ANCA-associated vasculitis : towards patient-tailored therapy

Berden, A.E.

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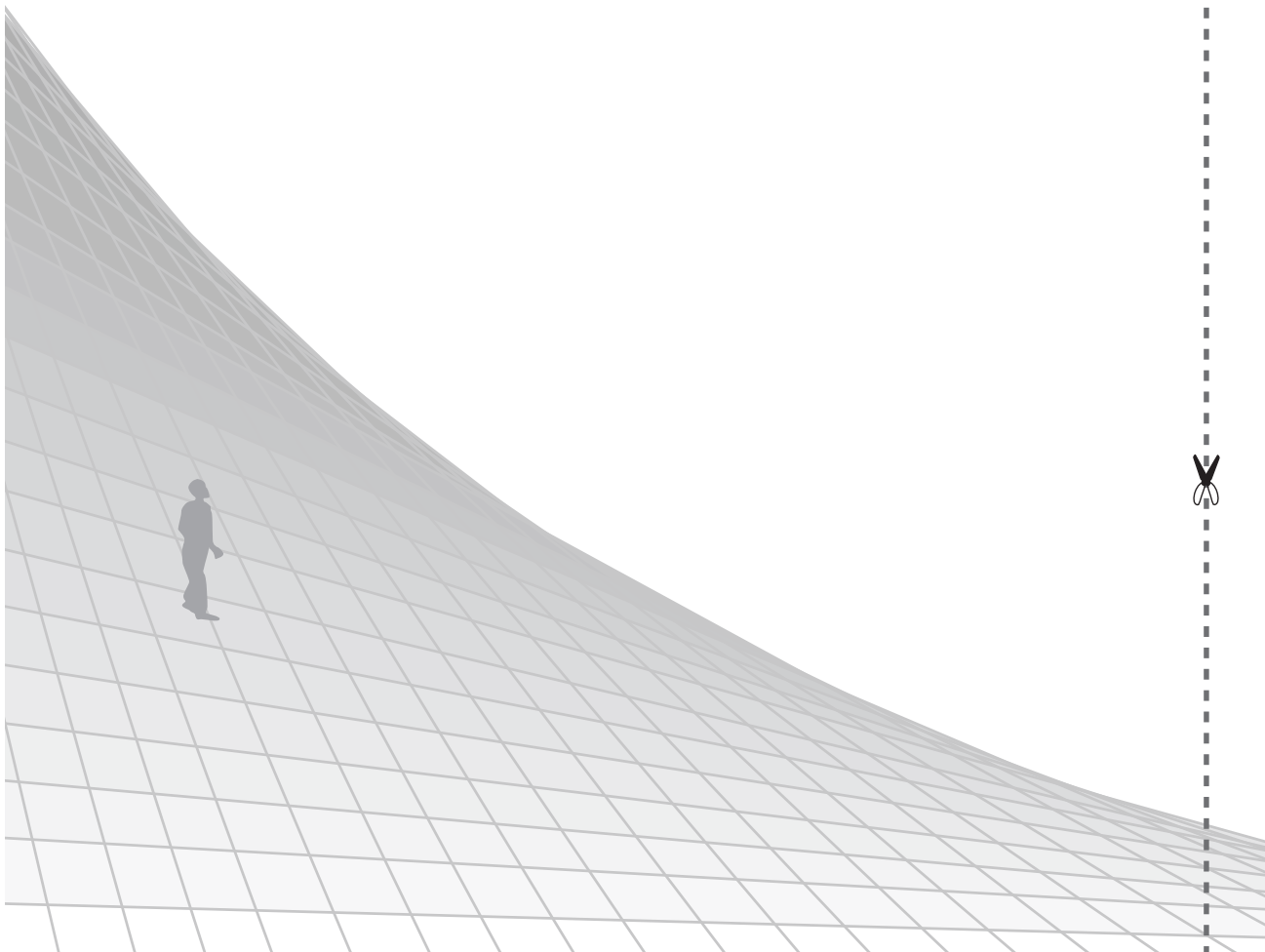
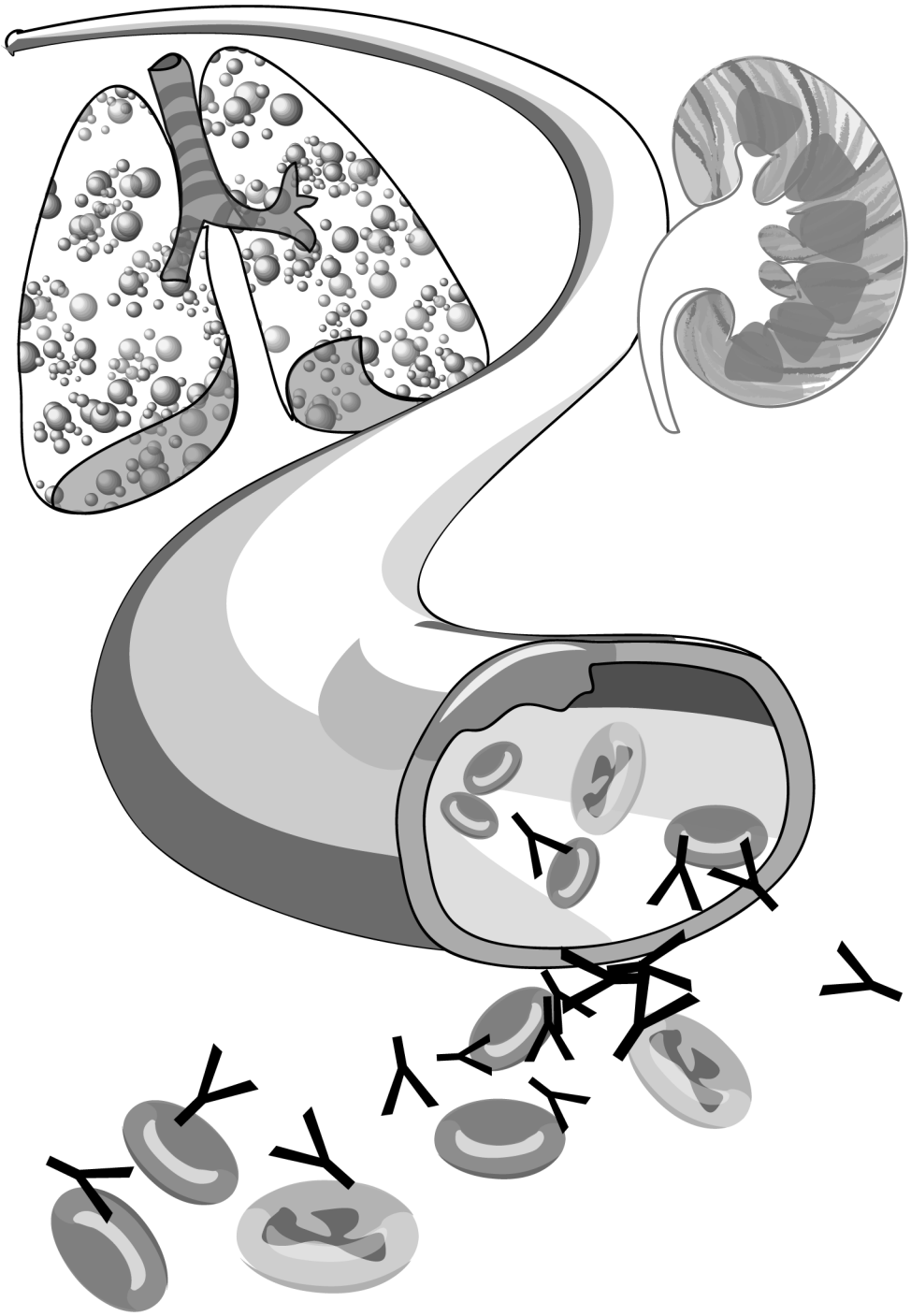
GENERAL INTRODUCTION, AIMS AND OUTLINE OF THIS THESIS





BRIEF INTRODUCTION

A HELICOPTER VIEW OF ANCA-ASSOCIATED VASCULITIS

Case presentation

A 59-year old woman presented with a 3-month history of malaise, epistaxis, arthralgia and constitutional symptoms. She had a productive cough and lost 5 kilograms of weight. The past medical history was unremarkable and she was not on any medication. On clinical examination the patient appeared pale and cachectic but not acutely ill. Further examination demonstrated no abnormalities; specifically the chest was clear on auscultation. Laboratory tests revealed an ESR of 109 mm/hour and a serum creatinine concentration of 355 µmol/L. Dipstick testing of her urine was strongly positive for hemoglobin and protein. Chest radiograph showed bilateral pleural effusion and multiple round shadows in both lung fields. A CT scan showed bilateral non-cavitating nodules. Abdominal ultrasound demonstrated an abnormally decreased cortex/medulla ratio in both kidneys. A nasal biopsy sample revealed necrotizing granulomatous inflammation, renal biopsy confirmed pauci-immune crescentic glomerulonephritis. An indirect immunofluorescence assay for antineutrophil cytoplasmic antibodies was positive with a cytoplasmic fluorescence pattern, and enzyme-linked immunoassay demonstrated high titer anti-proteinase 3 antibodies. A diagnosis of Wegener's granulomatosis with involvement of ear-nose-throat, lungs, joints and kidneys was made. Treatment consisted of cyclophosphamide and high-dose corticosteroids. Within 4 months all pulmonary lesions had resolved and serum creatinine decreased to 105 µmol/L.

This case presentation is classical for Wegener's granulomatosis (WG), one of the antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides. The etiology of WG, analogous to that of other vasculitic diseases, is largely unknown. Classifying the different vasculitides was and is therefore only possible using 'surrogate' classification criteria, such as clinicopathologic disease manifestations and abnormal laboratory parameters. A widely used classification system of the vasculitides is primarily based on the size of the vessels that are affected by the disease process. During the Chapel Hill Consensus Conference on the nomenclature of systemic vasculitis a distinction was made between large-vessel vasculitides, medium-sized vessel vasculitides and small vessel vasculitides.[1] The large-vessel vasculitides affect

the aorta or the largest arterial branches; the disease entities that fall into this category are Takayasu arteritis and giant cell (temporal) arteritis. Among the medium-sized vessel vasculitides, affecting the main visceral arteries and their branches, are classic polyarteritis nodosa and Kawasaki disease. The small vessel vasculitides can be divided into those associated with ANCA and those not associated with ANCA. ANCA are autoantibodies that recognize neutrophil and monocyte constituents, the classical ANCA-antigens are proteinase 3 (PR3) and myeloperoxidase (MPO). Henoch-Schönlein purpura is an example of small vessel vasculitis that is not associated with circulating ANCA. The ANCA-associated vasculitides are all small vessel vasculitides, and are thus characterized by inflammation of small arteries, arterioles, capillaries and venules throughout the body.

