High mobility group box-1 (hmgb1) in systemic vasculitides

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HIGH MOBILITY GROUP BOX-1 (HMGB1) IN SYSTEMIC VASCULITIDES – THE INTERPLAY WITH ACTIVE DISEASE, SPECIFIC ORGAN INVOLVEMENT AND THERAPY

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CHAPTER 1

Introduction to this thesis

Alexandre W. S. de Souza

Systemic vasculitides

Vasculitis is an inflammatory process that occurs within the vessel wall as the primary site of inflammation. The vasculitic process may affect vessels of any type or any size including capillaries, venules, arterioles, veins and arteries. The inflammation may result in damage and fibrinoid necrosis in the vessel wall, and when arteries are involved this process may result in irreversible changes of arterial walls such as stenosis, occlusion, dilation or aneurysm formation. When capillaries and venules are affected by the vasculitic process, weakening of vessel wall may ensue often leading to rupture of vessel wall and bleeding to surrounding tissues [1,2].

Disease manifestations of vasculitic syndromes are heterogeneous and usually include constitutional symptoms due to the systemic inflammatory process as well as dysfunction of organs and systems whose supplying vessels are affected by the vasculitic process [3]. The involvement of multiple organs and/or systems characterizes a systemic vasculitis while when only one organ is affected by the vasculitic process it is regarded as a single organ vasculitis [4]. Systemic vasculitis is considered primary when there is no known etiological factor identified but systemic vasculitis may be associated with infectious diseases (e.g., hepatitis C or HIV infection), drugs (e.g., anti-thyroid drugs, hydralazin and minocyclin), drug abuse (e.g., vasculitis associated with levamisole-adultered cocaine), systemic autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid vasculitis and Sjögren's syndrome) and cancer [5,6].

Several classification systems of systemic vasculitides have been developed since the first proposal in 1952 by Dr. Perla Zeek that included: hypersensitivity angiitis, allergic granulomatous angiitis, rheumatic arteritis, periarteritis nodosa and temporal arteritis [7]. In 1993, the first International Chapel Hill Consensus Conference (CHCC) aimed to develop a consensus for the names and definitions of the most common forms of vasculitis. Ten

vasculitic syndromes were included in the first CHCC and their definition was based on disease manifestations and histopathologic features [8]. More recently, a second International CHCC was held in order to update the initial vasculitis nomenclature and definition system based on advances in the understanding of epidemiology and pathophysiology of vasculitis. Systemic vasculitides are classified according to the size of vessels predominantly affected in large vessel vasculitis (LVV), medium vessel vasculitis (MVV) and small vessel vasculitis (SVV) (Table 1) [9].

Table 1. Names of vasculitides adopted by the 2012 International Chapel Hill Consensus Conference, adapted from [9].

Large vessel vasculitis (LVV)
Takayasu arteritis (TAK)
Giant cell arteritis (GCA)
Medium vessel vasculitis (MVV)
Polyarteritis nodosa (PAN)
Kawasaki disease (KD)
Small vessel vasculitis (SVV)
Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)
Microscopic polyangiitis (MPA)
Granulomatosis with polyangiitis (Wegener's) (GPA)
Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) (EGPA)
Immune complex SVV
Anti-glomerular basement membrane (anti-GBM) disease
Cryoglobulinemic vasculitis (CV)
IgA vasculitis (Henoch-Schönlein) (IgAV)
Hypocomplementemic urticarial vasculitis (HUV) (anti-C1q vasculitis)
Variable vessel vasculitis (VVV)
Behçet's disease (BD)
Cogan's syndrome (CS)
Single-organ vasculitis (SOV)
Cutaneous leukocytoclastic vasculitis
Cutaneous arteritis
Primary central nervous system vasculitis
Isolated aortitis
Others
Vasculitis associated with systemic disease
Lupus vasculitis
Rheumatoid vasculitis
Sarcoid vasculitis
Others
Vasculitis associated with probable etiology
Hepatitis C virus–associated cryoglobulinemic vasculitis
Hepatitis B virus–associated vasculitis
Syphilis–associated aortitis
Drug–associated immune complex vasculitis
Drug–associated ANCA–associated vasculitis
Cancer–associated vasculitis
Others

SVV is divided in two subgroups based on the deposition of immune deposits in the vessel wall as follows: antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis characterized by paucity or absence of immune deposits, and immune complex small vessel vasculitis with moderate to marked immune complex deposition on vessel walls. Furthermore, additional categories of vasculitis have been added to the current classification system such as variable vessel vasculitis, single-organ vasculitis, vasculitis associated with systemic diseases and vasculitis with probable etiology have been added (Table 1) [9].

The International CHCC nomenclature and definitions of vasculitis are not meant to be used as diagnostic or classification criteria. Thus, the diagnosis of a systemic vasculitis in patients with suggestive signs and symptoms needs to be confirmed by biopsy findings, serological markers (e.g. ANCA test, serum cryoglobulins) or by imaging studies as appropriate [1,3]. The American College of Rheumatology (ACR) in 1990 developed classification criteria for seven common forms of vasculitis: Takayasu arteritis (TAK), giant cell arteritis (GCA), polyarteritis nodosa (PAN), granulomatosis with polyangiitis (GPA) (formerly Wegener's granulomatosis), eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg-Strauss syndrome), IgA vasculitis (formerly Henoch-Schönlein purpura) and hypersensitivity vasculitis [10-16]. The 1990 ACR criteria for systemic vasculitides were developed to identify a homogeneous group of patients for inclusion in epidemiologic and therapeutic studies and not intended to be used as diagnostic tools in clinical practice [5,6]. The sensitivity of the 1990 ACR criteria for systemic vasculitides ranged from 71.0% to 95.3% while the specificity ranged from 78.7% to 99.7%. The criteria for EGPA, GCA and TAK were shown to present the best sensitivity and specificity [17].

In 2006, Watts et al. developed and validated an algorithm to categorize patients with ANCA-associated vasculitis (AAV) and PAN for

epidemiological studies. The four-step algorithm included patients with a clinical diagnosis of AAV or PAN. The ACR and Lanham criteria for EGPA and the ACR criteria for GPA were applied first, surrogate markers of GPA were used to distinguish GPA form microscopic polyangiitis (MPA), then MPA was classified using the CHCC definition and surrogate markers for renal vasculitis. Finally, the CHCC was used to classify patients with PAN [18]. More recently, an international multicenter collaboration is underway to develop a single set of validated diagnostic and classification criteria for systemic vasculitides [19].

Without appropriate therapy, systemic vasculitides are associated with a high mortality rate. Advances in the management of these conditions have converted systemic vasculitides into relapsing and remitting chronic diseases with periods of active disease and remission. In systemic vasculitides, significant morbidity may arise from permanent damage secondary to disease activity and therapy as well. In clinical practice, a systematic evaluation of patients with systemic vasculitis is important to check all possible organs and systems involved by the vasculitic process and to avoid missing new disease manifestations [20]. Currently, the Birmingham Vasculitis Activity Score (BVAS) is the most widely used instrument to measure disease activity, especially in SVV and in MVV [21]. BVAS was developed in 1994 for use in collaborative clinical trials in vasculitis, it was modified in 1997 to create a second version [22]. In 2001, BVAS was adapted to produce a specific instrument to evaluate disease activity in patients with GPA (BVAS/WG) and in 2009 BVAS was modified and validated again to create a third version with 56 items from 9 organs and systems [23,24]. Due to different disease manifestations, BVAS is not adequate to evaluate disease activity in TAK. Kerr's criteria and more recently the Indian Takayasu Activity Score (ITAS) have been used for this purpose [25,26].

High mobility group box-1

High mobility group box-1 (HMGB1) is a nuclear non-histone protein with 215 amino acid residues that comprises three distinct domains: two tandem HMG box domains (box A and B) and a 30 amino acid-long C terminal tail. Nuclear HMGB1 binds to DNA, modulates chromosomal architecture and regulates DNA transcription, repair and recombination [27]. Although HMGB1 is abundantly found within the nucleus, HMGB1 may also be translocated into the cytoplasm, where it is responsible for mediating cellular autophagy and also acts as a cytosolic sentinel for immunogenic nucleic acids by binding to DNA or RNA [28-30]. In addition, HMGB1 may be released by dying cells or secreted by activated cells to the extracellular environment. The derangement of cell permeability from necrotic cells results in the passive release of HMGB1 whereas apoptotic cells retain HMGB1 in the nucleus due to post-translational modifications that affect chromatin binding. However, when late apoptosis ensues (secondary necrosis) HMGB1 may be finally released by apoptotic cells [28]. Immunologically active cells can secrete HMGB1 upon activation by cytokines or TLR ligands. After cell activation, lysine residues of HMGB1 are acetylated and this chemical modification signals migration into the cytoplasm inside vesicles that merge with the plasma membrane and eventually HMGB1 is secreted into the extracellular milieu [28,31].

Once outside the cell HMGB1 acts as an alarmin or a danger-associated molecular pattern (DAMP) and displays different functions by binding to different receptor such as the receptor for advanced glycation-end products (RAGE), Toll-like receptor (TLR)-2, TLR-4 and TLR-9 [28,29]. The interaction between extracellular HMGB1 and its receptor triggers signaling events that mediate HMGB1 functions on cellular activation, differentiation and proliferation, cytokine production, chemotaxis and angiogenesis. Moreover, HMGB1 may complex with other molecules including interleukin

(IL)-1β, DNA, RNA, LPS and nucleosomes in order to act synergistically in the activation of their receptors [32].

