPO-ANCA [87]. Unfortunately the results from the capture ELISAs were lumped together and the only conclusion about them is that they performed better than the direct ELISAs.

In capture ELISA the sensitivity and specificity depends on the monoclonal antibody that is used to capture the antigen. The capturing antibody has to be carefully chosen and tested so as not to interfere with the binding of PR3-ANCA to PR3. In the study by Baslund *et al* [77] different monoclonal antibodies were analysed and the antibody giving the best sensitivity of the capture ELISA was chosen. In this way capture PR3-ANCA ELISA was found to be more sensitive than direct PR3-ANCA ELISA and IIF and thus the capture assay was recommended as a screening method for PR3-ANCA. To our knowledge, this is the only study evaluating the screening method of choice for PR3-ANCA.

Studies show that ANCA can be detected in many other conditions than vasculitis, e.g. connective tissue disease [82], pulmonary disease [88], human immunodeficiency virus, infections in cystic fibrosis, invasive amoebiasis, endocarditis, leprosy, tuberculosis and cocaine-induced nasal destructions [89-95]. This emphasise the fact that the diagnostic value of ANCA testing depends on the clinical setting. In a patient with low probability of vasculitis a positive ANCA test is not very helpful for differential diagnosis, while in a patient with signs of glomerulonephritis and pulmonary haemorrhage a positive ANCA test can support the diagnosis. Still, no assay has yet proved to be sufficient for the diagnosis of vasculitis as a substitute for the histological examination of a tissue biopsy.

Prognosis of systemic vasculitis

The prognosis for patients with WG and MPA was extremely poor before the use of immunosuppressive treatment, and if these diseases were left untreated patient survival was only 20% at 1 year [96]. As a result of the introduction of therapy with cyclophosphamide and corticosteroids patient survival has improved to 80% at 5 years of follow-up [97].

Outcome in systemic vasculitis

The 5 year patient survival has been reported to be around 80% in PAN [9], but as mentioned above reports on PAN often include patients with Hepatitis B associated disease as well as small vessel vasculitis. Renal survival has not been studied in patients with PAN.

Studies on outcome in ANCA associated vasculitis have reported 5 year patient survival to be between 63% and 81% [19,23,26,98-106]. In one report 1 year survival was only 52%, but in that study all patients had severe renal failure with serum creatinine levels above 500 µmol/litre at diagnosis [107], and in another study with patients who were dialysis dependent at diagnosis patient survival was also only 52% over a median follow-up period of 34 months [108].

When analysing patient survival in studies which only include patients with WG 1 year survival is reported to be between 93% and 99% [22,23] and 5 year survival between 74% and 79% [23,100,105]. The 1 year patient survival in MPA is reported to be between 71% and 85% [19,24,103,109] and 5 year survival is commonly found to be around 74% [25,103], but was as low as 45% in one study [19].

Renal outcome in ANCA associated vasculitis depends on the study design. Most studies that have analysed renal survival have only included patients with renal involvement and then 5 year renal survival is reported to be between 65% and 76% [98,99,104,105].

Factors predicting prognosis in systemic vasculitis

There still is a considerable mortality and in addition many patients suffer from disease damage, e.g. renal insufficiency, with a reduced quality of life. Are some patients at a greater risk of a poor outcome and should different groups of patients have different treatment? In order to tailor treatment the prognosis of each patient must be known.

Polyarteritis nodosa

In 1996 Guillevin *et al* performed a study on 342 patients with polyarteritis nodosa and Churg-Strauss syndrome evaluating different parameters for predicting the outcome in patients with PAN [10]. In this study also patients with MPA and HBV associated PAN were included since the CHCC definitions were not applied. Five prognostic factors were found to predict excess mortality: renal insufficiency (serum creatinine ≥140 µmol/l); proteinuria (>1 g/day); severe gastrointestinal involvement; cardiomyopathy; and CNS involvement. The authors proposed that treatment in PAN and Churg-Strauss syndrome should be based on this Five Factor Score (FFS). The patient survival was 69% over the whole study period with a mean follow-up of 88 months. Studies from the same group have confirmed the prognostic significance of FFS in predicting mortality in a mixed group of patients with MPA, PAN without HBV, HBV associated PAN and Churg-Strauss syndrome [9]. However, the FFS has not been validated in studies with PAN diagnosed according to CHCC definitions or EMEA classification.

Table 4. Selection of studies on patient survival and mortality risk factors in small vessel vasculitis.

Author	Year	n	Diagnosis: WG/MPA (incl RLV)/ CSS	ANCA: PR3-ANCA or c-ANCA/ MPO-ANCA or p-ANCA/neg	Follow-up, months mean or median	Patient survival	Mortality risk factors (at diagnosis)
Franssen (113)	1995	92	Nd	46/46/0	Nd	2y 86% PR3 78% MPO	Age, serum creatinine
Segelmark (150)	1995	99	73/26	99/0/0	48	4y 80%	PiZ (heterozygosity for α1-antitrypsin deficiency)
Matteson (106)	1996	77	77/0	Nd	85	5y 75% of general population	Histologic granuloma formation
Hogan (114)	1996	107	0/107	39/68/0	30	85% over study period	pulmonary hemorrhage, cANCA vs pANCA
Westman (18)	1998	123	56/67	73/43/0	55	69% over study period	Age, Serum creatinine
Guillevin (25)	1999	85	0/85	33/5/13,	70	5y 74%	proteinuria >1g/d, FFS
Hedger (112)	2000	128	pi RPGN	46/33/0	22	1y 68%	Age, dialysisneed
Cohen (111)	2000	94	pi GN	27/20/14	33	87% over study period	Age, male sex, respiratory tract involvement
Aasarød (105)	2000	108	108/0	83/8/7,	42	5y 74%	age, low serum albumine
Reinhold-Keller (22)	2000	155	155/0	130/5/20	84	2y 97%	age>50, nephritis (normal creatinine), renal failure, lung involement
Mahr (110)	2001	49	49/0	37/4/8,	22	2y 68%	age>57, serum creatinine>18.1mg/dl
Koldingsnes (23)	2002	56	56/0	37*/6 *	56	5y 79%	age,dialysis dependency, presence of organ damage (VDI)
Slot (104)	2003	85	AASV w GN	85/0/0	Nd	5y 73%	age>65 compared to <51, male sex
Booth (103)	2003	264	83/153/11	245*/0 *	37	5y 76%	age>60, serum creatinine>200μmol/l
Weidner (102)	2004	80	32/48	43/31/6	47	5y 81%	PR3-ANCA compared to MPO-ANCA,age >68 vs <56, serum creatinine>582μmol/l vs <299μmol/l
Little (101)	2004	86	31/50/2 (+3 GP)	35/34/10	41	5y 63%	Karnofsky score (functional status)
Bligny (100)	2004	93	93/0	67/10/4	54	5y 74%	age >52, ENT involvement associated with longer survival
Rihova (99)	2005	61	33/24/3	30/22/9	90	5y 78%	age>60, dialysisdependency
Harper (24)	2005	229	81/148	218*/0 * (ANCA pos)	Nd	1y 71%	age, serum creatinine>400μmol/l
Lane (19)	2005	99	57/24/18	Nd	26	5y 66% (5y:WG 76%, MPA 46%, CSS 68%)	age >65 (with inceasing risk per year of age), less risk without ENT
Pavone (26)	2006	75	36/16/23	29/25/16	42	5y 78%	cerebral involvement, hepatic involvement, serum creatinine >132μmol/l
Bakoush (98)	2006	83	37/46	40/43/0	54/70	5y 77%	age, MPA, high urine IgM excretion

Nd=no data available; RLV=renal limited vasculitis; CSS=Churg-Strauss syndrome; AASV w GN=ANCA associated systemic vasculitis with glomerulonephritis; GP=Goodpastures disease; * = ANCA positive/negative

ANCA associated vasculitis

In ANCA associated systemic vasculitis prognostic factors have been evaluated in many studies. In Table 4, factors that are found to predict patient survival in selected studies are summarised. The most frequently found prognostic factor is age: the higher the age, the greater the risk of death [18,19,22-24,98-100,102-105,110-113].

In Table 5, factors that are reported to predict renal survival in selected studies are summarised. The most frequently reported prognostic factor is serum creatinine at diagnosis [18,102,103,113,114].

Table 5. Selection of studies on renal survival and renal risk factors in small vessel vasculitis.