Apart from WG, the ANCA-associated vasculitides comprise the following disease entities: microscopic polyangiitis (MPA), renal-limited vasculitis (RLV) and Churg-Strauss Syndrome (CSS). Approximately 90% of patients with active, generalized WG, MPA or RLV have circulating ANCA prior to treatment, and most ANCA are specific for either PR3 or MPO.[2] CSS is often included as one of the ANCA-associated vasculitides, but in comparison to the other diseases it is far less "ANCA-associated", since a substantial percentage of patients with CSS are ANCA-negative.[3] The study of CSS is therefore beyond the scope of this thesis.

The overall incidence of ANCA-associated vasculitis is approximately 20/million, the peak age of onset is between 65-74 years of age, but disease manifestations can occur at any given age.[4] Generally, the disease is somewhat more frequent among men than among women, but when the disease becomes manifest at a younger age, women seem to be more frequently affected than men.[5] ANCA-associated vasculitis is most common in Caucasian populations.[5-7] Family members or twins of patients with WG are rarely reported to have disease manifestations, not supporting a strong genetic predisposition for the disease.[5] The incidence of WG has been reported to be higher in northern Europe, contrariwise, the incidence of MPA has been demonstrated to be higher in southern Europe and Japan.[4;6;8]

Patients with ANCA-associated vasculitis often have had prodromal signs such as 'flu-like' symptoms for several months prior to diagnosis, similar to the case presen-

tation. Early in the diagnostic process, it can be challenging for clinicians to pinpoint the diagnosis of their patient since various organs can be involved, not pointing to a specific disease. There is overlap in symptoms between the different ANCA-associated vasculitides and, in time, the symptoms may change. The diagnostic delay is well characterized by data from a large survey that was completed by 701 patients with WG: for most patients the time between disease onset and diagnosis was somewhere between 3-12 months. Only 7% of patients were correctly diagnosed on their first visit to a physician, approximately 50% of patients visited 4 physicians or more before the diagnosis of WG was made.[5]

For a proper diagnosis, histologic evidence of granulomatous inflammation or small vessel vasculitis/glomerulonephritis, or both, with matching clinical symptoms is required. A positive result on the ANCA tests (indirect immunofluorescence [IIF] and/or ELISA-method) is not sufficient for establishing the diagnosis, but can help considerably since histologic lesions in e.g. airway biopsies can be non-specific.
All ANCA-associated vasculitides are characterized by necrotizing inflammation of small- to medium-sized blood vessels and often, with the exception of RLV, multi-organ involvement. Renal involvement is common and holds the risk of severe renal damage that can lead to end stage renal failure (ESRF) in as short a period as a few days to a week. Most of the research described in this thesis is dedicated to renal disease, the renal biopsy and renal outcome.

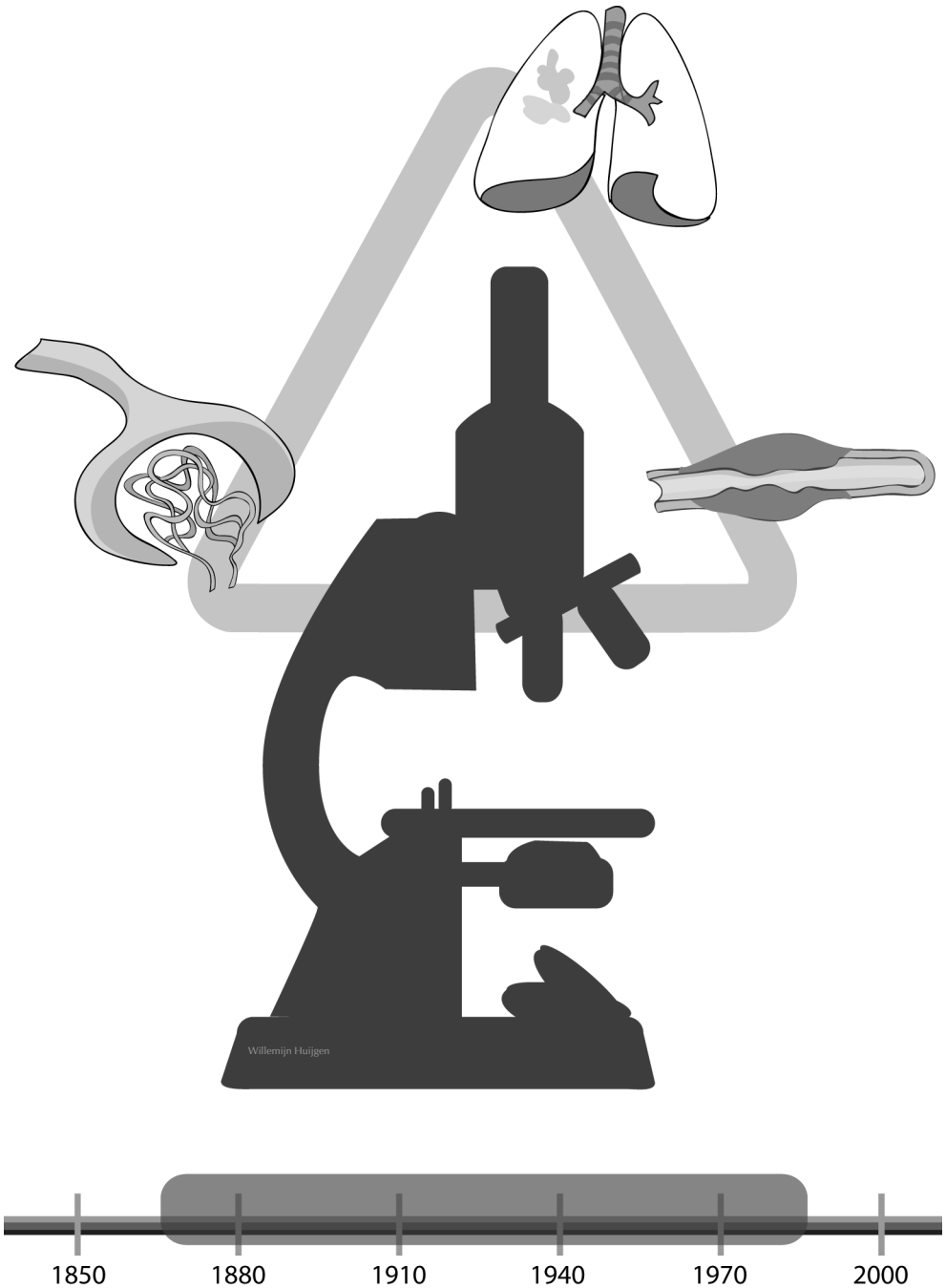
Regarding the etiology of ANCA-associated vasculitis, much is still uncertain, but with time more pathogenetic mechanisms will undoubtedly be unraveled. Currently, ANCA are thought of by most as a pathogenic factor. The most direct clinical evidence for their pathogenicity is found in the reported development of pulmonary-renal syndrome in a neonate, shortly after birth from a mother with MPO-ANCA-positive MPA, most likely because of transplacental transmission of maternal MPO-ANCA.[9;10] This clinical evidence is limited, however, in that it comprises only one case-report, and until today no sequelae have been reported. Moreover, a successful pregnancy and delivery of a healthy normal-term child, despite transplacental transfer of high levels of MPO-ANCA from a mother with MPA, has been reported as well.[11] At birth, MPO-ANCA levels in the child's venous blood were greater than 100 U/ml, but ANCA titers decreased gradually and MPO-ANCA was undetectable at the

age of 4 months (day 120). This child did not develop any clinical manifestations of vasculitis, and therefore this case supports that solely the presence of anti-MPO antibodies in the blood is not sufficiently pathogenic for disease development, and other factors are required.

With the development of the first mouse model in which injection of MPO-ANCA induced glomerulonephritis and vasculitis that was remarkably comparable to human disease, strong *in vivo* evidence for the pathogenicity of MPO-ANCA was obtained.[12] To date, an equally good model has not been successfully developed for PR3-ANCA.

The main issue regarding current therapies is their accompaniment by serious side effects. Over the course of time, ANCA-associated vasculitides have changed from mostly imminent life-threatening diseases[13] into chronic diseases, characterized by a lifelong chance of relapses. Combination therapy with cyclophosphamide and steroids is still considered standard induction therapy for generalized disease, while non-renal disease can also be treated with steroids and methotrexate. Trimethoprim-sulfamethoxazole (co-trimoxazole) is prophylactic against upper airway infections, but may also prevent relapses.[14]

The brief general introduction on the ANCA-associated vasculitides as we know them today ends here, and aims to provide the reader of this thesis with some immediate background information on the topic. The rest of the introduction aims to guide the reader through the history of the ANCA-associated vasculitides; from the first patient ever described in the literature, via the discovery of the association with ANCA, towards the here and now and ending in the future perspectives. At the very end of this introduction, the incentives for and aims of the studies described in this thesis are listed.


Willemijn Huijgen
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1880
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A TIMELINE OF ANCA-ASSOCIATED VASCULITIS

1866-1985: THE "PREHISTORY" OF SYSTEMIC VASCULITIDES, THE ERA BEFORE ANCA

Periarteritis nodosa & the benefit of steroids and alkylating agents

The earliest communications on necrotizing vasculitis are found in the German literature from the end of the 19th century onwards. It is generally accepted that the index case of necrotizing vasculitis was described in 1866 by the internist Adolf Kussmaul and the pathologist Rudolf Maier,[15] although the Viennese pathologist Rokitansky had previously described a similar case,[16] and other comparable cases may have been described in older literature still.

The index patient was a 27-year-old man, a tailor's journeyman, who presented with fulminant, systemic disease including renal involvement (historically termed 'Bright's disease'). At autopsy, numerous nodules were found along multiple muscular-type arteries throughout the body. Kussmaul and Maier attributed these vascular anomalies to "inflammation of the arteries affecting principally the perivascular sheaths, in which the media also had a part at least in its outer layers...and which often attacked neighboring tissues in the opposite direction, for example renal parenchyma, connective and muscle tissue".[17] These pathologic manifestations were the basis for establishing the diagnosis of 'periarteritis nodosa'.