Post-translational modifications of HMGB1 may also affect its functions. The immunological activity of HMGB1 is affected by the redox state of cysteine residues at positions 23, 45 and 106. When HMGB1 is in its fully reduced state (i.e., all-thiol HMGB1) it only induces chemotaxis by binding to CXCL12 and activating CXCR4. However, HMGB1 triggers cytokine production by the interaction with TLR4 when HMGB1 is partially oxidized with a disulfide bond formed at position 23 and 45, with a free thiol at position 106. The fully oxidized form of HMGB1 cannot induce either chemotaxis or cytokine production [31,33,34].

Due to its multiple functions in activating the immune system, the role of HMGB1 in the pathogenesis or as a biomarker has been evaluated in several systemic inflammatory and autoimmune diseases, in atherosclerosis, cancer and infectious disease [28,32,31,35]. In systemic lupus erythematosus (SLE), increased levels of HMGB1 were found in serum and in urine samples of patients with active systemic disease and nephritis, respectively [36,37]. Increased expression of HMGB1 was found in the cytoplasm and in extracellular sites of renal tissue from patients with lupus nephritis [37,38]. Furthermore, the release of HMGB1 is increased in the skin of SLE patients and this release is increased by ultraviolet B exposure and is related to the number of apoptotic cells [39]. In rheumatoid arthritis (RA), an increased concentration of HMGB1 is found in synovial fluid in comparison with patients with osteoarthritis while CD68-positive cells express HMGB1 in the synovium of RA patients [40]. Serum levels of HMGB1 in RA are associated with IL-6 levels, swollen joint count and acute phase reactants [41].

Patients with active idiopathic inflammatory myopathies present high cytoplasmic expression of HMGB1 in muscle fibers, infiltrating macrophages and in vascular endothelial cells, but this expression is dramatically

decreased after 3-6 months of high dose prednisolone [42]. Increased serum HMGB1 levels were found in patients with Sjögren's syndrome, systemic sclerosis, ankylosing spondylitis and juvenile idiopathic arthritis [43-46].

In systemic vasculitides, up to the start of this thesis, serum HMGB1 levels had been evaluated in patients with Kawasaki's disease (KD), Behçet's disease (BD), GPA and MPA. In KD, serum HMGB1 levels were very high in the early acute phase of the disease and decreased in the late acute phase and convalescent phase of KD [47]. Furthermore, serum HMGB1 levels were predictive of the clinical response to intravenous immunoglobulin [48]. In BD, serum levels were higher in patients than healthy controls and patients with intestinal involvement of BD presented the highest levels of HMGB1 [49]. In patients with AAV, serum HMGB1 levels had been evaluated in three cross-sectional studies. Firstly, serum HMGB1 levels were associated with disease activity in GPA but not MPA and a significant correlation between serum HMGB1 and BVAS was found in GPA [50]. Higher serum HMGB1 levels were found in GPA patients with predominantly granulomatous manifestations compared with GPA patients with predominantly vasculitic manifestations. A significant correlation was found between the volume of pulmonary granuloma and serum HMGB1 [51]. Finally, another study showed higher serum HMGB1 levels in AAV patients with biopsy-proven glomerulonephritis compared with those patients with a normal kidney biopsy. A significant decrease in serum HMGB1 levels was observed in seven patients who underwent a second kidney biopsy in remission 6-9 months after baseline [52].

Aims of this thesis

In this thesis we aimed to evaluate HMGB1 as a biomarker of disease activity in systemic vasculitides including GPA, MPA, TAK, GCA and Behçet's disease (BD). Furthermore, we also aimed to check associations between

serum HMGB1 levels and specific organ and system involvement and to assess the impact of therapy on serum HMGB1 levels in systemic vasculitides. Firstly, we reviewed the subject of HMGB1 in vascular diseases including systemic vasculitides and atherosclerotic disease in **chapter 2**. As previous cross-sectional studies had already evaluated serum HMGB1 levels in AAV patients (i.e. GPA and MPA), in **chapter 4** we decided to evaluate serum HMGB1 levels in a longitudinal study with AAV patients to assess fluctuations of HMGB1 at disease presentation, during remission, prior to and during relapses. We hypothesized that serum HMGB1 levels could be predictive of disease relapses in AAV. Moreover, we checked associations between serum HMGB1 levels at presentation and specific organ or system involvement in AAV and the presence of anti-HMGB1 antibodies in AAV patients with active disease. In **chapter 5**, we evaluated whether serum levels of HMGB1 and soluble RAGE (sRAGE) could be associated with subclinical atherosclerotic disease or with therapy in GPA. In this study, we also evaluated the *in vitro* effect of atorvastatin on the release of HMGB1 by human umbilical vein endothelial cells (HUVEC) activated with lipopolysaccharide (LPS). In **chapter 6**, we evaluated whether urinary HMGB1 levels were increased in AAV patients with active nephritis compared to healthy controls and whether levels decreased after achieving remission. In this study, we also evaluated associations between urinary HMGB1 levels and BVAS, urinary monocyte chemoattractant protein-1 (MCP-1), CD4+ T cells and effector memory T cells in urine and peripheral blood. To introduce this study, we wrote a review about the role of mononuclear cells, especially T cells, in endothelial damage in AAV in **chapter 3**. The role of serum HMGB1 as a biomarker of active disease in LVV (i.e. TAK and GCA) was evaluated in chapter 7 and the role of HMGB1 levels in BD was addressed in **chapter 8**. Finally, the summary and conclusions from all findings described in these studies are given in **chapter 9**.

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CHAPTER 2

HMGB1 in vascular diseases: Its role in vascular inflammation and atherosclerosis

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Abstract

The nuclear protein high mobility group box 1 (HMGB1) has been suggested to be involved in the pathogenesis of several vascular diseases such as systemic vasculitis and atherosclerosis. In systemic vasculitides including ANCA-associated vasculitis and Kawasaki disease, serumHMGB1 levels are higher in patientswith active disease compared to healthy controls. In atherosclerotic disease, HMGB1 displays increased expression in nuclei and cytoplasm of macrophages and smooth muscle cells in the atherosclerotic lesions, and is implicated in the progression of the atherosclerotic plaque. Experimental models of acute coronary syndromes and cerebrovascular accidents show that HMGB1 is not only involved in the amplification of the inflammatory response during acute ischemic injury, but also in the recovery and remodeling process after ischemia. Patients with acute coronary syndromes or stroke present significantly higher serum levels of HMGB1 than healthy controls and levels are associated with disease severity and mortality. Here we review clinical and experimental studies dealing with the role of HMGB1 in vascular diseases.

1. Introduction

High mobility group box 1 (HMGB1) is a nonhistone DNA-binding protein of 215 amino acid residues organized into three domains that include two tandem HMG box domains (A box and B box) arranged in an L-shape configuration, and a 30 amino acids long C-terminal tail [1,2]. HMGB1 is constitutively expressed in most cell types and it resides mainly in the nucleus under physiologic conditions where it acts as a structural component in complex with chromatin. HMGB1 facilitates the assembly of nuclear proteins and participates in DNA replication, recombination, transcription and repair [3,4].

HMGB1 can be actively secreted from immunologically competent cells when exposed to microbe-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs) or cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-1 and interferon (IFN)γ [5,6]. HMGB1 is also passively released from necrotic, damaged cells or from apoptotic cells. In contrast to HMGB1 released from necrotic cells, HMGB1 released from apoptotic cells does not induce significant inflammatory responses. This is due to the oxidation of cysteine at position 106 in the HMGB1 molecule by mitochondrial reactive oxygen species released inside apoptotic cells. This oxidation precludes a significant pro-inflammatory response by HMGB1 [7].

Biologically activeHMGB1 can be expressed on the plasma membrane or may be secreted into the extracellular milieu where it acts as a cytokine and interacts with the receptor for advanced glycation end products (RAGE) and toll-like receptors (TLR)2, TLR4 and TLR9 [4,8–11]. HMGB1may also bind to other proteins like CXCL12, syndecan, triggering receptors expressed on myeloid cells 1 (TREM1), and macrophage adhesion molecule 1 (MAC1) [12–15]. Furthermore, HMGB1 is a part of the nucleic-acid-sensing system and binds to immunogenic nucleotides in order to activate innate immune responses during microbial infection and tissue damage [16]. The interaction with TLR4 has been shown to be dominant in inducing the release of cytokines such as TNFα, IL-1, IL-6 and IL-8 by activated macrophages via activation of the inhibitor of kappa B kinase complex (IKK), including the kinases IKKα and IKKβ, leading to phosphorylation and degradation of the IκB, nuclear translocation of NF-κB, and enhanced expression of proinflammatory cytokine genes whose transcription is dependent on NF-κB [9,17]. Several other biological activities of extra-cellular HMGB1 have been described related to targeting different cells involved in inflammatory and immune responses. Upon binding to monocytes, HMGB1 induces transendothelial migration to inflammatory sites. With respect to dendritic cells (DC), HMGB1 induces maturation of immature DC and migration to lymph

nodes and also expression of MHC class II molecules and secretion of several proinflammatory cytokines including IL-1α, IL-6, IL-8, IL-12, TNFα and RANTES [18–20]. Ligation of HMGB1 to neutrophils induces cell activation through nuclear translocation of NFκB, resulting in the production of cytokines such as TNFα and IL-8, activation of NADPH oxidase, increased adhesion, and chemotaxis [21–24].