Author	Year	n	Diagnosis: WG/MPA (incl RLV)/ CSS	ANCA: PR3-ANCA or c-ANCA/MPO-ANCA or p-ANCA/neg	Renal involv-ement (%)	Follow-up, months mean / median	Renal survival	Renal risk factors at diagnosis
Andrassy (151)	1991	25	25/0	21/0/1	100	Nd/36	80%	Percentage of obsolescent glomeruli, degree of tubulointerstitial lesions
Franssen (113)	1995	92	Nd	46/46/0	75	Nd	2y 67%	Serum creatinine
Hogan (114)	1996	107	0/107	39/68/0	100	30/Nd	57%	Serum creatinine, race (Afro-american), presence of arterial sclerosis on renal biopsy
Westman (18)	1998	123	56/67	73/43/0	100	Nd/55	78% in survivin g	Serum creatinine, PR3-ANCA in capture ELISA, low blood thrombocytes
Booth (103)	2003	264	83/153/11	92% ANCA pos	100	Nd/37	72%	Serum creatinine
Weidner (102)	2004	80	32/48	43/31/6	100	Nd/47	77%	Serum creatinine>582 µmol/l vs ≤299 µmol/l, WG
Rihova (99)	2005	61	33/24/3	30/22/9	100	Nd/90	5y 69%	No found
Harper (24)	2005	229	81/148	95% ANCA pos	100	Nd	Nd	Age
Bakoush (98)	2006	83	37/46	40/43/0	100	54/70	1y 84% 5y 76%	High urine IgM excretion
de Lind van Wijngaarden (152)	2006	100	33/67	100% ANCA pos	100	12* (study period)	Nd	Amount of neutrophils in interstitial infiltrate, abnormal glomeruli

Nd=no data available; RLV=renal limited vasculitis; CSS=Churg-Strauss syndrome

Relapses in systemic vasculitis

Systemic vasculitides are inflammatory diseases in which disease activity fluctuates. In the majority of patients the disease is brought into remission with immunosuppressive drugs and no obvious disease activity can be detected [24,26,115]. In these patients medications can be tapered and in some cases eventually eliminated. However, many patients sooner or later experience relapses, which mean return of

disease activity. In other patients complete remission can not be induced and a chronic grumbling inflammation can persist for years. In most patients with ANCA associated vasculitis ANCA disappear once stable remission has been induced, but some have high levels of ANCA without signs of disease activity. The significance of this is still obscure. Some data indicate that vasculitis patients never become entirely free from the disease. It has been shown that cytokine patterns are somewhat disturbed in patients with ANCA associated vasculitis, independent of disease activity, as compared to healthy controls [116,117].

Relapses are defined in various ways in different studies. A common definition is recurrence of vasculitic activity after a period of complete or partial clinical remission has been achieved. One way to measure disease activity is with the Birmingham Vasculitis Activity Score (BVAS) [118]. This was created to measure the disease activity in patients with a variety of systemic vasculitides and it scores abnormality ascribable to the presence of active vasculitis. However, in patients with grumbling disease it can be difficult to discern if new activity has arisen. It can also be difficult to differentiate disease activity from infection, e.g. in patients with WG and nasal symptoms. The best way to diagnose a relapse is to have a histological confirmation of vasculitic activity in a tissue biopsy. However, this is often not possible to obtain before start of treatment and other signs and symptoms are usually employed as markers for vasculitic activity.

Table 6. Summary of selected studies on relapses in patients with PR3-ANCA positive small vessel vasculitis. PR3-ANCA determinations by direct ELISA and capture ELISA.

Assay and author, year (reference)	No. of relapses/no. of patients with relapses	Positive at relapse[a]	Rise in assay value at relapse[a]	Rise in assay value without relapse[b]
Direct ELISA				
Pettersson et al 1992 (153)	7/5	6/7	3/4	2/5
Dolman et al 1993 (154)	8/8	6/8	6/8	NA
De'Oliviera et al 1995 (127)	23/18	20/23	10/20	5/15
Kyndt et al 1999 (155)	30/13	Nd	10/30	7/17
Boomsma et al 2000 (121)	33/33	33/33	27/33	11/38
Arranz et al 2001 (156)	8/5	3/8	3/8	NA
Gisslén et al 2002 (157)	29/10	29/29	23/29	2/25
Segelmark et al 2003 (122)	21/14	19/21	10/13	NA
Capture ELISA				
Arranz et al 2001 (156)	8/5	7/8	6/8	NA
Gisslén et al 2002 (157)	29/10	29/29	29/29	6/35
Segelmark et al 2003 (122)	21/14	21/21	13/13	NA

[a] Number of relapse incidents assay positive at relapse/total number of relapses. [b] Number of patients without a relapse but with a rise in assay value/total number of instances with a rise in assay value. Nd = No data available; NA = Not analysed

In patients with ANCA associated vasculitis serial ANCA measurements for the monitoring of disease activity are commonly used. Already in the first report linking ANCA to WG it was stated that ANCA levels are related to disease activity [5]. Since then many studies have been performed to examine the relationship between ANCA levels and disease activity [119-123]. Views range from the opinion that ANCA levels should guide immunosuppressive therapy directly, even in the absence of any clinical symptoms, to the opinion that serial measurements of ANCA are of no value for clinical management [121,124-129]. In Table 6, selected studies on relapses in PR3-ANCA positive patients are summarised. These studies are based on serial measurements of PR3-ANCA in direct ELISA and/or capture ELISA. In all but one of them the vast majority of patients were ANCA positive at the time of relapse. In most cases a clinical relapse was associated with a rise in assay value, but rises could also be seen that were not associated with relapse. Patients with persistently elevated ANCA levels were also noted in most of the studies. If pre-emptive therapy on the basis of fluctuations in ANCA levels had been used in all these studies many patients would have been given unnecessary immunosuppressive therapy with risk of severe side-effects.

Relapses were thought to be uncommon with frequencies below 20% in studies including patients with HBV associated PAN [9,15,130], but in reports with more strictly applied diagnostic criteria the frequency is approximately 50% 14. In WG the reported relapse rates are between 43% and 64% [17,22,23,100,105,110,131] and in MPA between 34% and 42% [25,115].

In some studies with patients with WG and MPA prognostic risk factors for relapse have been reported. ANCA type (PR3-ANCA/c-ANCA versus MPO-ANCA) [103,115,132], lung involvement [115], ENT involvement [115] and gastrointestinal involvement [26] have been associated with a higher relapse rate while renal involvement [26] has been associated with a lower relapse rate.

Aims of the present studies

The overall aim of the studies on which this thesis is based was to improve our knowledge about the diagnostic and prognostic significance of ANCA in systemic vasculitis.

To this end the following specific goals were defined:

- To evaluate the clinical significance of a capture PR3-ANCA ELISA as a sero-diagnostic tool for patients with small vessel vasculitis (I).
- To investigate patient survival in ANCA associated small vessel vasculitis with renal involvement and analyse possible prognostic factors for patient outcome (II).
- To describe the symptoms, signs and clinical course of a cohort of patients with PAN that fulfil the CHCC definition and that do not have ANCA (III).
- To construct recombinant chimeric molecules in order to map the epitopes of monoclonal antibodies to PR3. Also to explore the clinical utility of these chimeric proteins for the diagnosis and serial follow-up of patients with PR3-ANCA positive vasculitis (IV,V).

Materials and methods

Patients and sera

Paper I

In this paper 4 different patient series were analysed.

A. Sera from 80 patients having typical c-ANCA by IIF and 40 IIF-negative sera in a routine laboratory at Statens Serum Institut in Copenhagen, Denmark.

B. Consecutive sera from 82 patients who had positive PR3-ANCA in capture ELISA at Wieslab, Lund, Sweden. Clinical data were retrieved for all patients and patients with small vessel vasculitis were subgrouped according to the CHCC nomenclature and for WG the criteria established by ACR.

C. Sera from 52 patients with biopsy-proven glomerulonephritis without small vessel vasculitis at the Department of Nephrology at Lund University Hospital. 14 patients had endocapillary glomerulonephritis and 38 had mesangioproliferative glomerulonephritis.

D. Sera from 48 consecutive patients with WG with renal involvement at the Department of Nephrology at Lund University Hospital. The diagnosis of WG implied granulomatous inflammation in the respiratory tract either histologically proven or strongly indicated by non-invasive diagnostic procedures.

Paper II

A cohort of 117 consecutive patients with biopsy-confirmed renal involvement of WG or MPA admitted to the Department of Nephrology at Lund University Hospital between 1971 and 1993 were followed until 2000 or death. Serum samples were drawn at the time of diagnosis. Subclassification into WG and MPA was performed according to the CHCC nomenclature. For the diagnosis of WG, the requirement was for granulomatous inflammation to be either proven histologically or strongly indicated by a non-invasive procedure.

Paper III

10 patients with a diagnosis of PAN treated at the Departments of Nephrology and Rheumatology at Lund University Hospital between 1990 and 2002 were followed until 2003. Patients were included in the study if they had disease findings compatible with systemic vasculitis with histological evidence of vasculitis affecting small or medium-sized arteries and/or with angiographic features of multiple microaneurysms and multiple stenoses and/or with an electromyography typical for mononeuritis multiplex. Patients were excluded if there were any signs of small vessel vasculitis.

Paper IV

Sera from 3 patients with WG with biopsy-confirmed renal involvement at the Department of Nephrology at Lund University Hospital and sera from 3 healthy blood donors at the Blood Donor Centre at Lund University Hospital.

Paper V

A cohort of 38 patients with PR3-ANCA positive vasculitis, WG or MPA, diagnosed between 1990 and 2003, were followed at the Departments of Nephrology at the University Hospitals in Lund and Malmö until 2005. The cohort was generated in January 2003 and followed prospectively until December 2005. Serum samples were drawn at each out-patient visit. In addition older samples drawn for other research studies or for clinical purposes were retrieved.

Ethics

Studies were approved by the local Ethical Committee at the Faculty of Medicine, Lund University.

Measurements of disease activity, disease damage and prognostic score

Birmingham vasculitis activity score (BVAS)

The BVAS was created by a group in Birmingham in 1994 to measure disease activity in patients with a variety of systemic vasculitides [118]. It scores abnormality ascribable to the presence of active vasculitis and the maximum possible score is 63.