Kussmaul and Maier were the first to distinguish this 'unique arterial disease' from arterial disease caused by infectious agents (such as syphilitic aneurysm), although it has to be said that they did speculate about an infectious cause at first as well, namely a nematode infestation,[18] but they discarded this explanation in their second report because true worms could not be detected upon further careful examination.[15] Of note, apart from the index case of necrotizing vasculitis, Kussmaul and Maier's landmark article also recounts a second patient in whom a muscle biopsy was taken, which could in fact have been the first biopsy in the history of medicine obtained in a living patient ever.[15;19] In any case, it was the first biopsy obtained in a living patient with suspected vasculitis, as well as the first muscle biopsy on a living patient ever (private communication). The patient in question most likely had myositis and not periarteritis nodosa.

At the end of the 19th century, periarteritis nodosa was considered a rare disease with an invariably fatal outcome.[20] Post-mortem examination of patients with periarteritis nodosa showed small nodules (size range: "millet seeds, hemp seeds, peas or even hazelnuts"[16]), filled with fibrin thrombi ("with an appearance from fresh dark red coagulum to rusty brown fibrous tissue"[16]) along small- and medium-sized muscular type arteries, predominantly at branching points, throughout the body but not in the lungs and the brain. These vascular lesions were considered to start off as foci of inflammation with fibrinoid necrosis (eosinophilic amorphous material) and would, via intima media ruptures, result in the formation of aneurysms. In classical periarteritis nodosa, capillaries were not involved. An unknown infectious agent was still thought by many to be responsible for the development of periarteritis nodosa at that time.[20]

In 1903 Ferrari suggested to change the name periarteritis nodosa into polyarteritis nodosa, after having shown that the disease did not only comprise inflammation of the outer coat of (medium-sized) arteries, but that all layers of the arterial wall were affected by the inflammatory process.[21] In that same year, 1903, Veszprémi and Jancsó first reported a case wherein the diagnosis was made based on microscopic findings,[20;22] however, they do describe macroscopic changes as well, especially in the coronary arteries and the arteries of the bowel.[19]

In 1923 Wohlwill described a form of microscopic polyarteritis in two patients,[23] but this report was largely forgotten,[24] even though it provided for the first time a comprehensive discussion of the differences compared to classic polyarteritis nodosa. Wohlwill used the term 'nodosa' in the title of his manuscript, but in fact had not found nodular lesions upon examination of his two cases. Evident macroscopic vascular changes were not found in either of the cases. The patients both had suffered from systemic disease including glomerulonephritis, and disease manifestations were evident in the smallest arteries, arterioles, capillaries and venules.[23] Wohlwill gives the following summary of his post-mortem findings on one of his patients "Arterien makroskopisch intakt. Mikroskopisch: Typische Veränderungen der Periarteriitis nodosa an den kleinsten Arterien der Muskeln und Nerven, des Darms, Gehirns, Hodens, des Herzens und der Nieren" [free translation: Arteries macroscopically intact. Microscopically: typical changes of periarteriitis nodosa of the smallest arteries of muscles, nerves, bowel, brain, skin, heart and kidneys].[23] Macroscopically, vascular disease

was not expected in either of the two cases described by Wohlwill, but the smallest arteries in different organs evidently showed typical changes of periarteriitis nodosa. At about the same time that a microscopic form of polyarteritis nodosa was recognized, cases of polyarteritis nodosa with lesions in the lungs were described as well.[25;26] One of the reported cases with lung involvement stood apart from the hitherto described 'classical polyarteritis nodosa' cases, because of the marked eosinophilic infiltrates that were seen in different tissues.[26]

In the same period two German pathologists, Heinz Karl Ernst Klinger and Friedrich Wegener, first recognized and described patients with necrotizing vasculitis and glomerulonephritis accompanied by necrotizing granulomatous inflammation of the respiratory tract, the disease entity that is currently known as WG.[27;28] In 1931 Klinger reported two cases with as he called it at the time "borderline variants of periarteriitis nodosa".[27] One patient had destructive sinusitis, nephritis, and disseminated vasculitis.[27] Five years later in 1936, and again in 1939,[28;29] Klinger's friend from medical school Friedrich Wegener explicitly defined this disease as a distinct clinical and pathologic entity.

In 1934 Wegener performed an autopsy on a 38-year-old man who had died from uremia after febrile illness.[24] Wegener described inflamed nasal mucosa and cartilage with destruction of the nasal septum. The patient had extensive ear-nose-throat symptoms and an evident saddle nose deformity. Histologic examination of affected tissue demonstrated that the inflammatory process was granulomatous and necrotizing. In the kidneys, necrotizing glomerulonephritis was evident. A few years later Wegener performed a post-mortem examination on a second patient, and autopsy findings strikingly resembled those of the first patient. This patient was a 36-year-old housewife, who had died after an illness characterized by chronic rhinitis and renal failure.[24] Wegener studied these (and other) cases thoroughly, and in 1939 published the full report on the clinicopathologic manifestations of three patients that has become most famous.[28] Wegener himself referred to the disease characterized by necrotizing granulomatous inflammation of the respiratory tract, focal necrotizing glomerulitis and a systemic vasculitis affecting arteries and veins as a unique rhinogenic granulomatosis. After World War II, isolated case reports linked the name of Wegener to this form of granulomatosis, and in 1954 Godman and Churg clearly established

the name Wegener's granulomatosis.[30] In this landmark article Godman and Churg reviewed 22 cases from the literature and 7 patients of their own. The diagnostic criteria that later became known as Wegener's triad are formulated in this article, namely 1) necrotizing granulomata of the (upper and/or lower) respiratory tract; 2) generalized focal necrotizing vasculitis affecting arteries and veins; 3) focal necrotizing glomerulitis (glomerulonephritis). In detail, Godman and Churg describe the characteristic histopathologic renal lesions to consist of "fibrinoid necrosis with destruction of one or more glomerular capillary loops associated with polymorphonuclear cell exudation" and refer to epithelial crescent formation of Bowman's capsule as a "healing stage".

Before long it was recognized that patients not necessarily fulfilled all the diagnostic criteria of the Wegener's triad, and Carrington and Liebow first adopted the term 'limited' WG for disease characterized by prevalent pulmonary lesions (with or without limited extrapulmonary manifestations) in patients who did not have signs of glomerulonephritis.[31;32] Soon hereafter, DeRemee and colleagues proposed the ELK classification for patients with WG, based on the presence of Ear-nose-throat, Lung and Kidney involvement. The concept of ELK was helpful in the management of those cases that did not match the strict criteria of Godman and Churg.[33] Those patients without glomerulonephritis clearly had a better prognosis than 'classical' patients who met all criteria of the triad.

Wegener had always disliked the eponym Wegener's granulomatosis,[34] and it has been suggested that the question whether the eponym should be continually used warrants balanced discussion within the scientific medical community.[24] In fact, recently an alternative name for WG was proposed, namely granulomatosis with polyangiitis, which can be abbreviated to GPA.[35]

Wegener had described 'his disease' as uniformly fatal. A natural history study of 56 patients reported by Walton in 1958 demonstrated that on average patient survival was approximately 5 months; 82% of patients did not survive the first year after diagnosis and > 90% of patients died within 2 years.[13] The major cause of death in these patients was 'uremia' caused by rapidly progressive renal failure, and the second most frequent cause of death was respiratory failure.[13] Walton postulated that 'the ulceration of the respiratory tract is primary and that the widespread lesions occur later

in the natural history of the disease'.[13] Furthermore, Walton discussed that the widespread granulomata found in WG resembled those found in allergic granulomatosis as described by Churg and Strauss in 1951,[36] while the necrotizing vascular lesions resembled microscopic polyarteritis[37] or Zeek's hypersensitivity angiitis.[20;38]

In the second half of the 20th century, WG was regarded as a hypersensitivity disorder,[13;39] and as a separate disease entity from 'classical polyarteritis nodosa' (affecting medium-sized arteries, generally without primary extravascular lesions) and the microscopic form of polyarteritis nodosa (affecting capillaries and veins, with lung involvement) or allergic angiitis.[20;37] In the etiology of polyarteritis nodosa, a role for hypertension has been proposed, as reviewed by Zeek in 1952.[20]

Antibiotics,[13] chelating agents (EDTA)[40] and local radiotherapy (upper respiratory tract lesions)[13;41] have early on been used to treat WG, but the first substantial improvement was made after the introduction of corticosteroids to dampen the immune response.[42;43] Around the same time as the benefit of corticosteroids was acknowledged and described in the literature, the first reports described the use of alkylating agents such as nitrogen mustard and chlorambucil in (limited) WG.[44;45] Fahey *et al.* actually were the first to report the use of cytotoxic chemotherapy in the form of nitrogen mustard in a 38-year-old man with WG already in 1954.[39]

In 1967 Hollander *et al.* reported a patient with extensive ear-nose-throat symptoms, pulmonary infiltrates, generalized muscle pains, fever, skin rash and abnormalities on urinalysis, who they first treated with high dose prednisone.[43] Despite the high dose of prednisone, however, this patient suffered a rapid deterioration of renal function. Consequently this patient received nitrogen mustard intravenously and after approximately one week the patient's renal function ameliorated and the patient recovered. Chlorambucil was then started and the dosage was gradually increased during the same time that the prednisone dose was tapered. After over 1 year of follow-up the patient was still considerably well. In reviewing the literature on treatment up to 1967, Hollander *et al.* remark that large daily doses of corticosteroids should be used early in the therapy of the disease, but that nevertheless corticosteroid therapy alone invariably failed to achieve remission in patients with advanced disease characterized by renal and pulmonary failure.[43] Patients who received corticosteroids in a termi-

nal phase of their disease clearly had no benefit of this therapy at all. Mean survival time reported in the survey by Hollander *et al.* in patients treated with corticosteroids was 12 ½ months,[43] which was a substantial improvement compared to the 5 months in Walton's natural history study.[13] In their review of the literature Hollander *et al.* concluded that radiotherapy, although some improvement of upper respiratory tract lesions was noted in some patients, was not found to be beneficial in patients with pulmonary and renal disease. Finally, what the paper by Hollander has become famous for, is that they review the four patients[43-45] who by that time had received alkylating agents and in whom apparent improvement was noted. Hollander *et al.* also posed the question whether alkylating agents should be used as a sole therapeutic agent or in combination with steroids, and they suggested long-term use of the alkylating agent they themselves studied, chlorambucil, for remission maintenance. In the period wherein the paper by Hollander was published, beneficial effects of azathioprine alone[46] or in combination with duazomycin A[47] were reported as well.