Several effects of HMGB1 on T-lymphocytes have been described indicating that HMGB1 may also play a role in adaptive immunity [25]. HMGB1 has been shown to induce proliferation of CD3+ naïve T cells which is inhibited by anti-CD3 monoclonal antibodies. Th1 polarization has been demonstrated upon stimulation of DC and T cells by the B box of HMGB1 leading to the production of IL-2, IL-12 and IFNγ whereas polarization of CD4+ T cells into a Th2 phenotype has also been described [19,25–27]. Moreover, in vitro stimulation of CD4+ T cells from patients with rheumatoid arthritis with increasing concentrations of recombinant HMGB1 induced the production of IL-17 indicating that HMGB1 may contribute to Th17 activation in these patients [28]. Regarding regulatory T cells (Tregs), HMGB1 has been shown to decrease the expression of CTLA4 and FoxP3, and to inhibit the secretion of IL-10 leading to decreased regulatory T-cell activity [29,30].

HMGB1 has an effect on B cells as well, since it has been demonstrated that HMGB1 in DNA containing immune complexes can stimulate cytokine production through interactionwith TLR9 and RAGE, activating plasmacytoid DC and B cells. Binding of HMGB1 to CpG oligodeoxynucleotides enhanced activation and cytokine production via RAGE and TLR9 [10]. Moreover, immune complexes containing HMGB1, DNA and IgG2a are also able to activate B cells through TLR9 in a RAGE-independent mechanism [31]. More recently, it has been demonstrated that HMGB1 is also released by activated plasma cells into the extra-cellular environment contributing further to the enhancement of inflammatory responses [32].

HMGB1 acts as a pro-inflammatory mediator that it is released after sterile injury or microbial invasion and activates immune competent cells to amplify inflammatory responses through the release of proinflammatory cytokines. The role of HMGB1 has been investigated in several systemic disorders such as sepsis, cancer, trauma, ischemia–reperfusion injury (e.g. stroke and acute myocardial infarction), acute respiratory distress syndrome, and chronic inflammatory and autoimmune diseases [11,33]. Here, we review the literature on circulating HMGB1, its expression in tissues, and its relation to specific disease manifestations and prognosis in inflammatory diseases of the vessel wall, in particular systemic vasculitis and atherosclerosis.

1.1. Methods to detect HMGB1 in serum, plasma and in tissues

HMGB1 can be detected both in serum and in plasma samples using a sandwich enzyme-linked immunosorbent assay (ELISA) or a Western blot technique [34]. HMGB1 levels may be 5 times higher when analyzed by Western blot technique compared to ELISA but levels correlated well between both assays [35]. The difference in HMGB1 levels between both techniques may be due to binding ofHMGB1 to several molecules such as phospholipids, thrombomodulin and proteoglycans resulting in lower levels measured by ELISA [34,36,37]. In addition, Urbonaviciute et al. [38] demonstrated that serum and plasma components may interfere with the detection of HMGB1 by ELISA as HMGB1 may bind to plasma/serum proteins, in particular IgG class immunoglobulins. Titers of IgG in sera were shown to correlate inversely with the detected amounts detected by ELISA of a defined dose of recombinant HMGB1 added to these sera. IgG1 is the isotype predominantly found to co-immunoprecipitate with HMGB1 [38]. Immunohistochemical staining has been used to detect HMGB1 in tissues in studies that evaluated HMGB1 in chronic autoimmune and inflammatory diseases, liver transplantation, and malignant tumors. Using this technique, both the intensity of HMGB1 staining as well as

its localization can be evaluated, that is intranuclear, cytoplasmic or extracellular [35,39–43].

2. HMGB1 and anti-HMGB1 antibodies in systemic vasculitis

Serum levels of HMGB1 have been studied in several systemic vasculitides including Kawasaki disease (KD), Henoch-Schönlein purpura (HSP) and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) [44–49], but not in patients with large and medium-sized vessel vasculitis or cryoglobulinemic vasculitis. Antibodies against HMGB1 have been detected in patients with SLE and sepsis [33,50]. Sato et al., utilizing a Western blot technique, could not find those antibodies in 22 patients with AAV and 8 patients with HSP [48]. We have tested anti-HMGB1 antibodies using an in house ELISA technique and detected antibodies in 3 out of 24 patients with active AAV (12.5%) and in one of 18 healthy controls (5.6%) (unpublished data).

2.1. HMGB1 in Kawasaki disease

Serum levels of HMGB1 have been tested in two studies that included 63 Japanese patients with KD. Patients in the early acute phase of KD presented the highest levels of HMGB1, decreasing significantly during the late acute phase and convalescent phase of the disease (Table 1). Even in the latter phase, serum levels of HMGB1 in KD were higher than in healthy controls and were comparable to levels in patients with sepsis. Gene expression of RAGE and CD74, the invariant chain of class II major histocompatibility complex, in peripheral blood mononuclear cells was also higher in the acute phase of KD [44].

The use of high-dose intravenous immunoglobulin (IVIG) in combination with aspirin has been shown to decrease the frequency of coronary artery aneurysms in children with KD, especially in those with a good response to therapy [51]. Eguchi et al. showed that levels of HMGB1

were a potential marker of poor response to IVIG therapy in KD, since its levels were significantly higher in poor-responders to IVIG when compared to those who had a good response (Table 1). Using a receiver operating characteristic (ROC) curve, HMGB1 levels as measured by ELISA showed the largest area under the curve (0.852), and the optimal cut-off point for predicting poor responsiveness to IVIG was 2.4 ng/mL, showing a sensitivity of 86% and a specificity of 86% [45]. HMGB1 levels were shown to correlate with leukocyte counts, a known prognostic factor for poor responsiveness to high-dose IVIG in KD [45,52]. As such, levels may be a surrogate marker for poor responsiveness to IVIG in KD.

2.2. HMGB1 in ANCA-associated vasculitis

Some studies have evaluated serum HMGB1 levels and HMGB1 expression in tissues in patients with AAV. HMGB1 levels were shown to be higher in patients with AAV compared to healthy controls, in particular in patients with active granulomatosis with polyangiitis (GPA) (Table 1) [48,49]. HMGB1 levels correlated with the Birmingham Vasculitis Activity Score (BVAS) ($r=0.49$; $p<0.005$) in GPA, while in microscopic polyangiitis (MPA) no significant differences were observed when levels of HMGB1 were compared between patients with active disease, patients in remission or controls (Table 1). Serum HMGB1 levels as measured by ELISA were significantly higher in patients with GPA when compared to MPA, and, using ROC curve analysis, a cut-off value of 4.4 ng/mL of HMGB1 was found to discriminate GPA patients from MPA patients. Sensitivity and specificity for active GPA were 84% and 83%, respectively [49]. However, no significant differences in median HMGB1 levels were found among patients with GPA, MPA and Churg-Strauss syndrome (CSS) in another study (Table 1) [47].

The relationship between specific organ involvement in patients with AAV and serum levels of HMGB1 has also been investigated. Patients with AAV and active biopsy-proven nephritis presented higher serum levels of

HMGB1 when compared to those with inactive kidney disease and healthy controls [47]. Otherwise, levels of HMGB1 were significantly higher in patients with GPA and predominantly granulomatous inflammation than in those with predominantly vasculitic manifestations (Table 1). A positive correlation was found between HMGB1 serum levels and volumes of pulmonary granulomatous tissue as measured by chest computed tomography volumetry ($r=0.761$; $p=0.0017$), suggesting that granulomatous inflammation is an important source of HMGB1 in GPA [46]. Increased extra-nuclear staining of HMGB1 has been found by immunohistochemistry in kidney biopsies from patients with AAV and active nephritis when compared to those with inactive disease [47]. Furthermore, endonasal biopsies from patients with GPA showed marked nuclear, cytoplasmic and extracellular staining for HMGB1 in granulomas [46]. Hence, the inflammatory process in the kidneys and in granulomatous tissue resulting in activated, damaged and even necrotic cells seems to be the source of the increased cytoplasmic and extracellular localization of HMGB1 in AAV.

Although HMGB1 serum levels and extra-cellular expression of HMGB1 are higher in patients with AAV and biopsy-proven active glomerulonephritis than in those without renal inflammation, the extent of granulomatous inflammation seems to have a major impact on HMGB1 production and/or release, even when the disease is confined to the ENT region and lungs [46].