Vasculitis damage index (VDI)

VDI was created in 1997 to aid in the separation of damage from disease activity in systemic vasculitis [133]. The VDI scores damage due to non-healing scars from any cause that has occurred since the onset of vasculitis. It is a cumulative assessment of organ dysfunction, damage or scarring and either remains stable or increases with time. Sixty-four items of damage are scored.

Five factor score (FFS)

The FFS was created by Guillevin *et al* in 1996 by evaluating different parameters for predicting outcome in patients with PAN and Churg-Strauss syndrome [10]. The factors found to predict excess mortality in that study were renal insufficiency (serum creatinine ≥140 μmol/l), proteinuria (>1 g/day), severe gastrointestinal involvement, cardiomyopathy and CNS involvement.

Definition of relapses

Clinical relapse was defined as return of vasculitic activity after achieving remission.

For small vessel vasculitis in paper II at least one of the following signs was required: (i) rapid rise in serum creatinine (excluding other causes of deterioration of renal function); (ii) new appearance of pulmonary infiltrates or haemoptysis without evidence of infection or malignancy; (iii) iritis; (iv) mononeuritis multiplex or vasculitis of the central nervous system or (v) active necrotizing vasculitis identified in any biopsy specimen. In paper V a return of vasculitic activity to an extent yielding a BVAS of 4 was required.

For PAN in paper III an occurrence of one the following was required: (i) general symptoms (malaise, fever, weight loss, myalgias, arthralgias) in conjunction with a rise in CRP, when infection and other causes were ruled out; (ii) gastrointestinal or renal infarction or bleeding from aneurysms, as seen at surgery or strongly suspected by angiography; (iii) new mononeuritis multiplex; (iv) a rapid rise in serum creatinine levels and blood pressure; (v) testicular pain; (vi) skin vasculitis; and (vii) uveitis, when (i-vii) were considered to be due to vasculitis.

In paper V clinical relapse was defined as return of vasculitic activity to an extent yielding a BVAS of 4 or more. A "serological relapse" was defined as an increase of 75% in the assay studied when comparing with the lowest result obtained during the preceding 12 month period. A "serological remission" was defined as a decrease of 50% of the value of the assay when compared with the most recent relapse. In both cases the difference in absorbance units had to be larger than the cut-off level for positive results, which in turn was based on the mean of 24 samples from healthy blood donors plus two standard deviations.

Statistical methods

The following statistical methods were used:

Chi-square method (using Stat view 1994 in paper I and GraphPad InStat version 3.0 in paper V), Spearman's rank correlation (using Stat view 1994 in paper I and GraphPad InStat version 3.0 in paper III), Fisher's exact test (using GraphPad InStat version 3.0 in paper V), multiple Cox regression analysis (using SPSS version 12 in paper II) and cumulative relative survival calculated by the method described by Hakulinen [134] (using Stata version 7.0 in paper II).

Antibody detection

Indirect immunofluorescence (IIF)

IIF was performed on ethanol-fixed leukocytes according to a previously described standard procedure [135]. In short, leukocytes from healthy blood donors were fixed on

glass slides in absolute ethanol. Patient sera were diluted in phosphate buffered saline (PBS) and bound IgG was detected by FITC-conjugated anti-human IgG.

Enzyme linked immunosorbent assay (ELISA)

Microtitre plates were coated with purified antigen (PR3 or MPO) and the assay was performed as described earlier [58]. In short, patient sera were diluted in PBS and bound IgG was detected by alkaline phosphatase-conjugated anti-human IgG. All assays were done in duplicate.

Figure 6. (A, B) The amino acid sequences for human PR3 (hPR3), human leukocyte elastase (ELA) and murine PR3 (mPR3) used as templates for the recombinant proteins. The signal peptides and propeptides are removed and a HindIII site is introduced at the 5' end and a NotI site is introduced at the 3' end. All the recombinant proteins were mutated at the active site by changing Ser to Gly (shaded) to create enzymatically inactive mutants. The cleavage sites for the different constructs are marked a, b, c, d and e (at points where unique restriction enzyme sites were found/introduced). (C) The chimeric constructs were named according to the origin of the respective proportion of the molecule, were H stands for hPR3, m for mouse PR3 and E for elastase. The cleavage sites for constructs made of thirds are a, b, d and e, and for the constructs made of halves the cleavage sites are a, c and e. For example the HHm construct comprise hPR3 from a to d and the last third, from d to e, is mPR3 and the Hm construct is composed of hPR3 from a to c and mPR3 from c to e.

Capture PR3-ANCA ELISA

The method described by Baslund *et al* was followed [77]. In short, microtitre plates were coated with the monoclonal antibody 4A3 before incubation with purified PR3. Patient sera were diluted in PBS and bound IgG was detected by alkaline phosphatase conjugated anti-human IgG. To exclude non-specific binding and rheumatoid factor binding, a control plate was coated with an unrelated monoclonal antibody of the same mouse IgG subclass and the absorbance values of this plate were subtracted from the absorbance values obtained on the anti-PR3 coated plate for each individual well.

Recombinant proteins

Generation of recombinant proteins

cDNA for human PR3, murine PR3 and human leukocyte elastase was mutated at the active site by changing serine at position 203 to glycine (in elastase at position 200) to create an enzymatically inactive mutant. This was achieved by point mutations in a polymerase chain reaction (PCR). These cDNAs were subcloned into the pcDNA-BM40-HisEK vector by inserting a HindIII restriction enzyme site at the 5' end of the constructs and a NotI site at the 3' end by PCR. Primers were constructed to exclude the signal peptides and propeptides, in order to make mature proteins. For technical reasons, when inserting the HindIII site, the first isoleucine in all the recombinant proteins was replaced by a leucine. All three amino acid sequences for these templates are shown in Figure 6.

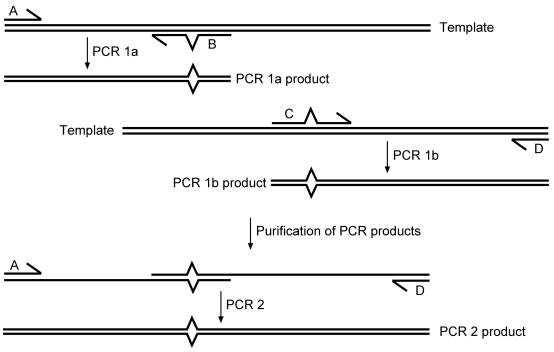

Figure 7. Double overhang splicing PCR. In step one, two reactions are performed and a mutation is introduced in both products. The two PCR1 products are then used as templates in step two.

These mutated cDNAs were then used as templates in an overlap extension PCR as described by Ho [136], shown in Figure 7. In addition to mature recombinant hPR3, mPR3 and HLE, six constructs consisting of thirds of human PR3 (hPR3) and mouse PR3 (mPR3), two constructs consisting of halves of hPR3 and mPR3 and six constructs consisting of thirds of hPR3 and HLE were made using different primers. The protein coding cDNAs were inserted in the pcDNA3-BM40-HisEK vector (Figure 8). All constructs were sequenced.

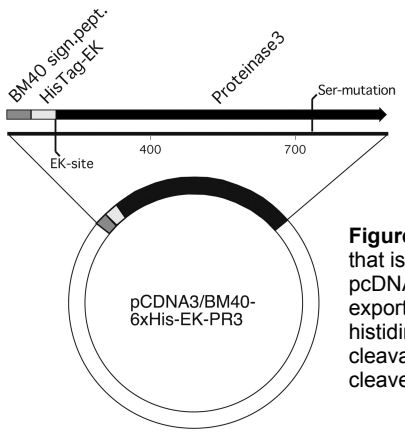

Figure 8. The expression vector pcDNA3-BM40-HisEK that is based on the mammalian CMV promoter-driven pcDNA3 vector. It adds a BM40 signal peptide for exporting proteins to the medium followed by a hexa-histidine tag for purification and an enterokinase D cleavage site N-terminal of the recombinant protein to cleave off the BM40 signal and histidine tag.

HEK-293 cells were transfected with plasmid and selection with geniticin was performed. Cells growing under selective conditions were expanded. During harvesting, the cells were kept in serum-free medium. The supernatants were purified using an FPLC column packed with Talon Superflow resin and the bound material was eluted and the fractions analysed for PR3 and 6xHis tag by ELISA. After concentration the recombinant proteins were analysed by silver-stained SDS-PAGE gels and by immunoblotting using antibodies against the His-tag. The His-tag and BM40 signal peptide were cleaved off and the recombinant proteins were re-analysed using silver-stained SDS-PAGE and immunoblotting using antibodies against the His-tag to check that the His-tag was cleaved off.

Detection of recombinant proteins

SDS-PAGE and immunoblotting

10% NuPAGE gels from Novex (San Diego, CA, USA) were run according to the supplier's recommendations. For immunoblotting, proteins were transferred to PVDF membranes using a semidry technique, and the antigens were detected with polyclonal anti-hPR3, anti-mPR3 or anti-HLE antibodies and visualised with alkaline phosphatase-conjugated antibodies.

ELISA

Microtitre plates were coated with purified recombinant protein. Monoclonal antibodies or human sera were diluted in PBS and bound IgG was detected by alkaline phosphatase-conjugated antibodies.