The therapeutic potential of alkylating agents became clear-cut after a series of publications by Fauci *et al.* from 1971 onwards on their experience with cyclophosphamide in patients with WG.[48-50] In a comprehensive study of 18 patients, 15 with generalized disease and 3 with limited disease, Fauci *et al.* elaborated on the clinical manifestations, and of particular interest to this thesis, on renal histopathology.[50] They described focal glomerulitis as the most common renal lesion, especially in patients wherein a timely diagnosis was obtained. Other common findings were proliferative glomerulonephritis, hyalinization of glomeruli and interstitial nephritis. Immunofluorescence microscopy was performed on one renal biopsy and a coarse granular glomerular staining pattern positive for IgG and C3 was demonstrated. This was in agreement with a small number of reports published around the same time that also described a coarse granular staining pattern.[51-53] Of the 18 patients Fauci *et al.* described, 1 patient did not receive cytotoxic chemotherapy and 2 patients received this therapy for less than 1 week. Of the remaining 15 patients, 14 received cyclophosphamide and 1 patient was started on azathioprine. The latter patient was a 31-year-old woman who wished to have children and by that time the risk of infertility with cyclophosphamide therapy for both men and women was already acknowledged.[54;55] This was not a problem that was evident for therapy with azathioprine.[56] Most of the patients had been started on corticosteroid therapy prior to starting cytotoxic chemo-

therapy. Patients were started on 1 to 2 mg/kg/day of oral cyclophosphamide or azathioprine. Three patients who presented with fulminant disease received intravenous cyclophosphamide, 2 to 4 mg/kg/day for the first couple of days (up to a week) and were later switched to oral cyclophosphamide. These initial dosages were maintained for 10 days to 2 weeks before dose adjustments were made where necessary. Among the 15 patients who received adequate cytotoxic therapy, 12 patients had generalized disease and a striking response of renal manifestations to cyclophosphamide was seen. Several patients had follow-up renal biopsies, and disappearance of disease activity in the kidneys could be visualized. Of the 15 patients, a total of 13 achieved complete clinical remission. One patient did achieve complete remission at first, but relapsed and developed ESRF, after which this patient elected to discontinue hemodialysis and died shortly afterwards. The other patient did not reach remission at any point in time at all and the disease relentlessly progressed over a course of 9 months, ending in death from renal failure. Taken together, cyclophosphamide clearly was a potent drug in treating disease manifestations of WG. Fauci *et al.* had previously described that cyclophosphamide, in the doses employed in WG, suppressed delayed hypersensitivity reactions and antibody responses to a new autoantigen without affecting established delayed hypersensitivity reactions.[48] Clearly cyclophosphamide was a potent drug, but the optimum duration of therapy was a matter of debate in the late 20th century. Views had rapidly been changing. At first WG was a uniformly fatal disease requiring long-term or even permanent cytotoxic therapy, but later considerable disease control could be achieved and, because of the adverse event profile of cytotoxic agents (notably leukopenia, risk of malignancies[57]), attempts were then made to taper and when possible stop therapy. Fauci *et al.* recommended in the 1970s that in patients who promptly responded to cyclophosphamide therapy, therapy should be tapered and stopped 1 year after all traces of disease activity disappeared. Regarding patients with an inadequate response and/or smoldering disease no clear recommendation was made, and this was left to best clinical judgment at the time. Adverse events were considerable with cyclophosphamide, but the side effect profile of high-dose long-term corticosteroid treatment was unfavorable as well, and it was evident that such a therapy regimen was undesirable too.[58]

By the end of the 20th century only limited advances in understanding the pathogenesis of vasculitis had been made, notably a hypersensitivity phenomenon was still

regarded likely, as was an autoallergic phenomenon, but no causative antigen or toxin had been isolated.

Fauci *et al.* did report that patients with severe sinus disease, common in WG, often developed secondary infections wherein *Staphylococcus (S.) aureus* was the most frequently cultured microorganism.[50] In these patients, appropriate antibiotic therapy proved highly successful,[50] but *S. aureus* was not considered to be the trigger for disease development, because it was regarded to be secondary to impaired drainage of damaged nasal sinus tissue. The respiratory tract was considered to be the initial site for a hypersensitivity reaction to develop, and subsequently there could be spreading to the kidneys, ultimately resulting in multi-organ involvement.

Although by the end of the 20th century many physicians treated patients with (classical) WG with high dose corticosteroids and cyclophosphamide, antimicrobial therapies were also still advocated by some. DeRemee and colleagues first observed improvement in a WG patient treated with antimicrobial drugs in 1975, and ten years later they reported salutary effects of treatment with these agents in a series of 12 patients. Particularly trimethoprim/sulfamethoxazole treatment was reported to be effective.[59] These results supported the use of co-trimoxazole in patients with (limited) WG, and interestingly this drug is still in use in present day treatment of WG. Finally, after the benefit of corticosteroids and alkylating agents had been documented, it was recognized that there could also be a substantial role for plasma exchange in the treatment of patients with systemic vasculitis.[60]

1960
1970
1980
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2000
2010

ANCA-associated vasculitis & ANCA-assay standardization

In 1985 European investigators (the Dutch/Danish Cattegat Study Group) first associated seropositivity for ANCA with WG.[61] This finding would later prove to have provided clinicians with a valuable diagnostic tool. A few years earlier, in 1982, the presence of ANCA was already reported in patients with pauci-immune glomerulonephritis by Davies and colleagues.[62] Earlier still, in 1980, Wiik (later a member of the Dutch/Danish Cattegat Study Group) reported on the existence of granulocyte-specific antinuclear antibodies, so-called GS-ANA, detectable in sera of patients with rheumatoid arthritis (RA).[63] At that point, it was already evident that the antigens these GS-ANA recognized were very heterogeneous, although a couple of distinct and reproducible staining patterns could be recognized on testing patient sera with IIF techniques.

The hallmark paper by van der Woude *et al.* that was published in 1985 described autoantibodies (IgG) in patients with WG that were in several ways similar to the GS-ANA described in RA. However, they were not directed against nuclear but against cytoplasmic components of neutrophilic granulocytes. Van der Woude *et al.* first named them ACPA - or anticytoplasmic antibodies - this term was later replaced by ANCA because they were directed against neutrophil (and monocyte) constituents. Comparable to the detection of GS-ANA, ANCA were detectable by IIF techniques, and several distinct patterns could be recognized. In the IIF procedure granulocytes of healthy donors are incubated with patient sera and evaluated by immunofluorescence microscopy. The first indirect immunofluorescent staining pattern detected testing serum of patients with systemic vasculitis was the cytoplasmic, or cANCA pattern.

For the time being, the nature of the ANCA-antigen(s) remained unknown, but the association of ANCA with WG reported in 1985 raised broad scientific interest in the topic. The first international meeting on ANCA, called the ANCA workshop, was held in Copenhagen in January 1988. In the year wherein the first ANCA workshop was held, a second type of ANCA was identified. This ANCA demonstrated reactivity to MPO, a lysosomal protein stored in azurophilic granules of neutrophils, and a somewhat different indirect immunofluorescent staining pattern was described. This

second staining pattern was designated as a perinuclear or pANCA pattern.[64] Shortly after the discovery of MPO-ANCA, the antigen that elicited the cANCA pattern on IIF was identified as well. Ludemann *et al*. were the first to conclude that cANCA were most probably directed against PR3, a neutrophil serine protease,[65;66] and this was confirmed almost instantly by others.[67-69]

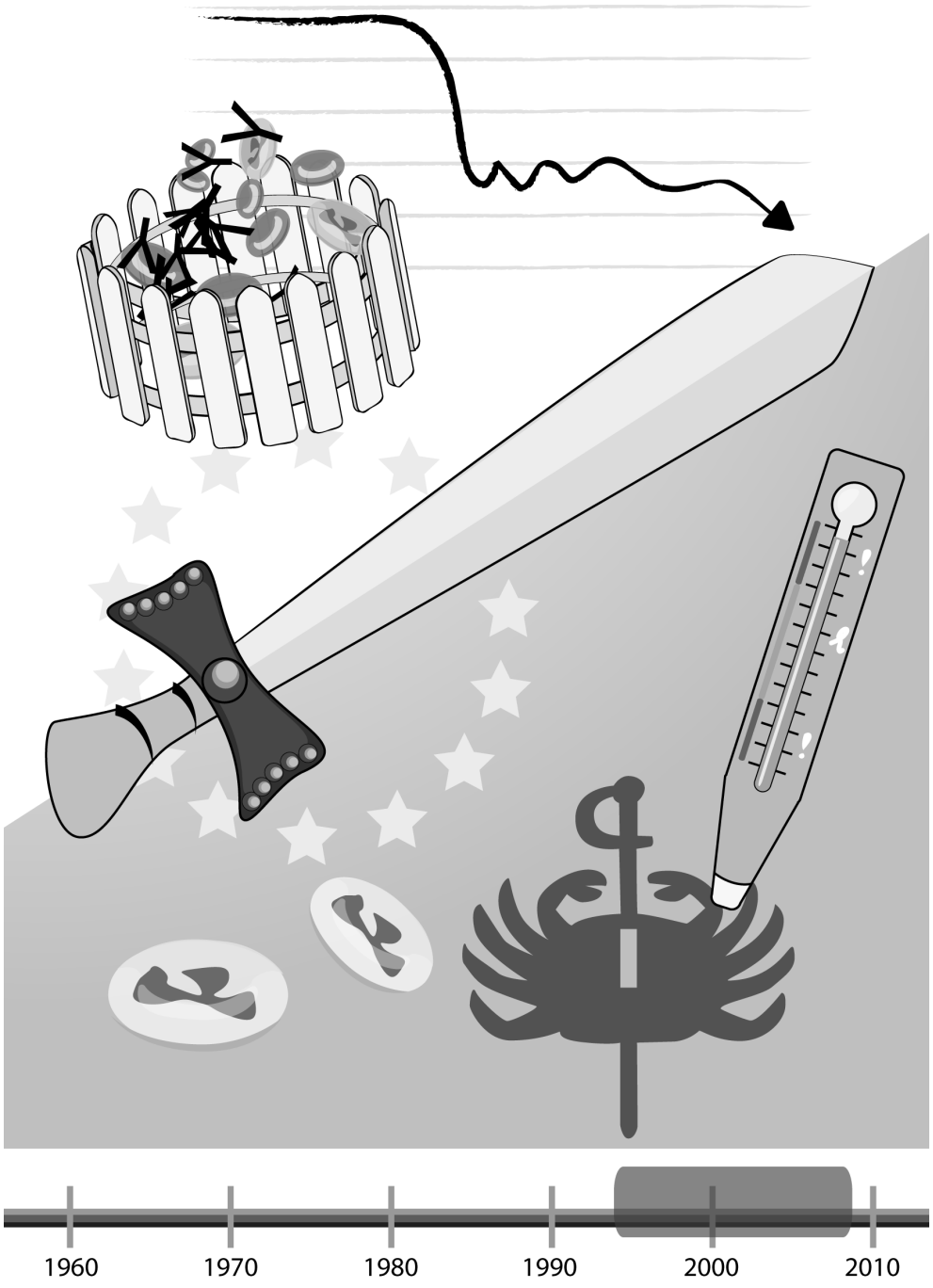
During the first International Vasculitis & ANCA workshop in 1988 several decisions were made on how to proceed with the development and subsequent standardization of ANCA tests, particularly considering antigen-specific solid phase assays (adopted from a letter by Rasmussen *et al*.[70]). First, Statens Seruminstitut in Copenhagen would provide an international reference serum, positive for cANCA. When performing standard IIF with ethanol-fixed, smeared or cytospun healthy human leucocytes, a sample would be considered positive for cANCA when there was uneven, granular staining of the neutrophil/monocyte cytoplasm, identical to that of the international reference serum. At the time of the first ANCA workshop, the pANCA pattern had just been discovered, and it was therefore decided that a second international standard reference serum should be obtained for pANCA as well. The pANCA pattern seen on IIF, obtained with patient serum that contains MPO-ANCA, is actually an artifact. During ethanol-fixation of neutrophils from a healthy blood donor, MPO is redistributed from its original location inside the neutrophils' primary granules towards the negatively charged nucleus,[71] resulting in a perinuclear instead of a granular, cytoplasmic staining pattern, and it is therefore distinguishable from the cANCA pattern obtained with PR3-ANCA. Antigen-specific ELISA techniques for ANCA detection would be further developed and standardized. Correct IIF interpretation was considered a challenge and it was therefore recommended that routine ANCA determination in centers using standard IIF techniques should only be applied after examination of > 1000 sera, and preferably only after confirmation of test results by experienced centers.[70]