3. HMGB1 in atherosclerotic lesions

Atherosclerosis is an inflammatory condition that affects the arterial wall and is characterized by progressive thickening due to the accumulation of lipids [53]. The early event in the pathogenesis of atherosclerosis is endothelial dysfunction as a result of endothelial injury possibly caused by elevated and modified LDL, free radicals from cigarette smoking, diabetes mellitus, hypertension and other risk factors for cardiovascular disease alone

or in combination. Endothelial injury leads to compensatory responses that alter hemostatic properties of endothelium and result in increased endothelial permeability and adhesiveness [53]. The increased endothelial permeability allows the internalization of lipids into the intima. LDL particles become trapped in the arterial wall and trigger the secretion of chemotatic factors and expression of adhesion receptors by endothelial cells favoring monocyte recruitment, adhesion and migration into the vessel wall. In the intima layer, monocytes differentiate into macrophages and start internalizing lipids. Then, macrophages are transformed into foam cells which in turn release growth factors, cytokines, matrixmetalloproteinases (MMP) and reactive oxygen species [54,55]. Smooth muscle cells migrate into the intima and synthesize collagen, elastin and proteoglycans. In advancing lesions, a central lipidic or necrotic core is formed consisting of dying macrophages and extra-cellular lipids with formation of a fibrous cap [54,56]. The ultimate complication of atherosclerosis is disruption of the atherosclerotic plaque leading to thrombosis and to the clinical manifestations of atherosclerotic disease [54,56].

Table 1. Clinical studies evaluating HMGB1 levels in patients with inflammatory vascular diseases.

Diseases	Methods for HMGB1 detection	Remarks	Reference
Kawasaki's disease	ELISA	HMGB1 levels were higher in the early acute phase (29.8±29.3ng/mL) when compared to the late acute phase (16.3±20.3ng/mL, $p<0.01$) and the convalescent phase (12.3±21.8ng/mL, $p<0.01$).	[44]
	ELISA	Mean HMGB1 levels were higher in patients who did not respond to high-dose IVIG than in those who had a good response (6.0ng/mL vs. 1.5ng/mL, $p<0.01$).	[45]
ANCA-associated vasculitis	Western blot	Higher HMGB1 levels were found in patients with biopsy-proven active nephritis than in patients with inactive disease (120±48ng/mL vs. 78±46ng/mL, $p=0.01$). No significant differences were found in median HMGB1 levels among patients with GPA, MPA and CSS (95.8ng/mL vs. 127.7ng/mL vs. 112.9ng/mL; $p=0.53$).	[47]
	ELISA	HMGB1 levels were higher in active in comparison to inactive GPA (11.6±8.8 vs. 4.8±3.3ng/mL, $p<0.001$). No difference between active and inactive patients with MPA (2.6±2.5 vs. 2.6±2.7ng/mL, $p=1.00$) and HC (3.0±2.8ng/mL, $p=0.9$).	[49]
	ELISA	HMGB1 levels were higher in patients with GPA and predominantly granulomatous disease than in those with predominantly vasculitic inflammation (6.4±4.5ng/mL vs. 3.8±2.8ng/mL, $p=0.01$).	[46]
Coronary artery disease	ELISA	HMGB1 levels were higher in patients with CAD in comparison to those without CAD either in non-diabetic (4.3±10.4 vs. 1.74±2.0ng/mL, $P < 0.001$) or in diabetic patients (7.6±15.1 vs. 3.3±8.2ng/mL, $p<0.001$).	[80]
Acute coronary syndromes	Western blot	HMGB1 levels were higher in patients with ACS than in controls (159.0±54.3 vs. 1.9± 2.0ng/mL, $p<0.001$).	[79]
	ELISA	Patients with acute MI present higher HMGB1 levels in comparison to controls (14.8±6.8ng/mL vs. 2.3± 1.0ng/mL; $p<0.0001$).	[83]
	ELISA	Plasma HMGB1 levels were higher in patients with ACS who died when compared to surviving patients [4.8 µg/L (range 3.1-7.5) vs. 2.9 µg/L (range 2.6-3.2 µg/L), $p<0.05$].	[82]
Stroke	Western blot	Higher HMGB1 levels in patients with stroke than in controls (218±18.8ng/mL vs. 16.8±10.9ng/mL, $p<0.001$).	[79]
Intracerebral hemorrhage	Western blot	Higher HMGB1 levels in patients with a poor outcome than in those with a favorable outcome (221.4±49.5 vs. 114.6±32.6ng/mL, $p<0.001$).	[104]

ACS – Acute coronary syndromes; CAD – Coronary artery disease; CSS – Churg-Strauss syndrome; ELISA – Enzyme-linked immune assay; GPA – Granulomatosis with polyangiitis; HMGB1 – High mobility group box 1; MI – Myocardial infarction; MPA – Microscopic polyangiitis.

In normal human aorta, HMGB1 is constitutively expressed in endothelial cells, smooth muscle cells and in CD68 positive macrophages

localized close to the intima as well as in microvessels within the adventitia [57]. In contrast to normal human arteries, in human atherosclerotic lesions from the aorta, carotid and coronary arteries the expression of HMGB1 is markedly increased in the nuclei and in the cytoplasm of macrophages and smooth muscle cells localized near the intima [57,58]. Intense HMGB1 expression has also been observed in areas adjacent to the necrotic core of atherosclerotic lesions [57].

HMGB1 may be released from several cell types in the atherosclerotic plaque including smooth muscle cells, endothelial cells, foam cells, macrophages and activated platelets [57,59,60]. Once released, HMGB1 induces several inflammatory effects on endothelial cells, smooth muscle cells and macrophages. Recombinant HMGB1 has been shown to activate vascular endothelial cells leading to expression and secretion of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, granulocyte colony stimulating factor (G-CSF), RAGE, TNFα, monocyte chemotatic protein 1 (MCP-1), IL-8, plasminogen activator inhibitor 1, and tissue plasminogen activator [61,62]. Regarding smooth muscle cells from atherosclerotic plaques, HMGB1 promotes their proliferation, migration to the intimal layer, their release of more HMGB1 as well as C-reactive protein, and their expression of MMP2, MMP3 and MMP9 [58,59].

The importance of HMGB1 in the development of atherosclerosis has been demonstrated in apolipoprotein E deficientmice fed with a high-fat diet. The administration of neutralizing monoclonal antibodies against HMGB1 attenuated atherosclerosis by 55%. Furthermore, anti-HMGB1 neutralizing antibodies led to a decrease in macrophage, DC, and CD4+ T-cell accumulation in atherosclerotic lesions, and to a reduced expression of VCAM-1 and MCP-1 [63].

Statins have been shown to attenuate the effects of HMGB1 on endothelial cells in two experimental studies. Yang et al. observed that atorvastatin is able to inhibit endothelial activation in vitro upon HMGB1

stimulation in a dose-dependent manner. In this in vitro study, incubation of endothelial cells with 10 μM atorvastatin reduced the expression of ICAM-1 and E-selectin, and inhibited HMGB1-stimulated leukocyte adhesion to endothelial cells. Moreover, atorvastatin also suppressed HMGB1-induced TLR-4 expression and NFκB nuclear translocation in endothelial cells [64]. Haraba et al. observed that HMGB1 serum levels were increased in golden Syrian hamsters with induced hyperlipidemia, and HMGB1 release and RAGE expression were increased in cultures of U937-cells exposed to hyperlipemic sera. In this study, fluvastatin reduced serum HMGB1 levels by 38.2% and led to a 1.46-fold reduction of HMGB1 mRNA expression in lung tissue [65].

In contrast, a beneficial effect of HMGB1 has been demonstrated after ischemic limb injury in diabetic and non-diabetic mice. HMGB1 expression was lower in ischemic limbs of diabetic mice and this lower expression was associated with a diminished perfusion recovery after injury. Administration ofHMGB1 significantly improved blood flow and capillary density in ischemic muscles of diabetic mice and this beneficial effect was associated with an increased expression of vascular endothelial growth factor (VEGF) [66].

3.1. HMGB1 in acute coronary syndromes

3.1.1. HMGB1 in experimental studies of ischemic heart disease

HMGB1 has been implicated as an inflammatory mediator in ischemic heart disease responsible for increasing damage to myocardial tissue. Indeed, ischemia–reperfusion injury of the heart induced increased myocardial expression of cytoplasmic HMGB1 and apoptosis in vivo. In vitro, anoxia–reoxygenation challenge induced an increase in intra-cellular levels and extra-cellular release of HMGB1 in isolated cardiomyocytes. Myocardial apoptosis was decreased following administration of HMGB1 box A, an antagonist of functional HMGB1 cytokine activity [67]. In an experimental model of ischemia– reperfusion injury of the heart in mice, HMGB1 has been demonstrated to be overexpressed in infiltrating leukocytes in the myocardium

and in left ventricle tissue lysates in the early phase whereas HMGB1 mRNA levels remained high in injured myocardium up to 7 days after ischemia/reperfusion injury, demonstrating de novo local production (Fig. 1). The administration of recombinant HMGB1 to mice worsened myocardial injury whereas treatment with HMGB1 box A, a specific HMGB1 antagonist, reduced infarct size and markers of tissue damage. The administration of recombinant HMGB1 or HMGB1 box A to RAGE-deficient mice had no effect, indicating an important role of HMGB1–RAGE interaction in ischemia–reperfusion injury of the heart [68]. Although considered to be a mediator of myocardial injury in ischemic heart disease, several studies have demonstrated beneficial effects of HMGB1 in experimental models of myocardial infarction (MI). Kitahara et al. observed smaller infarcted areas, improved cardiac function and higher survival rates after induced MI in transgenic mice with cardiac overexpression ofHMGB1when compared to control mice. The transgenic mice released more HMGB1 into the circulation after ligation of the left anterior descending coronary artery. These findings suggest a beneficial effect of released HMGB1 on the heart after MI [69].