Inhibition ELISA

Dilutions of monoclonal antibodies were preincubated with the different inhibitors, i.e. recombinant proteins, before being transferred to microtitre plates coated with native PR3. The amount of bound antibodies was visualised with alkaline phosphatase-conjugated antibodies. Results were considered positive when 50% inhibition was achieved.

Results and discussion

ANCA as a tool for diagnosing systemic vasculitis

Small vessel vasculitis (I)

The detection of ANCA has become a useful tool in the diagnosis of Wegener's granulomatosis and microscopic polyangiitis. However, the results obtained by IIF and ELISA do not always correlate and the reason for this discrepancy is not known. One possible explanation is that proteins are denatured during the process of antigen purification or during coating onto the solid phase, thereby hiding or destroying conformational epitopes on the antigen. To avoid this, a plate precoated with a monoclonal antibody can be used to capture the antigen, thereby presenting it in a native form. Baslund *et al* [77] evaluated three capture assays for the detection of PR3-ANCA based on three different monoclonal antibodies. They found that the antibodies represented three separate epitope areas on PR3 and that the assay using the monoclonal antibody 4A3 had the best sensitivity. The significance of this capture PR3-ANCA ELISA as a sero-diagnostic tool for patients with small vessel vasculitis was evaluated in paper I. The study compared the results obtained by IIF, direct ELISA and capture ELISA.

The diagnostic sensitivity for PR3-ANCA in the capture ELISA in patients with a clinical diagnosis of WG was assessed from 48 patients with an established diagnosis of WG having renal involvement. As shown in Table 7, 85% of the patients with WG were positive for PR3-ANCA by the capture assay at the time of diagnosis. The overall sensitivity for c-ANCA and p-ANCA was 75% and for the combination of direct ELISAs for PR3-ANCA and MPO-ANCA 85%. The highest diagnostic sensitivity (98%) was obtained by combining the capture ELISA for PR3-ANCA with the direct ELISA for MPO-ANCA.

Although a higher diagnostic sensitivity was found with the capture technique than with IIF, there was an almost equal specificity by the capture PR3-ANCA ELISA and c-ANCA at IIF. This was studied using disease control sera from 52 patients with endocapillary or mesangioproliferative glomerulonephritis. In patients with these forms of glomerulonephritis the clinical presentation of renal findings can be much the same as in WG or MPA, that is, microscopic haematuria, mild proteinuria, granular casts in the urine and decreased renal function. However, the treatment differs in these different groups, which underlines the need for a reliable serological test. Obviously it is more harmful to start immunosuppressive therapy in a patient with endocapillary glomerulonephritis, particularly if it is associated with a bacterial infection, which is not always clinically overt, than in a patient with extracapillary glomerulonephritis that is not associated with WG or MPA. ANCA have previously been demonstrated in some patients with various forms of glomerulonephritis not associated with vasculitis [137,138]. As shown in Table 7, "false positive" results were obtained with the capture ELISA in 5 out of the 52 sera, giving a specificity of 90%. Four of these five positive sera manifested PR3-ANCA levels at the lower range of

positivity (9, 10, 15 and 15 AU/ml). By the direct ELISA none of the sera had PR3-ANCA and two had MPO-ANCA, which gives a combined specificity for direct ELISA of 96%, and the combined specificity for IIF (c- and p-ANCA) was 83%.

To achieve a higher specificity for the capture ELISA an elevation of the cut-off level could be used. For example, a cut-off of 15 AU/ml instead of the 8AU/ml used in the present study would have resulted in a higher specificity (96%) but an unchanged sensitivity. However, when testing patients with SLE, rheumatoid arthritis, Sjögren syndrome and ulcerative colitis and healthy controls the specificity was very high at 99%.

One problem that can arise using the capture technique is unspecific background. In a study by Merkel *et al* [82] 23 of 26 sera from patients with a known PR3-ANCA positive vasculitis were found to be positive by their capture PR3-ANCA ELISA. However, the authors used the same monoclonal anti-PR3 catching antibody to control for background as well as for anti-mouse IgG, and the reactivity of the serum to the monoclonal antibody alone was subtracted from the reactivity to the monoclonal antibody-PR3 complex. Such a procedure may render "false low" results, since a previous study has shown that patients with WG have complexes of PR3 and PR3-ANCA and such complexes may readily attach to the monoclonal anti-PR3 alone [139]. To avoid this we used a control plate coated with an unrelated monoclonal antibody of the same mouse IgG subclass.

Table 7. Detection of ANCA by IIF (c-ANCA or p-ANCA), by direct ELISA for PR3-ANCA and MPO-ANCA, and by capture ELISA for PR3-ANCA in patients with Wegener's granulomatosis and control patients with glomerulonephritis but without vasculitis.

ANCA method	Disease control sera from patients with glomerulonephritis (n=52)	Patients with Wegener's granulomatosis (n=48)
IIF c-ANCA	3 (6%)	28 (58%)
IIF p-ANCA	6 (12%)	8 (17%)
IIF negative	43 (83%)	12 (25%)
Capture PR3-ANCA ELISA	5 (10%)	41 (85%)
Direct PR3-ANCA ELISA	0 (0%)	35 (73%)
Direct MPO-ANCA ELISA	2 (4%)	6 (12%)
ELISA ANCA negative	45 (86%)	1 (2%)

Number of positive patients (% of positive patients) in each assay.

In paper II sera from the time of diagnosis from 117 consecutive patients with biopsy-confirmed WG or MPA were tested in direct ELISA for PR3-ANCA and MPO-ANCA and in capture PR3-ANCA ELISA. In direct ELISA 65 were positive for PR3-ANCA, 39 for MPO-ANCA and 10 were negative, while 71 were positive

in the capture PR3-ANCA ELISA. Thus, by combining direct ELISA for MPO-ANCA with capture ELISA for PR3-ANCA for screening a sensitivity of 97 % was achieved in this cohort. The combination of direct ELISAs yielded a sensitivity of 92%.

PAN (III)

ANCA can be used as a tool to find ANCA positive vasculitis, but it could also be used to distinguish ANCA negative vasculitis, such as PAN. Classical PAN strictly adhering to the CHCC nomenclature, with patients lacking signs of small vessel vasculitis such as glomerulonephritis or capillaritis and indirect evidence such as ANCA, is described in paper III. To the best of our knowledge there is only one study before the present study that describes classical PAN strictly adhering to this nomenclature [14]. As in other studies using less restrictive inclusion criteria PAN typically developed subacutely, with the onset of constitutional symptoms over weeks to months. Only few had an acute onset, and in those cases the symptoms were severe with malignant hypertension, renal insufficiency and gastrointestinal involvement. The median BVAS at diagnosis was 17 (range 5-33). Even though we had excluded patients with glomerulonephritis, organ involvement at diagnosis was dominated by renal involvement. Seven patients (70%) had signs of renal involvement at the time of diagnosis, which is more than other studies have reported [13,15]. Two patients (20%) in our study developed end-stage renal disease during follow-up. Both had malignant hypertension and relapses with renal involvement. This shows that patients with PAN must be followed closely to avoid deterioration of renal function.

FFS varied from 0 to 3 (median 0). Patients with the highest FFS tended to have short diagnosis delay. The small size of this study precludes any firm conclusion, but it is notable that the only patient who died had an FFS of zero. Median VDI at 1 year was 1 (range 0-5) and at 5 years 2 (range 1-5). Even though the study was small we found a correlation between BVAS at diagnosis and high VDI at follow-up, both at 1 year ($p=0.0029$) and at 5 years ($p=0.0072$).

Seven patients had altogether 11 relapses. Eight of the relapses were seen in the same organs that were affected at the time of diagnosis. The median time from first relapse was 3.2 years (range 0.7-6.8). Within 5 years 57% of the patients had experienced a relapse. The relapse tendency for these PAN patients does not seem to differ from the cohort of 62 MPA patients described in paper II, as shown in Figure 9. These data contradict the earlier opinion that PAN is a self-limiting disease with few relapses.

We attempted to find all patients with a diagnosis of PAN living in our local catchment area during a 12-year period and estimated an annual incidence of 1.6 per million in our part of Sweden. All of these patients were treated and followed at the Departments of Nephrology and Rheumatology in Lund. The annual incidence is less than what many others have found, but this is probably due to our strict classification criteria. More recent reports from other countries have also shown a

relatively low incidence [16]. In comparison, MPA, which is reported to have an annual incidence of 8.4-11.6 per million [16], seems to be around five times more frequent[16].

Reports on ANCA negative small vessel vasculitis are rare. Three studies on ANCA negative small vessel vasculitis with renal involvement report that these patients have a shorter prodrome, less extrarenal involvement [112,140] and a higher prevalence of nephrotic syndrome [140,141]. One study found poorer renal outcome in these patients [140], while another study could not show any differences in patient or renal outcome between ANCA positive and ANCA negative patients and found that similarities between the groups were notable [112]. The relapse rate was 20% during a follow-up period of 18 months in one study [112], which is comparable to the PAN patients in our study.