The hallmark paper by van der Woude *et al.,*[61] and the proceedings at the first ANCA workshop resulted in a broad European collaborative project, aiming to standardize assays for ANCA detection as well as to explore the role of ANCA as a diagnostic tool in vasculitis. The EU collaborative project was brought into life in 1989.[70;72;73] At the start seven medical centers participated (Bad Bramstedt [Germany], Cambridge

[United Kingdom], Copenhagen [Denmark], Leiden [the Netherlands], London [United Kingdom], Paris [France], Raisdorf [Germany]). Soon, the number of participating centers doubled and these 14 centers (previous 7 and Barcelona [Spain], Brussels [Belgium], Groningen [the Netherlands], Heidelberg [Germany], Ioannina [Greece], Milan [Italy] and Stockholm [Sweden]) jointly became the European Vasculitis Study Group (EUVAS).

First funding came from the Bureau Central de Reference, an office of the European Union. The first study conducted by the EUVAS was called the EC/BCR study, and resulted in the development and standardization of solid phase assays (ELISAs) making use of purified PR3 and MPO that enabled antigen-specific ANCA detection.[72] The EC/BCR study was important in the development of the International Consensus Statement on Testing and Reporting ANCA, which advocated screening by IIF and confirmation of IIF positivity in PR3-ANCA and MPO-ANCA ELISAs.[74;75]

Also part of the EC/BCR project was the evaluation of renal biopsies for histopathologic predictors of renal outcome. A total of 157 biopsies of patients enrolled in the EC/BCR project for ANCA assay standardization were available for the analysis of clinicopathologic correlations. All renal biopsies were scored according to a standardized scoring protocol, developed specifically for the evaluation of renal biopsies of patients with systemic vasculitis.[76] This large clinicopathologic study showed that not so much active lesions such as cellular crescents, which traditionally received much attention, but the proportion of normal glomeruli proved a good predictor of renal function during follow-up.[77]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Reducing cumulative cyclophosphamide exposure: a double-edged sword

In 1994 a grant was obtained from the European Union BIOMED 1 programme, that enabled the EUVAS to conduct three randomized controlled clinical trials for vasculitis and one single limb trial, this project was named the ECSYSVASTRIAL. Apart from funding these clinical trials, the grant also enabled the EUVAS to develop methodology for conducting clinical trials for systemic vasculitis. The trials started patient recruitment in 1995, and the main goal of these trials was to compare established therapies for vasculitis with regard to efficacy and safety profiles. The three randomized controlled clinical trials were the NORAM trial, the CYCAZAREM trial and the MEPEX trial. The single limb trial was called SOLUTION.

The NORAM trial was designed to investigate if methotrexate could replace standard therapy with cyclophosphamide as induction treatment of patients with newly diagnosed, limited ANCA-associated vasculitis (serum creatinine levels < 150 µmol/l, without critical organ manifestations of disease). A total of 100 patients were randomized to receive either methotrexate or cyclophosphamide. Patients in both trial limbs received the same corticosteroid regimen. All drugs were tapered and stopped at 12 months, patient follow-up continued until 18 months. NORAM's primary endpoint was the rate of remission achieved at 6 months. At 6 months, the remission rate achieved in the methotrexate treatment limb (89.8%) was not inferior to that achieved in the standard therapy limb (93.5%). Therefore it could be concluded that methotrexate could in fact replace cyclophosphamide for induction treatment of patients with limited ANCA-associated vasculitis. However, after termination of treatment at 12 months, it was evident that methotrexate treatment was associated with more relapses (relapse rate of 69.5% at 18 months) compared to standard treatment with cyclophosphamide (relapse rate of 46.5% at 18 months). Relapse rates were considerable in both treatment arms, and therefore the results of NORAM supported prolonged continuation of immunosuppressive treatment. Summarizing, the result of the NORAM trial was as follows: methotrexate can substitute cyclophosphamide in patients with limited ANCA-associated vasculitis, but immunosuppressive therapy should not be stopped at 12 months. NORAM was published in 2005.[78]

The second trial was called CYCAZAREM, and aimed to assess whether azathioprine could substitute cyclophosphamide for remission maintenance purposes in patients with generalized ANCA-associated vasculitis (serum creatinine < 500 µmol/L). Remission was achieved in 144 of the 155 patients under study, after having received 3 to 6 months of standard induction therapy with cyclophosphamide and corticosteroids. These 144 patients were then randomly assigned to receive either azathioprine or cyclophosphamide remission maintenance therapy. Eleven relapses were documented in the azathioprine limb (15.5%) compared to ten in the cyclophosphamide limb (13.7%). Severe adverse event occurrence was not different between the limbs. Therefore, the conclusion of CYCAZAREM was that in patients with generalized vasculitis, cyclophosphamide could safely be substituted with azathioprine in the remission maintenance phase without increasing the occurrence of relapses. Substitution with azathioprine thus provided a means to safely reduce cumulative cyclophosphamide exposure. CYCAZAREM was published in 2003.[79] Comparable to the clinicopathologic study that was part of the EC/BCR project, renal histology was also evaluated with regard to renal outcome for the patients entered in the CYCAZAREM trial, who all presented with moderate renal involvement. This study demonstrated that an impaired baseline renal function and a high extent of chronic renal lesions present in the diagnostic renal biopsy (glomerulosclerosis, interstitial fibrosis, tubular atrophy) were strongly correlated to adverse renal outcome. Active lesions on the other hand, such as cellular crescents and fibrinoid necrosis, were predictive of renal function recovery, an indication that these active lesions may be reversible. Importantly, this histopathologic study demonstrated that a combination of baseline renal function and renal histology better predicted renal outcome than did baseline renal function alone.[80]

The third trial was the so-called MEPEX study in which patients with ANCA-associated vasculitis and severe renal disease (serum creatinine > 500 µmol/L) were all treated with standard combination therapy with cyclophosphamide and steroids, and additionally were randomized to receive adjunctive therapy with either intravenous methylprednisolone or plasma exchange. Providing ANCA are pathogenic, removing immunoglobulins by means of plasmapheresis or plasma exchange could in theory be an effective treatment modality. In the MEPEX study, a total of 137 patients with a new diagnosis of generalized ANCA-associated vasculitis (serum creatinine > 500 µmol/L) were randomized to receive either seven plasma exchanges or intravenous

methylprednisolone as adjunctive therapy. The primary endpoint of this study was dialysis-independency at 3 months. At 3 months, 49% of patients in the methylprednisolone group were alive and off dialysis compared with 69% of patients in the plasma exchange group, this difference reached statistical significance. Patients who were randomized to receive plasma exchange were at a 24% reduced risk for progression to ESRF at 1 year compared with patients who received methylprednisolone. Patient survival and severe adverse event rates at 1 year were no different between trial limbs. At 1 year, patient survival and severe adverse event rates were 76% and 48% in the group that received methylprednisolone, compared with 73% and 50% in the group that received seven plasma exchanges. Concluding, although patient survival was similar in both groups at 1 year, a clear beneficial effect of plasma exchange on renal recovery was demonstrated in this trial. MEPEX was published in 2007.[81] A companion article had previously described histopathology and its correlations with renal outcome in this group of patients presenting with severely impaired renal function.[82] One hundred renal biopsies taken at baseline were available for study, and a total of 39 histologic parameters were investigated. Both chronic and acute tubulointerstitial lesions (tubular atrophy and tubulitis) were negatively correlated to renal function at 1 year after baseline in these patients with severe renal involvement. Baseline renal function was positively correlated to renal function at 1 year. Importantly, the percentage of normal glomeruli was positively correlated to ameliorated renal function at 1 year as well as to dialysis-independency at follow-up. It was therefore concluded that the proportion of glomeruli that are not affected by the disease process at the time of diagnosis are fundamentally important in the prediction of renal outcome.[82]

The fourth trial was a single limb trial by the name of SOLUTION. This was an open study in which 15 patients with WG refractory to standard therapy with cyclophosphamide and corticosteroids were experimentally treated with antithymocyte globulin (ATG). Of these fifteen patients, four achieved complete clinical remission, nine achieved partial clinical remission and two patients died within a few days after the first ATG dose (causes of death: pulmonary hemorrhage and infection). It was concluded that ATG could be a therapeutic option for refractory WG, however, concurrent infections were a clear contraindication for this treatment.[83]

In 1999, when the first four EUVAS therapeutic trials were well underway, a second European Union grant was awarded, this time under the BIOMED 2 programme. The ECSYSVASTRIAL project involved three randomized clinical trials and one single limb study, and the second project for which funding was now obtained, under the name of AVERT, involved another three clinical trials aiming to ameliorate therapy regimens. These trials received the names CYCLOPS, IMPROVE and REMAIN.