The administration of HMGB1 to the myocardium some hours after ischemic injury has also resulted in better outcomes in experimental studies. Limana et al. administered purified HMGB1 into the peri-infracted left ventricle 4 h after permanent coronary artery ligation in C57BL/6mice. The procedure resulted in the formation of new myocytes within the infarcted area in association with proliferation and differentiation of endogenous cardiac c-kit+ progenitor cells. HMGB1-treated mice demonstrated a significantly better recovery of cardiac performance in the following weeks in comparison to non-treated mice [70]. The administration of HMGB1 to the myocardium three weeks after coronary artery ligation has also been shown to have beneficial effects in the study performed by Takahashi et al. In this study, HMGB1 was injected intra myocardially three weeks after left coronary artery ligation in female Sprague–Dawley rats resulting in a significant improvement in left

ventricular ejection fraction as observed 28 days later in comparison to non-treated rats. Moreover, accumulation of inflammatory cells, mainly DC, in the peri-infarcted area, cardiomyocyte hypertrophy and extra-cellular collagen deposition were all attenuated following HMGB1 administration when compared to control treated rats [71]. Limana et al. also found favorable outcomes when HMGB1 was administered to the peri-infarcted region three weeks after coronary artery ligation in female C57BL6 mice. Four weeks after treatment, there was an improvement in left ventricular function as well as a reduction of left ventricle volume, an increase in infarcted wall thickness and a reduction in collagen deposition in the myocardium compared to mice injected with denatured HMGB1. Furthermore, cardiac regeneration was seen with an increase in c-kit+ cell number, newly formed myocytes, and arteriole length density. HMGB1 also led to an enhancement in collagenase (MMP2 and MMP9) activity and a decrease in tissue inhibitor of metalloproteinase-3 (TIMP-3) levels indicating increased collagenolytic activity [72]. The effect of HMGB1 on proliferation and differentiation of cardiac c-kit+ stem cells has been demonstrated to be indirectly mediated through paracrine stimulation of cardiac fibroblasts to produce several inflammatory cytokines and growth factors [73]. In terms of angiogenesis in response to ischemia, HMGB1 stimulates homing of endothelial progenitor cells to ischemic tissues as well as endothelial cell migration, sprouting and neovascularization (Fig. 1) [74–76].

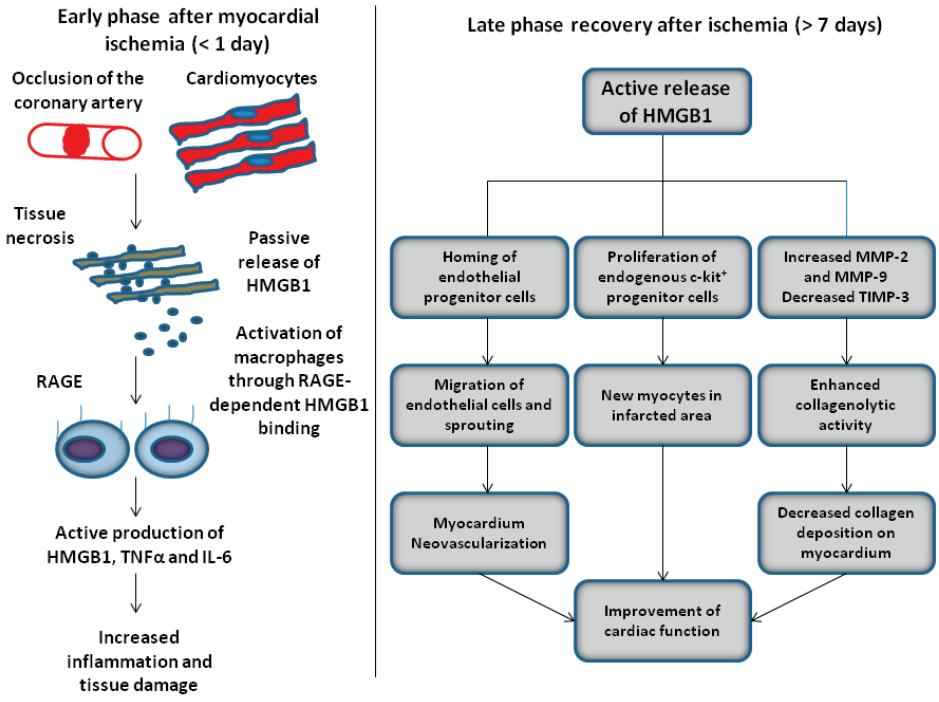

Figure 1. HMGB1 displays dual effects after myocardial ischemia. In the acute phase after ischemia HMGB1 is released by necrotic cells and activates macrophages via RAGE-dependent binding. Activated cells release HMGB1 and proinflammatory cytokines that amplify tissue damage caused by ischemia. HMGB1 mRNA levels remain high in injured myocardium up to 7 days after ischemia/reperfusion injury. In the late phase after ischemia, HMGB1 is essential for cardiac functional recovery contributing to tissue repair by stimulation of neovascularization, proliferation and differentiation of endogenous cardiac c-kit+ progenitor cells into myocytes and inhibition of excessive collagen deposition in the ischemic area.

More recently, Abarbanell et al. have shown a dose-dependent effect of HMGB1 on myocardial recovery after acute global ischemia/reperfusion injury in rat hearts using the Langendorff method. Either 200 ng or 1 µg of HMGB1 was administered 1 min after reperfusion and HMGB1 significantly improved myocardial functional recovery, decreased infarct size, and decreased levels of IL-1, IL-6, IL-10 and VEGF. Although HMGB1 dose

dependently decreased myocardial inflammation and infarct size, it did not result in a dose dependent improvement of left ventricular function after ischemia/reperfusion [77].

The previous findings indicate disagreement regarding the role of HMGB1 in ischemic heart disease. Differences in study design and the dose of HMGB1 used in these studies may account for the opposing effects of HMGB1 in ischemic hearts. Nevertheless, the results of experimental studies indicate that HMGB1 is not only a proinflammatory mediator during the early phases of ischemia–reperfusion injury, but also acts as an important factor for recovery in MI in the later phases. In ischemia–reperfusion injury, especially after reperfusion, tissue injury is elicited by the release of oxygen-derived free radicals leading to inflammation and myocardial apoptosis, which are all enhanced by HMGB1 [78].

3.1.2. HMGB1 in clinical studies of acute coronary syndromes

Increased levels of serum HMGB1 in comparison to controls were reported for the first time in a small study that evaluated 9 patients with acute coronary syndromes (ACS). HMGB1 levels did not correlate either to creatine phosphokinase (CPK) or to troponin levels [79]. Next HMGB1 serum levels were evaluated in a large study that included type 2 diabetic and non-diabetic patients with or without coronary artery disease (CAD) regardless of symptoms of ACS. CAD was defined as a luminal diameter narrowed ≥50% at a major epicardial coronary artery by angiography. HMGB1 levels were significantly higher in diabetic and non-diabetic patients with CAD compared to those without CAD (Table 1). A positive correlation between levels of HMGB1 and that of hsCRP, TNFα and IL-6 was found as well. Moreover, levels of endogenous secretory RAGE (esRAGE), a decoy receptor for advanced glycation end products and for HMGB1, were significantly lower in diabetic and non-diabetic patients with CAD. In multivariate regression

analysis, HMGB1 and esRAGE levels were independently associated with CAD either in diabetic or nondiabetic
patients [80].

In another study that evaluated patients with ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI), HMGB1 serum levels were significantly correlated with infarct size as measured by cardiac magnetic resonance imaging performed 2–4 days after MI for STEMI and NSTEMI, respectively (r^2=0.81 and r^2=0.74; p<0.001). Furthermore, cardiac magnetic resonance imaging was repeated 6 months after MI to estimate residual ventricular function and showed an inverse correlation between HMGB1 levels during MI and the residual ejection fraction both in STEMI and NSTEMI, respectively (r^2=–0.40 and r^2=–0.25; p<0.001) [81].

HMGB1 plasma levels have also been shown to be associated with increased mortality in patients with STEMI due to occlusion of the left anterior descending coronary artery successfully treated with primary percutaneous coronary intervention. In this study, 144 patients were evaluated and 13 patients suffered cardiovascular death after a median 10 months of follow-up. The average baseline levels of HMGB1 were higher in patients who died compared to surviving patients (Table 1). A doubling in HMGB1 levels increased the risk of mortality (hazard ratio: 1.75; 95% confidence interval: 1.1 to 2.8) [82].

Cirillo et al. demonstrated that HMGB1 serum levels were higher in patients with acute MI than in controls or post-infarct patients (Table 1), HMGB1 levels at the time of MI were significantly correlated with cardiopulmonary parameters, such as oxygen consumption at peak exercise (VO2peak) and the slope of increase in ventilation over carbon dioxide output (VE/VCO2slope), as well as with Doppler echocardiographic parameters, peak creatine kinase-MB (CK-MB), and troponin I levels [83]. In this study, tissue factor pro-coagulant activity in vitro was progressively increased after

stimulation of human coronary artery endothelial cells with increasing doses of HMGB1 indicating that HMGB1 may have a role in inducing microvascular thrombosis [83]. In another study performed by the same group, post-infarct HMGB1 levels were lower in patients who underwent a 6-month exercise-based cardiac rehabilitation program. In trained patients, lower HMGB1 levels were significantly associated with improvement in VO2 peak and heart rate recovery as well as with reduced left ventricular end-diastolic volume and wall motion score volume, indicating improvement in cardiopulmonary and autonomic function along with favorable cardiac remodeling [84].