ANCA as a tool for detecting relapses (V)

Serial ANCA measurements for the monitoring of disease activity are commonly used in patients with ANCA associated vasculitis. However, views range from the opinion that ANCA levels should guide immunosuppressive therapy directly, even in the absence of symptoms, to the opinion that serial measurements of ANCA are of no value for clinical management [121,124-127]. In paper V direct ELISA using native PR3, recombinant PR3 and chimeric mouse/human PR3 and capture ELISA using native PR3 are evaluated in the serial follow-up of 38 patients with PR3-ANCA positive vasculitis. All patients were positive at time of diagnosis for either direct ELISA (87%) and/or capture ELISA (97%) using native PR3 (Table 8). Only 50% of the patients were positive in the direct ELISA with recombinant PR3 and only between 16% and 47% of the patients were positive in the direct ELISA using different chimeric PR3 at the time of diagnosis.

All patients in this study responded to treatment and went into clinical remission. In most cases this was accompanied by a reduction in ANCA levels. To compare the different assays in their ability to detect such a decrease we predefined the term “serological remission” if the level decreased by at least 50% within 12 months after the initial flares of disease. A serological remission was seen in 73% of the cases with direct ELISA using native PR3 and 92% in capture ELISA (Table 8). For the assays with different recombinant PR3 the figures varied between 0 and 42%. However, since with some constructs there were only a few samples positive at the time of diagnosis there was no possibility to observe any fluctuations in levels.

Serum or plasma samples were available from 29 of the 36 instances of relapses occurring during the entire study period. As shown in Table 8, positive results were obtained in 97% of the cases with the capture ELISA and in 83% with the direct ELISA using nPR3. The chimeric recombinant proteins with the best reactivity in this analysis were only positive in 9 out of 29 instances (31%).

The clinical utility of an assay is not merely determined by the degree of positivity at relapse. Many samples were positive also in remission and to compare the ability to detect relapses we defined a “serological relapse” as an increase of 75% in the

absorbance value, when comparing with the lowest values obtained over the preceding 12 month period. The analysis was limited to one relapse per patient, to avoid the bias stemming from having multiple data sets from the same individual. Sets of samples were available from 19 of the 22 patients who experienced a relapse. The number of relapses detected with the various ELISAs based on the chimeric proteins varied from 0 to 4 (22%) (Table 8). ELISAs based on native PR3 performed better; direct ELISA detected 9 (47%) and capture ELISA 25 (79%) of the relapses.

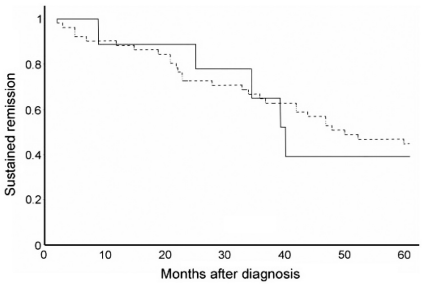

Figure 9. Cumulative sustained remission (censored for death) during 5 years of follow-up for 10 patients with PAN (solid line) and 62 patients with MPA (dashed line).

To investigate if rises in ANCA levels precede clinical relapses we analysed samples drawn 3 months (±1 month) prior to a clinical relapse and compared them with samples drawn during the preceding 12 month period. The assay was considered to predict a coming relapse if an increase of more than 75% OD (optical density) could be detected. The analysis was limited to one set of samples per patient and suitable sample sets could be retrieved from 15 patients. As shown in Table 8, the ability to detect relapses was limited; the capture assay was best but predicted only 67% of the relapses. The best chimeric proteins only reacted in one instance.

ANCA as a prognostic marker in small vessel vasculitis

Capture PR3-ANCA ELISA (II, V)

Patients with small vessel vasculitis still have a considerable morbidity and mortality and prognostic markers for these diseases have been sought in many studies. The most frequently found prognostic factor for patient survival is age [18,19,22-24,99,100,102-105,110-113] and for renal survival serum creatinine at diagnosis [18,102,103,113,114]. In paper II we aimed at investigating patient survival at 10 year follow-up of a cohort of 117 patients with small vessel vasculitis and with renal involvement confirmed by biopsy, comparing the patient cohort with the general population of the same catchment area, and analyzing possible prognostic factors for patient outcome.

The median duration of follow-up for the whole cohort was 92 months. 1 year survival was 85%, 2 year survival 82%, 5 year survival 74% and 10 year survival 52%. There was a statistically significant decrease in patient survival compared to the general population, matched for age and gender in the same geographical region. Both men and women had a decreased survival and Figure 10 shows the cumulative relative survival for women and men, respectively. The dramatic decrease in patient

Table 8. Samples from different time points in direct ELISA with 10 recombinant PR3 and native PR3 and in capture ELISA with native PR3. The number of available sets of samples for the respective analysis is indicated as n.

	Positive at diagnosis n=38 % (n)	Serological remission within 12 months n=26 % (n)	Serological relapse 3 months before clinical relapse n=15 % (n)	Positive at relapses n=29 % (n)	Serological relapse at clinical relapse n=19 % (n)
rHPR3	50 (19)	42 (11)	20 (3)	48 (14)	26 (5)
rmPR3	8 (3)	4 (1)	0	21 (6)	5 (1)
mmH	39 (15)	27 (7)	0	28 (8)	5 (1)
Hmm	26 (10)	4 (1)	7 (1)	28 (8)	16 (3)
HHm	37 (14)	31 (8)	0	31 (9)	22 (4)
mHm	16 (6)	0	0	3 (1)	0
HmH	18 (7)	8 (2)	0	17 (5)	11 (2)
mHH	47 (18)	31 (8)	0	24 (7)	16 (3)
Hm	37 (14)	15 (4)	7 (1)	31 (9)	22 (4)
mH	34 (13)	27 (7)	0	21 (6)	5 (1)
nHPR3	87 (33)	73 (19)	40 (6)	83 (24)	47 (9)
Capture ELISA	97 (37)	92 (24)	67 (10)	97 (28)	79 (15)

survival during the first year has also been described by others [23,104,110]. However, to our knowledge this is the first study that presents patient survival in ANCA associated vasculitis with renal involvement as cumulative relative survival.

In the multiple Cox regression analysis four predictive factors for patient survival were found: age, diagnosis of MPA versus WG, B-thrombocyte count and PR3-ANCA levels >550U by the capture ELISA. The risk of dying was increased by 5.8% for each year older in age (relative risk [RR]=1.058) and as mentioned above age is the most frequently found risk factor for patient survival in studies of small vessel vasculitis. The risk of dying was nearly twice as high for patients with MPA as for those with WG (RR=1.917). This finding is difficult to correlate to other studies due to disparate ways of differentiating these two diseases from each other in different studies. In this study the diagnosis of WG required the presence of granulomatous disease, histologically verified or strongly suspected by X-ray. Thus, patients with sinus involvement, epistaxis or haemoptysis, but without the above-mentioned criteria, received a diagnosis of MPA. For the third finding, that high levels of B-thrombocytes were associated with a better prognosis, we have no explanation. There was no correlation between the levels of B- thrombocytes and the levels of either B-leukocytes, B-haemoglobin or C-reactive protein.

The most interesting finding was that a very high level of PR3-ANCA measured by the capture ELISA at the time of diagnosis was a factor that predicted poorer patient

survival as well as poorer renal survival. The risk of dying was 2.7 times higher for those with a high PR3-ANCA level (p=0.001) and the risk for reduced renal survival was 2.2 times higher in this group (p=0.024). We speculate that this could indicate that the epitope on PR3, recognised by the capture method, might be of importance in the pathogenesis of the disease, contrary to the epitope recognised by the direct ELISA.

At the end of follow-up 39 (33%) had developed end-stage renal failure. In addition to a high level of PR3-ANCA measured by the capture ELISA the serum creatinine at baseline predicted a poorer renal survival; the higher the serum creatinine, the poorer the renal survival. This has been shown previously [18,102,103,113,114], but we did not find elevated serum creatinine to be a predictor of poor patient survival in our multivariate analysis, as has been reported by others [103,110,113]. Further analysis of our results revealed that there was a covariation of serum creatinine and age, i.e. older patients were often referred with a higher serum creatinine.

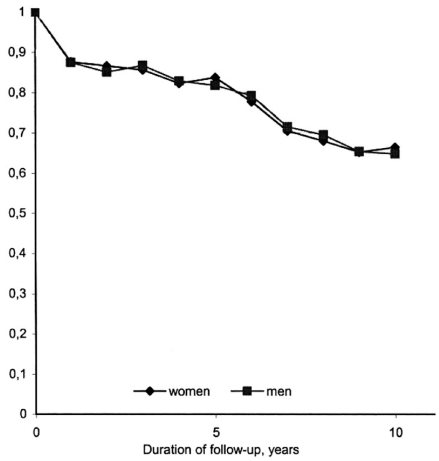

Figure 10. Cumulative relative survival of patients with WG (n=55) and MPA (n=62) with renal involvement for men and women respectively.

The prognostic value of the capture PR3-ANCA ELISA was further investigated in paper V. To see if a high PR3-ANCA level by capture ELISA at diagnosis was predictive of relapse we compared two equally large groups, each comprising of 19 patients, according to ANCA levels. For this analysis the levels were 11-750U in the low level group and 806-23200U in the high level group (Table 9). In the group with low PR3-ANCA levels 7 patients had one or more relapses and in the high level group 15 experienced relapses, which is a statistically significant difference (p=0.02). When dividing the patients into groups with high and low ANCA levels in direct ELISA no difference in relapse rate was seen (Table 9).