While the CYCAZAREM trial already demonstrated that azathioprine was a good substitute for cyclophosphamide as remission maintenance therapy, investigators still sought ways to further reduce cumulative cyclophosphamide dose, because of the association of cyclophosphamide exposure with the development of malignancies. The CYCLOPS trial compared pulse cyclophosphamide to daily oral cyclophosphamide for remission induction, to assess whether pulsed intravenous cyclophosphamide administration could reduce cyclophosphamide exposure in the remission induction phase. The 149 patients with newly diagnosed generalized ANCA-associated vasculitis who were enrolled in the CYCLOPS trial were randomized to receive pulse or daily oral cyclophosphamide until 3 months after clinical remission was reached. All patients were then switched to azathioprine for remission maintenance therapy. Patients who did not enter remission by 9 months were from then on treated according to best local practice. Summarizing, according to protocol patients received cyclophosphamide for a minimum of 6 and a maximum of 12 months and were then switched to azathioprine until the end of follow-up at 18 months. CYCLOPS' primary endpoint was the time to remission, and this was not different between trial limbs. The relative number of patients who entered remission was comparable between the treatment arms as well. Cumulative cyclophosphamide dose was higher in the daily oral group than in the experimental pulse group. In agreement with this finding, the rate of leucopenia was lower in the pulse group. Relapse rate was not a study outcome, and follow-up was limited. It remains to be seen if the benefit obtained with pulse cyclophosphamide of a reduced cumulative cyclophosphamide dose is accompanied with an increased risk of relapse. CYCLOPS was published in 2009.[84] The patients recruited into NORAM, CYCAZAREM, MEPEX and CYCLOPS were all included into a long-term follow-up study. Results on long-term patient survival and renal outcome are described in this thesis in **Chapters 2 and 3**.

The second trial that was launched with support from the European Union BIOMED 2 programme was called REMAIN. Because high relapse rates pose a considerable problem in the clinical management of patients with ANCA-associated vasculitis, the EUVAS designed a trial to investigate prolonged remission maintenance therapy. Where previous trials limited azathioprine remission maintenance therapy to 18 months after study entry, REMAIN continues maintenance therapy with azathioprine and prednisolone up to 4 years after trial entry in the experimental limb, while remission maintenance in the 'standard' limb is withdrawn between 18-24 months after diagnosis. The primary endpoint of REMAIN is the relapse rate, patient recruitment has been completed and results are expected in 2012.

The third trial under the BIOMED 2 programme was called IMPROVE and compared mycophenolate mofetil (MMF) with azathioprine for remission maintenance therapy in ANCA-associated vasculitis with renal involvement. Patient recruitment was completed in 2004. The IMPROVE trial showed, against prior beliefs, that MMF was a less potent drug than azathioprine for maintaining disease remission. In this study, adverse event rates were similar in patients treated with MMF or azathioprine.[85]

A couple of years later, another three randomized controlled clinical trials were started by the EUVAS. This time not with funding from a European Union grant, but in cooperation with the pharmaceutical industry. These trials were investigator-initiated. Among the trials were the RITUXVAS trial (launched in 2006) and the MYCYC trial (launched in 2007). The latter trial, MYCYC, investigates the potential of mycophenolate mofetil as an agent for remission induction therapy, the first patient was recruited in June 2007.

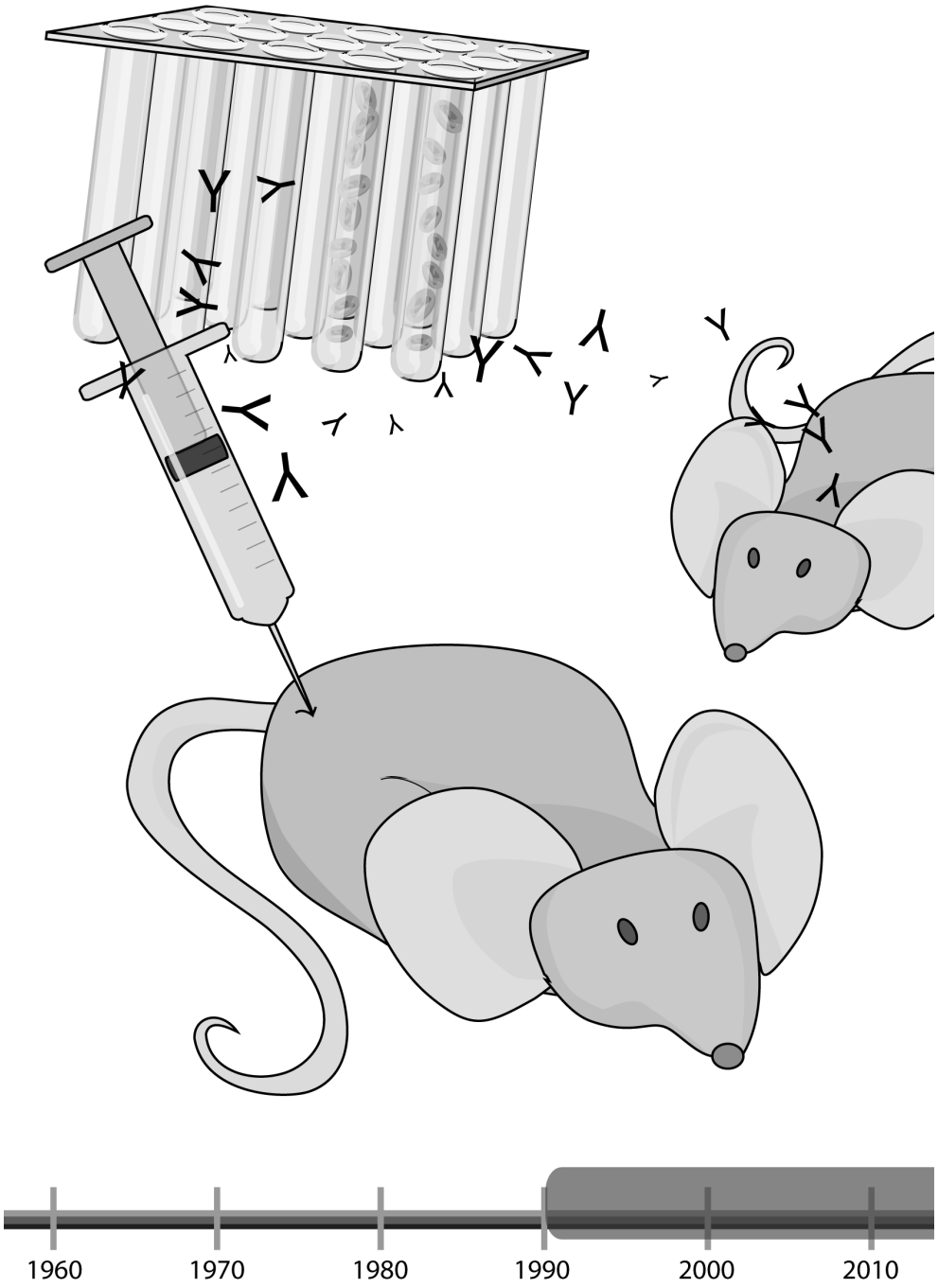
Although standard therapy with cyclophosphamide and corticosteroids clearly is effective in establishing remission in the majority of patients with ANCA-associated vasculitis, relapse rates remain high. Apart from studying prolonged remission maintenance therapy (REMAIN study) new treatment modalities are continually developed and require exploration in clinical trials. One of the candidate drugs for new effective treatment of ANCA-associated vasculitis was rituximab. Rituximab is a genetically engineered chimeric murine/human monoclonal antibody directed against the antigen CD20 (human B-lymphocyte-restricted differentiation antigen, alternatively

named Bp35 or B1). CD20 is expressed on the surface of pre-B cells and mature B lymphocytes, but not on pro-B cells or plasma cells, and regulates early step(s) in B cell activation and differentiation.[86] Rituximab consists of murine light- and heavy-chain variable regions and human kappa light-chain and IgG1 heavy-chain constant regions. The *in vitro* mechanism of action is as follows: the Fab (fragment antigen-binding) domain of rituximab binds CD20 on B cells and the Fc (fragment crystallizable region) domain recruits immune effectors to induce B cell lysis. Complement-dependent-cytotoxicity might be one mechanism via which rituximab mediates B cell lysis, *in vitro* studies demonstrated long ago that rituximab can bind human C1q and mediate complement-dependent B cell lysis.[87] Rituximab treatment results in peripheral blood B cell-depletion that is sustained for approximately 6-18 months, without affecting the plasma cell population.[88] In 1997-1998 this drug was licensed in the United States/Europe for the treatment of B cell lymphomas,[89-92] and in 2006 rituximab was licensed for the treatment of rheumatoid arthritis.[93-97] The good results obtained with rituximab in rheumatoid arthritis incited a series of studies in other autoimmune diseases, including systemic lupus erythematosus[98-100] and, as described, ANCA-associated vasculitis. One of those studies was the RITUXVAS trial conducted by the EUVAS.

The RITUXVAS trial was designed to test if a treatment regimen based on rituximab could induce a higher rate of sustained remission in patients with ANCA-associated vasculitis with renal manifestations compared to standard therapy primarily based on cyclophosphamide. Additionally, rituximab treatment should provide a means to reduce cumulative cyclophosphamide dosage. In short, this trial demonstrated a rituximab-based regimen not to be inferior to standard intravenous cyclophosphamide therapy. However, contrary to what was hypothesized, rituximab therapy did not lead to a reduction in early severe adverse events when compared to standard therapy.[101] A companion article on correlations of renal histology to outcome in patients entered in the RITUXVAS trial is described in this thesis in **Chapter 4**.