Taken together, these studies have shown that levels of HMGB1 are higher in patients with subclinical CAD and in those with overt ACS than in controls. HMGB1 levels have also been shown to be a prognostic factor in ACS as HMGB1 levels correlate with larger infarct size, transmurality, residual cardiac function, and mortality.

3.2. HMGB1 in ischemic stroke

Sudden arterial occlusion by thrombus formation or embolism leads to immediate loss of oxygen and glucose in cerebral tissue. This result in excitotoxicity, oxidative damage and neuronal death in the area affected by severe focal hypoperfusion while in the surroundings of the ischemic core, in the so-called penumbra area, neurovascular dysfunction is observed rather than neuronal death. Eventually, in the area affected by ischemic injury microvascular damage and blood–brain barrier dysfunction develop together with post-ischemic inflammation which may be detrimental and promote cell death in the early phases of stroke. HMGB1 can, however, also be beneficial and contribute to tissue remodeling during the recovery process [85,86].

3.2.1. HMGB1 in experimental studies of ischemic stroke

HMGB1 has been shown to be widely expressed throughout the normal rat brain, mainly in the nuclei of neurons and oligodendrocyte like cells

[87]. HMGB1 moves from the nucleus to the cytoplasm in neurons and astrocytes challenged with necrotic stimuli, and is then released passively into the extracellular space in the early phases after stroke [87,88]. Increased expression of HMGB1 is observed in the ischemic brain hemisphere and its levels increase aswell in plasma and cerebrospinal fluid after an ischemic injury [89]. The immediate increase in extracellular HMGB1 is a result of passive release of HMGB1 due to excitotoxity- and ischemia-induced neuronal death secondary to brain ischemia [88]. HMGB1 expression starts to decrease 3 h after middle cerebral artery occlusion (MCAO)/reperfusion and is further reduced one day after ischemic injury. Two days after MCAO/reperfusion, HMGB1 expression in brain tissue starts to increase again and it peaks around 4 days after MCAO/reperfusion [87]. This delayed expression of HMGB1 is observed in activated microglia, astrocytes and in microvascular structures, and results from active production of HMGB1 by activated cells (Fig. 2) [87–90].

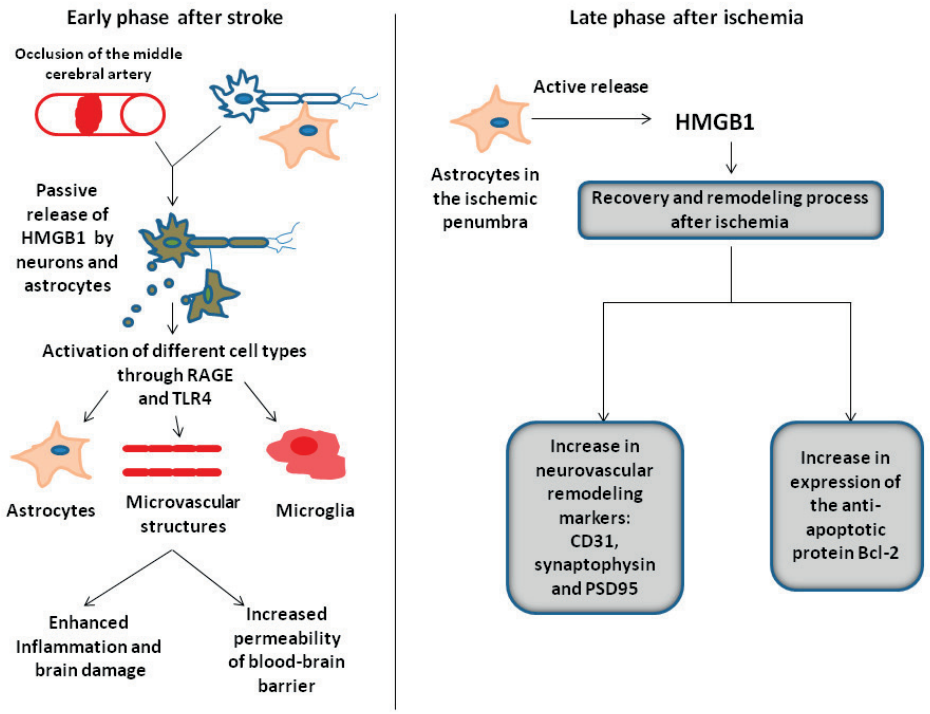

Figure 2. Passive release of HMGB1 stimulates inflammation in the acute phase and active release of HMGB1 stimulates recovery in the late phase after stroke. HMGB1 is passively released into the extracellular space by dying neurons and glial cells in the acute phase after ischemia. Extracellular HMGB1 activates several cell types through TLR4 and RAGE, especially the microglia, and enhances post ischemic inflammation and increased permeability of the blood–brain barrier. The acute effects of HMGB1 after brain ischemia lead to more brain edema and damage. In the late phase after brain ischemia, HMGB1 is released actively by activated astrocytes in the ischemic penumbra area. It contributes to the recovery and remodeling process of the brain through stimulation of vascular repair, neurite outgrowth and expression of Bcl-2 to protect neurons from apoptotic stimuli.

In the acute phase after stroke, the inflammatory process amplifies the initial ischemic injury and HMGB1 plays an important role in the induction of inflammation in ischemic brain tissue mainly through microglial activation, the hallmark of brain inflammation. RAGE and TLR4 have been shown to be the main receptors that exert HMGB1 effects in brain tissue affected by ischemia [90–92]. The importance of HMGB1 in the development of acute inflammation

after ischemic injury has been highlighted in studies that evaluated inhibition of HMGB1 with different agents. Blockade of HMGB1 signaling with short hairpin RNA in the post-ischemic brain suppressed infarct size, microglial activation and induction of proinflammatory mediators [89]. The use of anti-HMGB1 neutralizing antibodies in experimental models of MCAO/reperfusion led to a remarkable reduction in infarct size and an improvement in neurologic deficits in treated rats. Anti-HMGB1 antibodies also prevented the increase in permeability of the blood–brain barrier protecting the recipient from brain edema, inhibited activation of microglia and expression of TNFα and induced nitric oxide synthase (iNOS), while suppressing the activity of MMP9 [93,94]. Furthermore, the administration of several agents such as atorvastatin, minocycline, edaravone, cannabinol, niaspan and Tricin 7-glucoside was also shown to inhibit HMGB1 expression in brain ischemic tissue during the acute phase after stroke in experimental models of MCAO, so alleviating cerebral injury [95–100].

In the late phase after ischemia, recovery and remodeling take place in the brain. The interaction between neurons, glial cells, endothelial cells and extracellular matrix is important for neurovascular repair. In this recovery phase, reactive astrocytes not only contribute to glial scarring but also secrete several trophic factors thatpromote neurogenesis, synaptogenesis and angiogenesis after stroke [85]. It has been demonstrated that activated astrocytes concentrated in the ischemic penumbra area express HMGB1 2 days after stroke in parallel with an increase in neurovascular remodeling markers such as CD31, synaptophysin and PSD95. Metabolic inhibition of these astrocytes with fluorocitrate suppressed HMGB1 expression as well as neurovascular remodeling markers in astrocytes and resulted in worsening of behavioral recovery in mice after stroke [87,101]. These results indicate that HMGB1 produced by astrocytes is important for neurovascular repair in the brain after stroke. The HMGB1 receptor RAGE promotes neurotrophic effects in the nervous system upon stimulation by HMGB1 and S100 family proteins

that induce neurite outgrowth, activation of transcription factor NFκB and increased expression of the anti-apoptotic protein Bcl-2 (Fig. 2). Nonetheless, hyperactivation of RAGE by high concentrations of its ligands promotes neuronal apoptosis [102].

In summary, experimental studies of stroke have shown that HMGB1 presents dual effects after ischemic injury in the brain. HMGB1 acts as a proinflammatory mediator in the acute phase that amplifies damage in ischemic tissue through the activation of microglia, enhancement of inflammation and increase of permeability of the blood–brain barrier. In contrast, in the late phase after ischemic injury HMGB1 contributes to the recovery and remodeling process stimulating neurovascular repair mainly by astrocytes in the affected brain.

3.2.2. HMGB1 in clinical studies of cerebrovascular accidents

In contrast to experimental studies of ischemic stroke, few studies have evaluated HMGB1 in patients with ischemic stroke. Higher HMGB1 levels were described in patients with cerebral vascular ischemia within 24 h after the onset of symptoms in comparison to control subjects (Table1) [79]. HMGB1 levels in patients with stroke remain significantly higher than in control subjects up to 14 days after the ischemic event while levels of the natural inhibitors of HMGB1, soluble RAGE (sRAGE) and esRAGE, remain indistinguishable from control subjects within 48 h following stroke. HMGB1 levels in patients with stroke are significantly correlated with IL-6 levels but not with the extent of brain tissue destruction as assessed by CT morphometry. Moreover, patients with stroke present an increased proportion of activated CD4+ T-cells in peripheral blood expressing CD25 or HLA-DR when compared to controls [103]. Due to the similarity between the kinetics of serum HMGB1 and the kinetics observed for the absolute number of CD4+ T-cells expressing HLA-DR, the authors raised the hypothesis that HMGB1 acts

as a link between brain tissue destruction by ischemic injury and the activation and Th1 priming of T-cells [103].