Together with the findings that high PR3-ANCA levels measured by capture ELISA are prognostic for patient survival and for renal survival in paper II this strengthens our notion that in patients with small vessel vasculitis PR3-ANCA against the epitope that is exposed by the capture ELISA are somehow associated with the pathogenesis of these diseases. We conclude that patients with high levels of these PR3-ANCA should be observed more attentively to find signs of disease activity early, before permanent organ damage has occurred.

Recombinant antigens as tools for epitope mapping

Construction of chimeric PR3 for epitope mapping (IV, V)

Several studies have been carried out to map the epitopes recognised by PR3-ANCA. It has been shown that ANCA recognise conformational epitopes on PR3 [61] and subsequently studies using synthetic peptides, in order to find linear epitopes, have been inconclusive [69-72]. An alternative approach is to express different parts of the antigenic molecule in a homologous but nonantigenic framework. This approach was used successfully in Goodpasture's disease to locate epitopes on the NC1 domain of the α3 chain of type IV collagen by exchanging parts of it with parts of the nonantigenic α1 chain [142]. In paper IV and V we used the same strategy to express epitopes on PR3.

For the nonantigenic framework we chose human leukocyte elastase (HLE) and murine PR3 (mPR3) that are similar to human PR3 (hPR) in both sequence and shape. The amino acid sequences of these three proteins are shown in Figure 6. The expression of recombinant antigens was done in human embryonic kidney cells (HEK-293) that are known to provide a complete machinery for post-transcriptional modifications and that also secrete large amounts of protein to the medium.

In paper IV we started out using HLE, which has a 53% sequence homology with hPR3, as the framework molecule. Six different constructs were made, consisting of one third of the sequence from one molecule and two thirds from the other molecule. Despite several attempts we were only able to produce three of these hPR3/HLE proteins in sufficient amounts. We do not think that this was for technical reasons since we made different vectors and tried several transfection and culture conditions.

Table 9. Clinical characteristics at diagnosis and outcome for all patients and groups of patients; divided according to results in capture ELISA and direct ELISA against native PR3 at the time of diagnosis.

	All	Capture ELISA		Direct ELISA	
		Low	High	Low	High
Number of patients	38	19	19	19	19
Male/female	22/16	8/11	14/5	10/9	12/7
Age, years†	58 (10-78)	59 (16-80)	57 (10-77)	57 (23-78)	59 (10-80)
WG/MPA	34/4	17/2	17/2	16/3	18/1
Creatinine, μmol/L†	89 (47-777)	78 (47-684)	123 (62-777)	86 (62-641)	92 (47-777)
BVAS†	19 (5-32)	15 (5-29)	21 (9-32)	17 (5-32)	20 (6-30)
Patients with relapse % (n)	58 (22)	37 (7)*	79 (15)*	58 (11)	58 (11)

† Median (range). BVAS = Birmingham vasculitis activity score. * (p=0.02)

The most probable explanation is that these chimeric molecules were malfolded with consequent degradation in the endoplasmic reticulum. Instead, we decided to use mPR3, which has a 65% sequence homology with hPR3, and this approach was more successful. All six chimeric h/mPR3 molecules were produced and exported to the culture medium, and they appeared to have the correct molecular weight by Western blot. The purified recombinant proteins were tested in ELISA and different mouse anti-hPR3 monoclonal antibodies differed in their binding pattern to these proteins, but no distinct region for their binding could be identified. It was also demonstrated that PR3-ANCA from patients with WG would bind to these chimeric hPR3/mPR3 proteins.

From a pilot study with the h/mPR3 proteins we gained an indication that PR3-ANCA positive patients with active small vessel vasculitis have ANCA against the C-terminal part of PR3. In order to investigate this further we also produced another two chimeric proteins consisting of halves of hPR3 and mPR3.

Epitope specificity of PR3-ANCA using chimeric h/mPR3 (V)

In paper V the chimeric h/mPR3 were used as antigens in ELISA for the diagnosis and serial follow-up of 38 patients with PR3-ANCA positive vasculitis. Their performance was compared with capture and direct ELISA based on native PR3 purified from human neutrophils.

None of the recombinant proteins offered any improvement in diagnostic yield compared to standard methods for ANCA detection. Recombinant hPR3 was only recognised by serum from 50% of the patients with small vessel vasculitis at the time of diagnosis, while 87% were positive using native PR3 in direct ELISA and 97% in capture ELISA. Results using different h/mPR3 varied between 16% and 47%, and a construct having the human sequence in the C-terminal end showed the highest reactivity. There are several possible reasons for these results. First there might be trivial technical reasons, the purity and amounts of coated material perhaps being suboptimal in our ELISAs, but this is not likely to be the major explanation. It is more probable that the HEK 293 cells have not processed the proteins correctly and the recombinant proteins are aberrantly folded or that they lack some critical posttranslational modification. The expression of recombinant PR3 in HEK 293 cells has been studied in detail by Sun *et al* [67] and it was found that the N-terminal activation dipeptide of PR3 is not cleaved off in this cell type and that some PR3-ANCA only react with PR3 after cleavage of this propeptide. This was circumvented in our study by constructing cDNA without the propeptide. Another finding in that study was that the recombinant PR3 secreted into the culture media had an approximate molecular mass of 32-38 kDa and after removal of the aspargine-linked sugar moieties the molecular mass was reduced to about 29 kDa, which indicates that the recombinant PR3 was glycosylated. The same group has recently shown that glycosylation occurs at both aspargine-linked glycosylation sites (Asn-102 and Asn-147) in human neutrophil PR3 and when expressing recombinant PR3 in HMC-1 cells unglycosylated PR3 gets secreted preferentially into the media

supernatants [53].This unglycosylated variant was not recognised by all PR3-ANCA positive sera in capture ELISA, but on the other hand a subset of ANCA displayed increased binding to recombinant PR3 without a glycan at Asn-147. This shows that there is heterogeneity in the ANCA response and that ANCA are sensitive to minimal changes in the conformation of PR3. We have not analysed to what extent our recombinant proteins are glycosylated and a part of the differences between our recombinant PR3 and native human PR3 might be due to disparate glycosylation.

Although the chimeric constructs had a low sensitivity to detect active disease other interesting findings could be brought out of the study in paper V. When analysing the results of individual assays we observed that reactivity shifted during the course of disease. When analysing samples drawn from one exacerbation episode the autoantibodies seemed to be directed to the same target, but when the patients relapsed, sometimes many years later, often a different set of epitopes seemed to be targeted. In order to describe and compare the reactivity patterns we pooled the results from the chimeric assays and divided the reactivities into three main patterns: a C-terminal, an N-terminal and an intermediate pattern. A major change in pattern, that is from N-terminal pattern to C-terminal pattern or vice versa, was identified for 11 patients. One of them changed his reactivity pattern 3 times. The other 10 patients all exhibited only one shift, and interestingly in all ten patients this shift was from C-terminal pattern to N-terminal pattern. Epitope shifts in WG have been reported in a small study where it was shown that patients could inhibit their own PR3-ANCA [76]. In this study samples from the time of diagnosis were made to compete with samples from relapse in the same patient. With this method epitope spreading was observed in two patients while in two others epitope narrowing was seen. If PR3-ANCA is the consequence of molecular mimicry based on shared epitopes with a certain as yet unidentified organism, and if relapses are a consequence of reexposure to this organism, it is strange that patients react with different epitopes at relapse.

Another interesting finding was that the reactivity pattern at the time of diagnosis seemed to reveal prognostic information. 17 patients exhibited the predominant C-terminal pattern of reactivity at the time of diagnosis, 9 patients exhibited the predominant N-terminal pattern while 12 patients were considered to have an intermediate pattern. As shown in Table 10, we found no differences in basal clinical characteristics from the time of diagnosis when comparing patients with different patterns of reactivity. However, there was a higher tendency for relapse among the patients with the C-terminal pattern. During a mean follow-up of 8.6 years 82% of the patients with the C-terminal pattern experienced at least one relapse, while the corresponding figure was 22% during a follow up of 8.8 years among the patients with the N-terminal pattern and 50% among the patients with the intermediate pattern ($p=0.01$). In spite of the different relapse tendencies we did not record any significant difference in VDI at 5 years, ESRD and patient death during follow-up. However, it must be stressed that this was a serendipitous finding and the statistical significance can be questioned as no such hypothesis was formulated in advance.

Table 10. Clinical characteristics at diagnosis and outcome for all patients and groups of patients; divided according to reactivity pattern at diagnosis.

	All	Reactivity pattern at diagnosis		
		C-pattern	N-pattern	I-pattern
Number of patients	38	17	9	12
Male/female	22/16	12/5	3/6	7/5
Age, years†	58 (10-78)	57 (10-78)	52 (23-75)	59 (26-78)
WG/MPA	34/4	16/1	8/1	10/2
Creatinine, μmol/L†	89 (47-777)	85 (47-777)	82 (55-547)	121 (47-684)
BVAS†	19 (5-32)	21 (7-32)	15 (6-25)	17 (5-29)
Patients with relapse % (n)	58 (22)	82 (14)*	22 (2)*	50 (6)*

† Median (range). BVAS = Birmingham vasculitis activity score. *(p=0.01)

The findings that patients who exhibit a C-terminal reactivity pattern at the time of diagnosis and also patients who have a high level of PR3-ANCA measured by the capture ELISA experienced more relapses might have a common origin. We speculate that the epitope on PR3 that is exposed by the capture ELISA is present in the C-terminal part of the PR3 molecule. When analysing correlations, the chimeric PR3 with mPR3 in the N-terminal half and hPR3 in the C-terminal half (mH), showed the best correlation with the capture assay. Furthermore, these findings are compatible with the notion that a diagnostically and prognostically important epitope is present in the C-terminal part of the PR3 molecule, but that only part of this epitope is present on our construct with human PR3 sequence in the C-terminal end.