A last trial worth mentioning here is maybe the most ambitious EUVAS trial to date, under the name of PEXIVAS. PEXIVAS can be regarded as a double trial, because it is designed to confirm and further explore the benefit of adjuvant plasma exchange as well as, for the first time, compare a low dose to a standard dose of corticosteroids. A

total of 500 patients will be enrolled in this trial, and the EUVAS will cooperate with the Vasculitis Clinical Research Consortium (USA).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From ANCA-induced neutrophil degranulation to a successful animal model

After the association of ANCA with the small vessel vasculitides was well confirmed, many researchers set off to explore the pathogenic potential of ANCA. Although the pathogenesis of ANCA-associated vasculitis has not been completely elucidated, a key role for neutrophils in the acute injury to the blood vessel wall was soon recognized and has been firmly established over the years.

ANCA are directed against neutrophil constituents and activated neutrophils are abundantly present in the initial lesions encountered in ANCA-associated vasculitis. Priming of circulating neutrophils by cytokines, possibly during infection, is thought to underlie local neutrophil accumulation in ANCA-associated vasculitis. Priming with an agent such as TNF-α *in vitro* causes PR3 and MPO to be expressed on the neutrophil cell membrane, where it becomes accessible to ANCA. While PR3 is normally regarded to be localized intracellular under resting conditions, it has also been detected at the surface of unactivated, freshly isolated neutrophils that were not primed by cytokines. This demonstrates that these antigens may be expressed at the neutrophil surface under physiological conditions as well. The proportion of resting neutrophils that expressed PR3 on the surface proved stable within individuals in time, but was highly variable among different individuals.[102] Membrane expression of PR3 seems to be genetically determined,[103] and elevated levels of membrane PR3 expression have been detected in patients with ANCA-associated vasculitis and, moreover, have been correlated with disease activity.[104;105] A substantial, genetically determined, subset of membrane $PR3^+$ neutrophils might be a risk factor for developing ANCA-associated vasculitis.[106] While evidence for the mechanisms behind membrane PR3 expression is accumulating, to date, the mechanisms that underlie membrane expression of MPO are less clear. *In vitro* evidence demonstrated already in the early '90s that ANCA can activate primed neutrophils, via interaction with PR3/MPO on the neutrophil membrane. Neutrophils activated by ANCA degranulate, produce reactive oxygen species and release proteolytic enzymes.[107;108] For the activation of neutrophils by ANCA, apart from ANCA-antigen binding, Fc receptor and β2-integrin engagement are required.[107;108] ANCA are thought to interact with their respective antigens via their Fab domain and with Fc receptors via their Fc domain.

These interactions activate several downstream signal transduction pathways, such as the p38 mitogen-activated protein kinase and the phosphatidylinositol-3-kinase pathways. These pathways culminate in the neutrophil respiratory burst and damage to the endothelium ensues. Neutrophils that are activated by ANCA can directly interact with endothelial cells via β2-integrins and adhesion molecules expressed by activated endothelium. In this way, ANCA stimulate neutrophil cytotoxicity towards endothelial cells.[109]

Neutrophil apoptosis is a cardinal mechanism to prevent excessive tissue damage caused by activated neutrophils. Activated neutrophils will normally undergo apoptosis, and will then be cleared by macrophages. In the healthy situation, macrophage phagocytosis of apoptotic neutrophils is a non-inflammatory process. Several observations have been made regarding apoptotic neutrophils in ANCA-associated vasculitis. It has been demonstrated that PR3 and MPO can be externalized at the plasma membrane during neutrophil apoptosis,[110] and that ANCA can accelerate neutrophil apoptosis.[111] In the former study, apoptotic neutrophils could be divided into two subsets, with only one subset demonstrating PR3 and MPO externalization. Interestingly, PR3 and MPO externalization was not dependent on neutrophil priming. Therefore these results provided a novel mechanism, independent of priming, by which ANCA could gain access to their respective antigens. In the latter study, although apoptosis of TNF-α primed neutrophils was accelerated by ANCAs, phagocytic recognition and clearance was reduced. While several studies have confirmed that ANCA interfere with neutrophil apoptosis, it is unknown what the effect of this interference is *in vivo,* and whether neutrophil apoptosis might provide a therapeutic target.

During the '90s, *in vitro* studies identified ANCA-induced neutrophil degranulation as a key pathway that leads to tissue damage in ANCA-associated vasculitis. The first successful experimental model of ANCA-associated vasculitis was developed by Xiao *et al.* and published in 2002.[12] This was a mouse model of MPO-ANCA-associated vasculitis. In this experimental model, purified murine anti-MPO IgG was systemically administered to recipient wild-type mice. The murine anti-MPO IgG was acquired from MPO-immunized MPO-knockout mice. Upon MPO-ANCA administration the wild-type mice developed hematuria and proteinuria. Moreover, the histopathologic lesions found in the kidneys of these mice were comparable to those seen in

renal biopsies of patients with ANCA-associated glomerulonephritis.[12] Soon after the development of the mouse model, a rat model of MPO-ANCA-induced vasculitis was developed as well. Rats were immunized with human MPO, and subsequently developed anti-human MPO-ANCA. The MPO-ANCA that developed against human MPO cross-reacted with rat MPO, and rats developed vasculitis.[112]

Although the rodent models for MPO-ANCA-induced vasculitis and glomerulonephritis provide good *in vivo* models, that in several ways mimic human disease, there are limitations. One limitation of the mouse model developed by Xiao *et al.*[12] is that there is only one single injection of MPO-ANCA. This implicates that only acute disease manifestations can be studied, long-term follow-up is not possible, and disease progression cannot be monitored. Because of this, researchers aimed, and still aim, to fine-tune the animal models, and make adjustments that facilitate the answering of additional research questions that could not be answered using the existing models.

An example of this is found in a study that used an experimental mouse model that was a variant of the 'original'. In the adjusted model, MPO-knockout mice (that did not express MPO in/on their cells) were immunized with MPO, and consequently developed MPO-ANCA. Hereafter the mice underwent bone marrow irradiation. The bone marrow harbors the stem cells from which, via the myeloid lineage, neutrophils develop. Bone marrow-irradiated mice subsequently received a bone marrow transplant from either another MPO-knockout mouse, or from a wild-type mouse that did express MPO in/on its cells. Transplantation of bone marrow derived from a wild-type MPO+/+ mouse in a MPO-knockout mouse with circulating MPO-ANCA gave rise to disease manifestations. Contrariwise, transplanting bone marrow from a MPO-knockout mouse did not lead to disease development. These *in vivo* data illustrate that MPO-expressing cells derived from the bone marrow, in particular neutrophils, are indispensable for the development of MPO-ANCA-associated vasculitis.[113]

Another example of how variants of the *in vivo* mouse model for MPO-ANCA-associated disease increased understanding of the pathogenesis, is found in a study with this model that demonstrated that the genetic background of the mice used has a considerable effect on the severity of the disease manifestations, as assessed by histo-

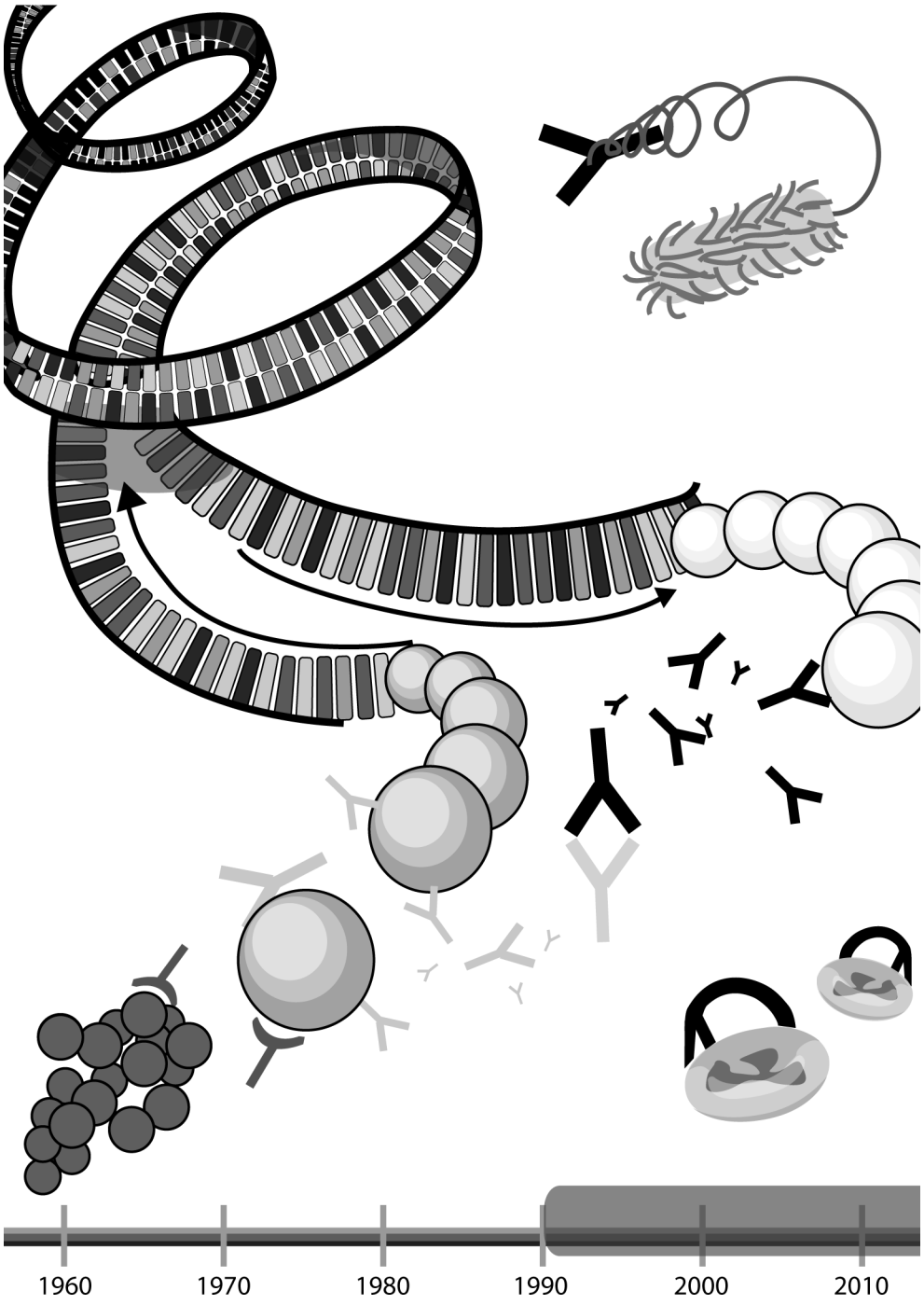
pathologic examination, that occur after systemic MPO-ANCA administration.[114]