Zhou et al. observed higher levels of HMGB1 in patients with intracerebral hemorrhage (ICH) when compared to controls. HMGB1 was associated with the severity of stroke since patients with ICH and poor outcome had higher levels of HMGB1 than those with a favorable outcome (Table 1). There was a significant correlation between HMGB1 levels and the National Institutes of Health Stroke Scale (NIHSS) at day ten after stroke, and with the modified Ranking scale score at 3 months. HMGB1 was also correlated with IL-6 and TNFα levels in patients with ICH [104].

4. Conclusion

HMGB1 has been implicated in the pathogenesis of inflammatory vascular diseases including systemic vasculitis and atherosclerotic disease. High serum HMGB1 levels have been found in patients with ANCA-associated vasculitis and Kawasaki disease as a reflection of active disease. Furthermore, HMGB1 is expressed in atherosclerotic lesions by several cell types and contributes to the progression of the atherosclerotic plaque. HMGB1 levels are significantly increased in patients with subclinical CAD and in those who develop acute ischemic events in cardiac and cerebral vascular beds. Experimental studies show that HMGB1 has a dual effect, amplifying the inflammatory response as well as damage in the acute phase and participating in tissue remodeling during the late phase after ischemic injury. Targeting HMGB1 may be an attractive therapeutic modality for inflammatory vascular diseases.

Take-home messages

- HMGB1 levels are increased in the acute phase of Kawasaki disease and this has been regarded as a marker of poor response to intravenous immunoglobulin therapy.

- Increased levels of HMGB1 are detected in patients with ANCA associated vasculitis with active disease and this is correlated both with granulomatous manifestations and with biopsy-proven active renal involvement.
- HMGB1 is expressed in the majority of macrophages and in some intimal smooth muscle cells in atherosclerotic lesions, and is implicated in the progression of the atherosclerotic plaque.
- Deleterious and beneficial effects of HMGB1 have been described for HMGB1 in experimental models of atherosclerotic disease, since HMGB1 is involved in the amplification of the inflammatory response during acute ischemic injury but also in the recovery and remodeling process after ischemia.
- Serum HMGB1 levels are significantly higher in patients with acute coronary syndromes and stroke in comparison to control subjects.

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CHAPTER 3

Are monuclear cells predominant actors of endothelial damage in vasculitis?

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Introduction

Antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) constitute a group of disorders characterized by autoimmune necrotizing inflammation of small blood vessels, which leads to systemic organ damage [1]. This group of systemic vasculitides includes Granulomatosis with poliangiitis (GPA), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS). These disorders are predominantly associated with the presence of circulating ANCA that are directed against proteins in cytoplasmic granules of neutrophils. ANCA with specificity for proteinase-3 (PR3-ANCA) are associated particularly with GPA, whereas ANCA with specificity for myeloperoxidase (MPO-ANCA) are predominant in MPA and to a lesser degree in CSS [2]. Although it remains unknown how these conditions develop, it has been postulated that ANCA in vivo bind to surface expressed autoantigens (PR3 or MPO) on pre-activated (primed) neutrophils, which enhances neutrophil degranulation and the release of toxic products that cause endothelial damage ultimately leading to necrotizing vasculitis.

In vivo experimental studies have clearly demonstrated that MPO-ANCA are pathogenic factors [3,4]. An immunopathogenic role for MPO-ANCA has also been strongly suggested by the occurrences of neonatal MPA in a child born to a mother with a history of MPO-ANCA-associated pulmonary-renal syndrome [5]. In contrast to MPO-ANCA, in vivo evidence is limited for a direct vasculitic pathogenicity of PR3-ANCA. More recently, Little et al. [6] injected PR3-ANCA containing human IgG into mice with a chimeric human-mouse immune system including human neutrophils. These mice developed glomerulonephritis (in a minority paucimune crescentic) and, in a few, pulmonary capillaritis, but granulomatous inflammation, characteristic for human PR3-ANCA GPA, was not observed [6]. It has been demonstrated that $CD4^+$ T-cells are the key player in the generation of granulomatous response.

For instance, CD4-deficient mice did not generate typical mononuclear granulomatous lesions following *Mycobacterium tuberculosis* infection [7]. In humans, the extent of granuloma formation was correlated with peripheral CD4 T-cells counts in HIV patients with mycobacterial infection [8,9]. This suggests a primary role of cell-mediated immunity in initializing granuloma formation.

Role of CD4 T_{EM} cells and their effector cytokines (IL-17 and IL-21) in ANCA-associated vasculitides

In AAV, neutrophil-mediated tissue damage has been considered an important part of disease pathogenesis. However, several observations support a key role of T-cells in disease manifestations as well. The important role of $CD4^+$ T-cells in the expression of crescentic glomerulonephritis (CG) has been demonstrated by Ruth et al. [10]. They induced experimental anti-MPO-associated CG by immunizing C57BL/6 mice with human MPO followed by subsequent challenge with anti-glomerular basement membrane antibodies (anti-GBM). Mice depleted of $CD4^+$ T-cells at the time of administration of antimouse GBM developed significantly less glomerular crescent formation and less cell influx when compared with control mice. These data provide convincing evidence that $CD4^+$ T-cells are crucial in granuloma formation and glomerulonephritis. Studies in AAV-patients also support this notion. It has been shown that MPO-induced proliferation of peripheral blood mononuclear cells from MPA-patients was completely lost after the depletion of $CD4^+$ T-cells, but not after depletion of $CD8^+$ T-cells [11]. In addition, IgG subclass distribution of ANCA (IgG1 and IgG4) implies isotype switching of ANCA for which $CD4^+$ T-helper cells are required [12].

In line with this observation, an altered phenotype of $CD4^+$ T-cells has been found in AAV-patients. An expanded population of $CD4^+$ T-cells lacking

the costimulatory molecule CD28 was observed in peripheral blood and in granulomatous lesions of patients with GPA [13,14]. These $CD28^{-}CD4^{+}$ T-cells display upregulation of the T-cell differentiation marker CD57 and show intracytoplasmic perforin expression indicating cytotoxic potential of these cells [13]. A more detailed analysis of T cells showed that expansion of $CD4^{+}$ T-cells in GPA occurred within the $CD4^{+}$ effector memory population (T_{EM}) characterized by being positive for CD45RO and negative for the lymphoid homing receptor CCR7 [15]. The generation of these $CD4^{+}$ T_{EM} cells needs a strong and persistent trigger [16], which suggests that T-cells in GPA are in a persistent state of an ongoing immunological trigger, also during remission. Defect in regulatory T-cell function, found in GPA-patients, may also contribute to the expansion of $CD4^{+}$ T_{EM} cells [17,18]. Surprisingly, these $CD4^{+}$ T_{EM} cells are decreased in number during relapsing disease [15,19]. They are supposed to migrate then into lesional tissues. In accordance, infiltrating T-cells in lung lesions and glomeruli were shown to consist mainly of $CD4^{+}$ T-cells with a memory phenotype [14,20,21]. Indeed, our cross-sectional and follow-up studies confirmed migration of $CD4^{+}$ T_{EM} cells during active renal disease into the diseased organs [19]. We observed a remarkable increase in $CD4^{+}$ T_{EM} cells in the urinary sediment with a concomitant decrease of circulating $CD4^{+}$ T_{EM} cells of GPA-patients with active renal involvement [19]. These urinary $CD4^{+}$ T_{EM} cells decreased or disappeared from the urine during remission, which might reflect their role in renal injury. In line with this, Wilde et al. demonstrated an expansion in a specific subset of circulating T_{EM} cells in GPA expressing CD134 and reported $CD134^{+}$ T_{EM} cells in active lesions, which support their migration to inflamed sites [22]. Importantly, CD134 costimulation was shown to program $CD4^{+}$ T-cells to express lytic molecules and to perform cytotoxic function [23]. This may indicate that these T_{EM} cells have a major role in tissue injury in AAV. Furthermore, Ordonez et al. found that AAV-patients exhibit an expanded $CD45RC^{Low}$ $CD4^{+}$ T-cell population that is a source of IL-17 [24].

Over the past few years, Th17 cells have challenged the classical Th1/Th2 paradigm, and have been implicated in a growing number of autoimmune and inflammatory diseases [25]. It has been reported that IL-17 enhances the production of autoantibodies, and induces CXC chemokine release and expression of adhesion molecules responsible for the recruitment of neutrophils to the site of inflammation [26-28]. This cytokine also promotes the production and release of IL1-β and TNF-α by macrophages [29], which are essential for triggering the translocation of PR3 on the surface of neutrophils. Thus, IL-17 is likely involved in the recruitment of neutrophils and other immune cells to the site of inflammation, which contribute to granuloma formation and also to create conditions for ANCA-induced neutrophil-dependent endothelial cell lysis. In GPA patients, skewing toward Th17 cells and increased serum IL-17A as well as increased MPO and PR3 specific Th17 cells were reported.