Conclusions and future perspectives

Screening for ANCA and monitoring disease activity with ANCA

In this thesis it has been shown that capture ELISA is a sensitive tool for detection of PR3-ANCA in patients with small vessel vasculitis. It is more sensitive than IIF and direct ELISA, but if the cut-off level is based only on healthy controls, the amount of "false positive" in samples from other diseases can be unacceptably high. With a cut-off level yielding equal specificity to IIF and/or direct ELISA the sensitivity is still superior. However, a combination of two methods is still required to achieve maximum sensitivity and specificity. One important question is how to combine assays, and which assay to use for screening and which for confirmation. The assay to use for screening has to be the most sensitive and should not fail to find any patients with ANCA and small vessel vasculitis. Nevertheless it is important for it to have a reasonable specificity. For the assay used for confirmation the most important quality has to be specificity. In this way the vast majority of patients with disease will be found in the first step together with some patients who do not have the disease, and in the confirmatory step most of the patients without disease will be screened out. There will always be a group of patients with inconclusive results (positive in the first step and negative in the second step) who will need further examinations to exclude a diagnosis of vasculitis.

If the WG patients in paper I had been screened with IIF and the positive results confirmed by capture PR3-ANCA ELISA and direct ELISA for MPO-ANCA only 36 (75%) would have been positive in the first as well as in the confirmatory step. If the testing procedure had been performed the other way around, starting with the ELISAs, 47 (98%) would have been positive in the first step and 34 of these (71% in total) would have been positive in the confirmatory step. In this case, one patient (2%) would not have been found, and 13 (27%) of the patients positive in the capture ELISA would have been negative in follow-up IIF test, requiring a more extensive examination.

The standard way in most autoimmune laboratories is to screen with IIF and confirm the results with direct ELISA. From the results in this thesis we conclude that screening with capture ELISA for PR3-ANCA based on the monoclonal antibody 4A3 in combination with direct ELISA for MPO-ANCA provides a very high sensitivity and a good specificity, and the combination of these two assays should be used as the first step. The samples that are positive in these tests should then be confirmed in a second step with IIF. The results from a recent epidemiological study by our group underscore the problem involved in using IIF for screening, where only 28 (12%) out of 228 patients initially positive in IIF were subsequently classified as cases with ANCA associated vasculitis while the corresponding figure for ELISA was 65% [143].

Monitoring disease activity with ANCA testing is usually done in the same way as when screening, i.e. the laboratories use the same set of assays, but the clinical setting is different. In this case the disease is known and the clinician wants information about significant fluctuations in ANCA levels.

We concluded that the chimeric constructs did not offer any improvement when trying to diagnose relapse by serological assays. However, the results in paper V indicated that both direct ELISA and capture ELISA based on native purified PR3 have a potential for this. In order to interpret a rise in the levels shown in these assays it is necessary to know how often fluctuations occur in ANCA levels without any evident change in disease activity. During the prospective phase of the study samples from 7 clinical relapses were recorded. The total number of serological relapses was 14 using the direct assay and 29 using the capture assay. When considering "false positive" increases in ANCA levels it must be kept in mind that when studying a whole series of ANCA determinations graphically there is more information conveyed in such a series than can be borne out of a simple formula, for instance because some patients have a slow rise in ANCA levels with a relapse occurring after more than one year. Nevertheless, the cut-off levels for the capture PR3-ANCA ELISA used for diagnosing vasculitis seem to make the assay too sensitive for detection of relapses. It has been shown that by requiring a larger increase in ANCA levels for the diagnosis of relapse, e.g. a fourfold increase instead of twofold, the likelihood of detecting a true relapse is higher at the expense of decreased sensitivity [144]. Possibly a combination of two different assay would be the best alternative. We have not analysed which combination of assays would be the optimum, but our results indicate that the capture assay investigated in this thesis should be part of such a solution. In clinical practice it might prove adequate to use the capture assay to monitor patients during the course of the disease and when significant rises occur to use additional assays to confirm the findings.

The problem of the new definition of an old disease

The term PAN has been in use for more than hundred years as a diagnostic term for patients with systemic vasculitis. In the meantime, however, so many separate types of vasculitis have been individually described that the original blanket concept of PAN now seems to lack some of its original usefulness. This raises the question of how far we can still trust older literature with respect to clinical features, natural history and response to treatment. Many authors of reviews and book chapters claim that they adhere to the Chapel Hill consensus conference definition of PAN, yet they still cite exclusively studies using older definitions without highlighting this dilemma. We addressed this problem by describing 10 patients with strict adherence to the new definition. We hope this will encourage others to describe their patients in order for the scientific community to ascertain the characteristics and course of this disease. This is the only way to eventually improve management.

Is there an immunodominant epitope?

The glomerular basement membrane is composed of a large amount of different molecules. Still, in Goodpasture's disease a single epitope in the NC1 domain of the α3 chain of type IV collagen is the target of pathogenic antibodies [145]. In parallel, neutrophils harbour hundreds of different proteins, yet patients with small vessel vasculitis only produce antibodies against two of these proteins; PR3 and MPO. Mice seem to produce monoclonal antibodies against a restricted number of epitopes on PR3 [75]. Similarly, PR3-ANCA seem to recognise a limited number of epitopes on PR3 and it has been shown that PR3-ANCA can compete for epitopes [73,76,77]. Our hypothesis is that there are one or a few dominant epitopes for PR3-ANCA.

Our studies have shown that high levels of PR3-ANCA in capture ELISA based on the monoclonal antibody 4A3 have prognostic significance for patient survival, renal survival and relapses in patients with small vessel vasculitis. This prognostic information has not been conveyed by other assays. Our interpretation is that this assay presents a dominant epitope that is recognisable by a large amount of PR3-ANCA in patients with the most severe forms of small vessel vasculitis.

It is important to have a good capturing monoclonal antibody that does not compete for epitopes recognised by some PR3-ANCA. 4A3 seems to be very good, but maybe there is an even better way to expose this epitope. An interesting way of presenting PR3 is via a carboxy-terminal c-myc tag. Lee *et al* [68] have constructed a capture ELISA with recombinant PR3 with a C-terminal c-myc extension that is bound to anti-c-myc coated ELISA plates. This recombinant PR3 seems to be recognised reasonably well by PR3-ANCA, but the sensitivity and specificity of this assay has not been evaluated. We plan to provide our chimeric recombinants with such a tag to see if this would lead to a greater sensitivity and a better diagnostic yield.

Another idea how to present PR3 epitopes stems from our recent findings that CD177 and PR3 are co-expressed on the cell surface of subsets of neutrophils [146]. Both proteins are increased in parallel and exhibit a dynamic plasma membrane expression with rapid internalisation and re-expression. It has also been demonstrated that both molecules interact physically [147]. If CD177 actually is the membrane receptor for PR3 on neutrophils then this protein would be a capturing molecule for both native and recombinant PR3 in ELISA. If PR3-ANCA exerts its pathogenic effect through activation of neutrophils, such a presentation of PR3 would mimic the situation in vivo and thus hold an interesting diagnostic potential.

Since we found that patients with ANCA against the C-terminal part of PR3 are more prone to relapse and that our assay with recombinant PR3 with the C-terminal part of the molecule being human PR3 was the one that correlated best with capture PR3-ANCA ELISA, we speculate that the prognostic epitope is located in the C-terminal part of PR3. If this part could be better exposed maybe an assay with even better prognostic significance could be created. This would be valuable for tailoring treatment in patients with small vessel vasculitis. Patients with a lower risk of

relapse could be selected for a milder treatment and side effects could be diminished, while high risk patients could be treated more aggressively and observed more vigilantly to find signs of disease activity early before permanent organ damage has occurred. These are of course only speculations and maybe there is no such thing as a dominant epitope. However, it would be interesting to explore this issue further and construct improved chimeric PR3 that could be tested using either a c-myc extension or with CD177 as a capturing molecule.

Diagnostiska och prognostiska aspekter på anti-neutrofila cytoplasmatiska antikroppar vid systemisk vaskulit

(Popularised summary in Swedish)

Systemisk vaskulit är ett samlingsbegrepp för en grupp sjukdomar med gemensamma symptom. Vas är det latinska ordet för kärl och –itis betyder inflammation, dvs. med vaskulit menar man kärlinflammation. Systemisk innebär att kärlinflammationen kan drabba flera olika organ i kroppen. Vaskulitsjukdomarna tillhör gruppen autoimmuna sjukdomar, vilket betyder att kroppens immunförsvar angriper de egna kroppsvävnaderna. Immunförsvarets normala uppgift är att angripa och oskadliggöra angrepp utifrån, såsom bakterier och virus, samt försvara kroppen mot förändrad kroppsegen vävnad, t ex tumörer. Immunförsvaret kan normalt skilja på kroppseget och främmande, men vid de autoimmuna sjukdomarna har det blivit fel på denna förmåga och immunförsvaret uppfattar kroppsegen vävnad som främmande och angriper denna. Vid systemisk vaskulit angrips blodkärl i olika delar av kroppen och symptomen varierar beroende på vilka organ som drabbas. Vaskuliter indelas ofta efter vilka förändringar man ser vid mikroskopisk vävnadsundersökning och storleken på drabbade kärl samt vilka organ som drabbas.