The rodent models for MPO-ANCA-associated disease have in recent years also implicated a role for complement in the pathogenesis of the ANCA-associated vasculitides. Because human ANCA-associated glomerulonephritis is pauci-immune, with virtually no complement deposition in the renal tissue, for long the general assumption was that there was no role for complement in this disease. However, studies on the mouse model developed by Xiao *et al.* demonstrated that induction of glomerulonephritis with MPO-ANCA required activation of complement. Depleting complement by using cobra venom factor completely blocked development of vasculitis and glomerulonephritis in these mice. Detailed studies in mice deficient for the complement common pathway component C5, classical & lectin pathway component C4 and alternative pathway component Factor B, illustrated that MPO-ANCA-induced necrotizing crescentic glomerulonephritis in experimental models depends on an intact alternative complement pathway. In short, C4-deficient mice developed necrotizing crescentic glomerulonephritis like wild-type mice, indicating that blocking the classical and lectin pathway did not prevent disease. In contrast, transgenic mice deficient for C5 or factor B were completely protected from disease induction. Possibly ANCA-induced neutrophil activation causes release of factors that activate the alternative complement pathway.[115]

In patients, the role of complement is inconclusive. Although ANCA-associated glomerulonephritis is by definition pauci-immune, this in fact does not rule out a role for complement in the disease process. By immunohistochemistry C3d, factor B, factor P and the membrane attack complex (the final product of complement activation) could be detected in glomeruli and small blood vessels upon examination of biopsies from patients with MPO-ANCA-associated vasculitis. Mannose-binding lectin and C4d were not detected in these biopsies. Together these findings support a role for the alternative pathway of the complement system not only in experimental models, but also in human pauci-immune MPO-ANCA-associated vasculitis.[116]

Concluding, a number of excellent experimental models have been developed for MPO-ANCA-associated vasculitis which have increased insight into the etiology of this disease entity, but it is uncertain to what extent parallels can be drawn to PR3-

ANCA-associated vasculitis, for which good models are lacking.[117] The approach Xiao *et al.* used to develop their MPO-ANCA mouse model has been tried for PR3-ANCA as well. However, passive transfer of PR3-ANCA into wild-type mice did not lead to vasculitic lesions in the lungs or kidneys, and did not provide a good mimic of human disease.[118]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T cells, anti-hLAMP2 antibodies and complementary PR3

Approximately 90% of patients with generalized WG, MPA and RLV have circulating ANCA prior to treatment.[2] Certain drugs (e.g. propylthiouracil and hydralazine[119]) can induce ANCA, particularly high titers of MPO-ANCA, and subsequently lead to the onset of disease manifestations, providing clinical support for the pathogenic potential of ANCA. Further clinical evidence for the pathogenicity of ANCA is found in the beneficial effects of plasma exchange treatment. Extensive *in vitro* and *in vivo* experimental data (previously described) also support a pathogenic role of ANCA. Nevertheless, since their discovery the role of ANCA in the pathogenesis of small vessel vasculitides has been the subject of debate, and it cannot even be ruled out that ANCA are an epiphenomenon.

Over the years it has become evident that abnormalities in cellular immunity have a role to play in the pathogenesis of the ANCA-associated small vessel vasculitides, as reviewed in **chapter 5** of this thesis. Regarding humoral immunity, apart from classical ANCA, other autoantibody-responses have been described. It is questionable if the classical ANCA directed against PR3 or MPO are rightly given so much attention. Over time, other antigens such as lactoferrin and elastase have been implicated in ANCA-associated disease, but no real breakthroughs were achieved. Yet another intriguing antigen has more recently been described, namely human lysosomal-associated membrane protein-2 (hLAMP-2).[120;121] hLAMP-2 is expressed on lysosomes and endosomes and it shuttles between these vesicles and the cell membrane.[122] In neutrophils, hLAMP-2 is an integral component of the membranes of MPO- and PR3-containing intracellular vesicles. Autoantibodies against hLAMP-2 give positive results on IIF tests, analogous to the positive results obtained on IIF with sera containing MPO- and PR3-ANCA. Up to 30% of the hLAMP-2 protein can at any time be expressed at the cell surface, where it could directly interact with circulating autoantibodies. hLAMP-2 protects lysosomal membranes from autodigestion, functions in the surface presentation of intracellular antigens, mediates adhesion of inflammatory cells, and plays a role in the traffic of lysosomes and endosomes. Kain *et al.* investigated the prevalence of autoantibodies directed against hLAMP-2 in sera from 84 patients with active (new onset or relapse) pauci-immune necrotizing crescentic glomerulonephritis. ANCA were detected on standard IIF in 80/84 patient sera. By

specific ELISA, ANCA were detectable in 70/84 sera. MPO-ANCA were detected in 38 sera and PR3-ANCA were detected in 39 sera, including 7 sera with antibodies against both MPO and PR3. Using a specific ELISA for hLAMP-2, antibodies against hLAMP-2 were detected in 78 sera, indicating that 93% of patients harbored these antibodies and that these novel 'ANCA' were more frequent in this cohort than the classical MPO- and PR3-ANCA. Kain *et al.* also provide substantial *in vitro* and *in vivo* data that underline the pathologic potential of these novel ANCA. First, *in vitro* experiments demonstrated that anti-hLAMP-2 antibodies can activate neutrophils and can kill human microvascular endothelium.[121] Second, intravenous injection of hLAMP-2-specific rabbit IgG (that cross-reacts with rat LAMP-2) in 15 Wistar Kyoto (WKY) rats led to the development of hematuria, proteinuria, severe renal leukocyte infiltration, focal capillary necrosis and crescents.[121] In the kidneys of rats that were sacrificed 2 hours after intravenous injection of anti-hLAMP-2 rabbit IgG, minimal deposition of rabbit IgG was detected. No rabbit IgG could be detected in the kidneys of rats that were sacrificed at later time points.[121]

Since 1985, the million-dollar-question in 'ANCA-associated research' has been and still is: "Why and how do ANCA develop?". In the case of anti-hLAMP-2 antibodies, a plausible mechanism based on molecular mimicry has been proposed. Kain *et al.* describe that anti-hLAMP-2 antibodies recognize two major epitopes, and that one of these epitopes is highly homologous to the bacterial protein FimH. FimH is an adhesin (adherence factor) that is located at the tips of the fimbriae of Gram-negative bacteria such as *Escherichia (E.) coli*. Fimbriae are proteinaceous appendages that bacteria use to adhere to other bacteria, host cells or the surfaces of non-living objects. The adhesin FimH that is found on the top of these fimbriae further enables bacteria to attach to host epithelia. Kain *et al.* immunized 10 WKY rats with a recombinant FimH fusion protein, and demonstrated that nine of these FimH-immunized rats developed autoantibodies to rat LAMP-2 as well as pauci-immune necrotizing crescentic glomerulonephritis.[121] Further research into FimH demonstrated that 9 of the 13 most recently included patients in the cohort under study had been exposed to pathogens expressing FimH during the months prior to onset of pauci-immune necrotizing crescentic glomerulonephritis (8 *E. coli* infections and 1 *Klebsiella pneumoniae* infection).

While molecular mimicry has regularly been implied as a mechanism by which autoimmunity may occur, a few years ago a serendipitous finding led to the postulation of a rather novel theory involving so-called autoantigen complementarity. Patients with antibodies recognizing PR3 were shown to harbor antibodies to a peptide translated from the antisense DNA strand of PR3 as well. This 'antisense peptide' was called complementary PR3 or cPR3. Immunizing mice with the complementary PR3 peptide resulted in the production of antibodies against this peptide, but also against PR3. These findings led to the hypothesis that the autoimmune response is not incited by the self-antigen or the autoantigen, but rather by a protein that is complementary in surface structure to the autoantigen. This complementary protein would be a protein homologous or identical to the amino acid sequence of translated antisense RNA from the noncoding strand of the autoantigen gene. Such a protein could elicit an immune response with the formation of antibodies, since it could be considered a 'foreign' protein. These antibodies could elicit an anti-antibody or anti-idiotypic response, resulting in the formation of antibodies against the autoantigen counterpart of the complementary protein.[123;124] Interestingly, antibodies recognizing the complementary protein of PR3 also recognized plasminogen.[125] Seropositivity for these anti-plasminogen antibodies was correlated to thromboembolic events.

While the last 20 years have greatly increased insight into possible pathogenic mechanisms, the puzzle is far from completed. The work described in this thesis extends some of the prior work mentioned in this text.

Thesis outline

The **second chapter** describes long-term survival data concerning 535 patients who participated in the NORAM, CYCAZAREM, MEPEX and CYCLOPS trials conducted by the EUVAS. The **third chapter** describes clinical determinants of renal outcome in the same patient cohort. The emphasis in these chapters is on the results of multivariable models, developed to detect baseline patient characteristics that can provide reliable prognostic information to treating physicians. **Chapter 4** comprises a clinicopathologic study performed on renal biopsies of patients experimentally treated with a rituximab-based regimen within the EUVAS RITUXVAS trial. Specific attention is paid to the presence of B cell, T cell and plasma cell infiltrates in the diagnostic renal biopsy and the relation of these infiltrates to renal outcome under rituximab treatment. **Chapter 5** reviews known disturbances in cellular immunity in vasculitis, illustrating that there is more to ANCA-associated vasculitis than humoral immunity. In **chapter 6** the presence of anti-plasminogen antibodies is described in two independent patient cohorts, one from the United Kingdom and one from the Netherlands. These antibodies had previously been detected in a North American cohort of patients with PR3-ANCA vasculitis. **Chapter 6** confirms anti-plasminogen antibodies to be present in a considerable number of patients with ANCA-associated vasculitis, and extends prior North American results to patients with MPO-ANCA as well. The **last chapter** before the general discussion comprises a first proposal for a histopathologic classification of ANCA-associated glomerulonephritis. **Chapter 7** illustrates that a simple classification schema comprising only four histologic classes correlates well with renal outcome in a first validation exercise. Finally, the results described in this thesis are summarized and discussed in **chapter 8**.

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