Vid vaskulit är det vanligt att njurarna angrips och man räknar med att ungefär 5 % av alla dialyspatienter har någon form av vaskulit som orsak till sin njursvikt. Det är också vanligt att lungor, övre luftvägar, hud och nerver drabbas. Kärlinflammationen vid dessa sjukdomar är aggressiv och tidigare var dödligheten mycket hög vid vaskulit. Numera behandlas vaskulitsjukdomar med immundämpande mediciner, men dödligheten är fortfarande förhöjd jämfört med befolkningen i övrigt och dessutom är det vanligt med organskador. Det är därför viktigt att diagnosen ställs tidigt så att skador kan förhindras.

En egenskap hos vaskulitsjukdomar är att de tenderar att komma och gå i skov. Ungefär hälften av patienterna får ett återfall i vaskulitsjukdomen inom fem år. Tyvärr finns det inga bra sätt att vid diagnostillfället förutsäga vem som senare kommer att få skov.

Vid de så kallade småkärlsvaskuliterna har patienterna oftast speciella typer av immunförsvarsproteiner, kallade anti-neutrofila antikroppar (ANCA) i blodet. Dessa kan vara riktade mot två olika proteiner som finns i de vita blodkropparna, proteinas 3 (PR3) eller myeloperoxidas (MPO), och antikropparna kallas PR3-ANCA eller MPO-ANCA beroende på vilket protein de reagerar med. Tack vare att man kan upptäcka dessa antikroppar i blodet kan man lättare ställa diagnosen och starta behandling, förhoppningsvis innan bestående organskador inträffat. ANCA försvinner vanligen när sjukdomen går tillbaka med hjälp av immundämpande behandling och återkommer ofta i samband med skov. Man vet idag inte om det är antikropparna i sig som skadar kärlen vid vaskulit eller om de har en annan roll i sjukdomsprocessen.

Det finns olika metoder att mäta ANCA i blodet och det kan skilja i känslighet (sensitivitet) och mätsäkerhet (specificitet) mellan olika metoder. Detta beror troligtvis på att olika mätmetoder presenterar molekylerna som antikropparna är riktade mot (antigen) på olika sätt. Det specifika området på antigenet där antikropparna fäster kallas epitop och dessa epitoper tror man kan döljas av vissa mätmetoder, vilket då ger metoden en sämre sensitivitet och antikroppar från vissa patienter med sjukdomen kommer inte att hittas. Olika metoder kan också skilja sig i mätsäkerhet (specificitet), och en sämre specificitet kan innebära att man får positivt resultat trots att personen inte har ANCA.

I denna avhandling utvärderades i arbete I en ny mätmetod, capture ELISA, för upptäckt av PR3-ANCA hos patienter med så kallad småkärlsvaskulit. I den mer allmänt använda metoden, direkt ELISA, för mätning av PR3-ANCA binder man PR3 i en plastplatta och låter patienternas blod reagera med detta och påvisar sedan närvaron av ANCA med hjälp av ett färgämne på en tredje antikropp (se Figur 5). I capture ELISA binder man i stället först en monoklonal antikropp mot PR3 i plastplattan och fångar upp PR3 med hjälp av denna. På detta sätt kan PR3 presenteras i en mer naturlig konfiguration.

Vi fann att capture ELISA för PR3-ANCA mätning har en ökad sensitivitet jämfört med tidigare standardmetoder och samtidigt en god specificitet, dvs. att fler patienter med PR3-ANCA hittades med denna metod och att man inte fick en stor mängd falskt positiva patienter (med positiva mätsvar utan att ha sjukdomen) i testen. Vi hävdar därför att denna metod bör vara en del av utredningen av alla patienter med misstänkt småkärlsvaskulit.

I arbete II och V fann vi att patienter med småkärlsvaskulit och njurkärlsinflammation med en stor mängd PR3-ANCA i blodet mätt med capture ELISA hade en sämre överlevnad, sämre njuröverlevnad och fler återfall än patienter med lägre nivåer av PR3-ANCA mätt med denna metod. Denna information om framtida sjukdomsförlopp kunde inte fås med hjälp av den mer allmänt använda metoden, direkt ELISA, för mätning av PR3-ANCA. Detta är ytterligare en anledning till att använda capture ELISA för detektion av PR3-ANCA. Om man redan när diagnosen ställs kan förutsäga vilka patienter som kommer att riskera en svårare sjukdom och risk för skov så skulle man kunna skräddarsy behandling och uppföljning och vara mer vaksam på nya sjukdomstecken. Patienter med en bättre prognos kunde få en mildare och kortare behandling. Den immundämpande behandlingen som används har nämligen inte bara fördelar, det finns också risk för biverkningar. På kort sikt finns risk för svåra infektioner, som till och med kan vara dödliga hos en person med nedsatt immunförsvar, och på lång sikt är risken för bland annat cancer och sterilitet ökad.

PR3-ANCA kan vara riktade mot olika epitoper på PR3. Vi tror att PR3-ANCA mot en eller några få epitoper har mest betydelse för sjukdomsförloppet och att man i capture PR3-ANCA ELISA metoden finner även dessa antikroppar. Därför ville vi försöka utveckla en metod som ännu bättre kunde detektera de PR3-ANCA som skulle kunna förutspå sjukdomsförloppet. För att kunna göra detta måste man veta

var på PR3 epitopen för dessa PR3-ANCA är lokaliserad. I syfte att endast presentera vissa epitoper på PR3 byggde vi med hjälp av rekombinant DNA teknik sammanfogade (chimära) molekyler av PR3 från människor (hPR3) och PR3 från möss (mPR3), dvs. vi ersatte delar av hPR3 med mPR3. De flesta människor bildar inte antikroppar mot mPR3 och de områden av hPR3 som byts ut mot mPR3 tänker man sig som ”en vit fläck på kartan” för PR3-ANCA. Detta är ett tidskrävande detektivarbete och det krävs att man framställer många olika chimära molekyler och testar med PR3-ANCA från många olika patienter i olika stadier av vaskulitsjukdomen för att så småningom förhoppningsvis kunna ringa in den dominanta epitopen.

I arbete IV visade vi att det är möjligt att framställa chimärt PR3 av hPR3 och mPR3 och att dessa chimära molekyler känns igen av monoklonala antikroppar mot PR3. I arbete V använde vi dessa chimära molekyler och undersökte hur PR3-ANCA från patienter med småkärlsvaskulit i reagerade med dessa chimära h/mPR3. Vi samlade in blodprover från 38 frivilliga patienter i samband med varje mottagningsbesök de gjorde och fick på så sätt prover från en och samma patient i olika skeden av sjukdomsförloppet. Även tidigare tagna prover som fanns sparade på forskningslaboratoriet eller analyslaboratoriet Wieslab från diagnostillfället och framåt letades fram. Alla dessa prover analyserades sedan tillsammans med var och en av de chimära h/mPR3. Resultatet av dessa försök var att vi fann att patienter ofta har ANCA mot en del av PR3 vid insjuknandet, men att de vid skov oftast bildar ANCA mot en annan epitop, s.k. epitope shift. Alla patienter har dock inte ANCA mot samma epitop vid insjuknandet. Vi fann att patienter med höga nivåer av ANCA mot en viss del av PR3 vid insjuknandet senare fick skov betydligt oftare än de med ANCA mot andra delar av molekylen. Detta är mycket intressanta fynd, men metoden är ännu inte kliniskt tillämpbar. Förhoppningsvis kan fortsatta studier utveckla metodiken och så småningom ge oss ett nytt verktyg för uppföljning av patienter med småkärlsvaskulit.

Som jämförelse till patienterna med småkärlsvaskulit studerades i arbete III 10 patienter med en form av vaskulit som inte bildar ANCA. Sjukdomen kallas polyarteritis nodosa (PAN) och angriper små och mellanstora kärl. PAN är mindre vanligt förekommande än småkärlsvaskulit, endast omkring 10-15 personer insjuknar årligen i PAN i Sverige medan ungefär 300 drabbas av ANCA-associerad vaskulit. PAN är också betydligt mindre studerat än småkärlsvaskulit. Tidigare har definitionen av PAN varit oklar och då inkluderades i denna sjukdomsgrupp även patienter med t ex hepatit som gav en PAN-liknande sjukdomsbild. Vi fann att 70 % av PAN patienterna i vårt sjukvårdsdistrikt hade njurengagemang vid diagnosen och att 57 % av patienterna fick ett skov inom fem år. Patienterna följdes i genomsnitt 6 år och 2 av patienterna utvecklade med tiden njursvikt och fick börja med dialysbehandling. Våra data motsäger den tidigare rådande uppfattningen att PAN är en ganska mild form av vaskulit som sällan ger skov eller svåra organskador. Dessa patienter måste följas upp med vaksamhet för att förebygga skov och ytterligare skador.

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