The most convincing experimental evidence that Th17 cells contribute to the pathophysiology of AAV comes from a recent study by Gan et al. [30]. They studied the effect of IL-17A, the key Th17 effector cytokine, on the development of necrotizing glomerulonephritis (NG) mediated by anti-MPO autoimmunity in IL-17A deficient mice in comparison with C57BL/6 wild-type mice. Both mice were immunized with MPO and developed cellular and humoral autoimmune responses to MPO. Glomerular injury in those mice was induced by injecting a low dose of heterologous anti-GBM, which triggered NG by recruiting neutrophils to glomeruli. They found that MPO-immunized C57BL/6 wild-type mice showed significant glomerular injury, whereas the glomeruli in IL-17-deficient mice were nearly completely protected due to diminished neutrophil accumulation and MPO deposition. This suggests a crucial role of IL-17A in renal tissue injury. Besides IL-17, Th17 cells can produce IL-21, a cytokine that is produced primarily by T follicular helper (T_{FH}) cells and is required for B-cell class switching and antibody production, and which induces differentiation of B-cells towards plasma cells by

synergizing with B-cell activating factor (BAFF) [31,32]. We have recently demonstrated that IL-21 producing T_{FH} were significantly increased in peripheral blood of GPA-patients [33]. The role of IL-21 in vasculitis was previously suggested by Chen et al. [34]. In their study, mice deficient in interferon regulatory factor-4, a protein that inhibits IL-17A production, rapidly developed large-vessel vasculitis and showed increased IL-21 synthesis in addition to increased IL-17A production. Moreover, a role of IL-21 in recruitment of Th17 cells to inflamed tissues has been reported by Caruso et. al. [35] by showing that IL-21 induces gut epithelial cells to secrete macrophage inflammatory protein-3α (MIP-3α), a chemokine that mediates Th17 cell homing to the skin, joints, and mucosal tissues. Given that endothelial cells are known to produce MIP-3α, it is possible that IL-21 in GPA-patients enhances the migration and accumulation of Th17 cells into the vascular wall resulting in inflammation. Besides, IL-21 was shown to enhance granzyme B expression and increase perforin-mediated cytotoxicity by human $CD8^+$ T-cells and NK cells [36-38]. It is therefore conceivable that IL-21, together with IL-17, can contribute to vessel injury and disease progression in GPA-patients.

Taken together, activated $CD4^+$ T_{EM} cells and their effector cytokines (IL-17 and IL-21) are believed to be inducers of tissue injury, and serve as effector cells in the pathogenesis of AAV.

How and when do $CD4^+$ T_{EM} cells attack endothelial cells in ANCA-associated vasculitis

Over the past decades, considerable research effort has been directed toward investigating and elucidating the pathogenic role of T-cells in endothelial injury in AAV. According to the aforementioned findings, we hypothesize that $CD4^+$ T_{EM} cells act as a key trigger of disease expression

and relapse and are an important player in endothelial injury in AAV. So the question arises: How can $CD4^+$ T_{EM} cells attack endothelial cells and when?

At the functional level, $CD4^+$ T_{EM} cells shown to mimic NK cells by their cytotoxicity and surface expression of the NKG2D molecule [39]. NKG2D is an activating C-type lectin-like homodimeric receptor, which differs from other NKG2 members as it apparently lacks an antagonist and substitutes for CD28-mediated costimulatory signalling in $CD28^-$ T_{EM} cells [40]. One of the NKG2D-ligands is the major histocompatibility complex class-I chain-related molecule A (MICA), which is usually absent on normal cells, but expressed upon cellular injury and stress on target cells such as fibroblasts, epithelial cells, and endothelial cells [41]. The expression of MICA on the surface of endothelial cells makes this polymorphic molecule a possible target in vasculitis. It has been shown that $NKG2D^+CD4^+$ T-cells can kill target cells that express MICA via NKG2D-MICA interaction [42]. Many clinical studies have shown that that the presence of MICA on kidney or heart transplant samples after transplantation is associated with acute or chronic allograft rejection [43-46]. Importantly, NKG2D was anomalously expressed and preferentially detected on circulating $CD4^+$ T_{EM} cells in GPA-patients [47]. It has been reported that IL-15 is the major inducer of $NKG2D^+CD4^+$ T-cells expansion in GPA [48]. In addition, MICA is upregulated in peritubular endothelium and glomerular epithelial cells in AAV-patients during active renal disease. Strikingly, Capraru and colleagues have shown that both NKG2D and MICA are expressed in granulomatous lesions in GPA, but not in disease controls [49]. Therefore, it is likely that killing mechanisms via NKG2D-MICA interaction contribute to vessel injury and disease progression in AAV-patients.

Based on aforementioned finding, we can postulate that expanded population of $CD4^+$ T_{EM} cells, resulting from persistent activation of Th-cells by PR3 or MPO, upregulate their NKG2D protein and migrate to the peripheral

blood, and remain in the circulation during remission. When the disease becomes active, MICA protein will be upregulated on several vascular endothelial cells, especially in the kidney, which attracts T_{EM} cells to the inflammatory areas. The MICA protein on the target cells can bind to NKG2D on T_{EM} cells, which in turn enhances their cytotoxic function, that is killing target cells in a perforin and granzyme dependent way which results in vasculitis. Accordingly, selective targeting of $NKG2D^{+}CD4^{+}T_{EM}$ or inhibiting MICA-expression without impairing other parts of cellular immunity might have value in the treatment of AAV.

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CHAPTER 4

Is serum HMGB1 a biomarker in ANCA-Associated vasculitis?

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Abstract

Background: Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are systemic inflammatory disorders that include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), Churg-Strauss syndrome and renal limited vasculitis (RLV). Extra-cellular high mobility group box 1 (HMGB1) acts as an alarmin and has been shown to be a biomarker of disease activity as well as an autoantigen in systemic lupus erythematosus (SLE) and, possibly, in AAV. This study aims to assess antibodies against HMGB1 and HMGB1 levels as biomarkers for AAV disease activity and predictors of relapsing disease.

Methods: AAV patients with active disease and healthy controls (HC) were evaluated for anti-HMGB1 antibodies while serum HMGB1 levels were measured longitudinally in AAV patients at presentation, during remission, prior to and at relapses.

Results: HMGB1 levels were similar between AAV patients at presentation (n=52) and HC (n=35) (2.64±1.80ng/ml vs. 2.39±1.09ng/ml; $p = 0.422$) and no difference regarding HMGB1 levels could be found among AAV disease subsets (GPA: 2.66±1.83ng/ml vs. MPA: 3.11±1.91ng/ml vs. RLV: 1.92±1.48ng/ml; $p = 0.369$). AAV patients with renal involvement had lower HMGB1 levels than patients without renal involvement at presentation (2.35±1.48ng/ml vs. 3.52±2.41ng/ml; $p = 0.042$). A negative correlation was observed between HMGB1 levels and 24-hour proteinuria ($\rho = -0.361$, $p = 0.028$). Forty-nine AAV patients were evaluated for HMGB1 levels during follow-up and no differences were observed between relapsing and nonrelapsing patients ($p = 0.350$). No significant increase in HMGB1 levels was observed prior to a relapse comparing to the remission period and changes in HMGB1 levels were not associated with an increased risk for relapse in AAV. Positivity for anti-HMGB1 antibodies was low in patients with active AAV (3 out of 24 patients).

Conclusion: Serum HMGB1 levels at presentation are not increased and are lower in patients with renal involvement. Relapses are not preceded or accompanied by significant rises in HMGB1 levels and changes in HMGB1 levels are not related to ensuing relapses. Anti-HMGB1 antibodies are present in only a few patients in AAV. In contrast to SLE, HMGB1 is not a useful biomarker in AAV.

Introduction

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are primary systemic vasculitides affecting small and medium-sized vessels, and are associated with ANCA against proteinase 3 (PR3) and myeloperoxidase. AAV include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), Churg-Strauss syndrome, and isolated pauci-immune necrotizing crescentic glomerulonephritis also designated as renal limited vasculitis (RLV) [1,2]. Disease relapses are common in AAV and occur in up to 60% of patients, especially in patients with GPA and PR3 ANCA [3-7]. Risk factors for relapses in AAV include the persistence of PR3 ANCA after induction of remission, upper and lower airway involvement, cardiovascular involvement, and chronic nasal carriage of *Staphylococcus aureus*, particularly strains that express the toxic shock syndrome toxin-1 superantigen gene [3,5,6,8]. A recent meta-analysis showed that the rise in ANCA titers or their persistence during remission is only modestly associated with an increased risk of relapses in AAV patients [9]. There is thus an unmet need for biomarkers predicting which AAV patient is prone to relapse.

High-mobility group box-1 (HMGB1) is a nuclear protein that binds DNA and modulates chromosomal architecture. Once released into the extracellular space, after cell death or upon activation, HMGB1 acts as a danger associated molecular pattern or as an alarmin and stimulates inflammatory and immunological activities that include cytokine production, chemotaxis, cell proliferation, angiogenesis and cell differentiation. HMGB1

has to bind to the receptor for advanced glycation endproducts (RAGE) and toll-like receptor (TLR)-2, TLR-4 and TLR-9 in order to exert its actions [10,11].

In systemic lupus erythematosus (SLE), serum HMGB1 has been shown to be a biomarker of disease activity, especially in patients with lupus nephritis. Moreover, patients with active lupus nephritis present higher HMGB1 levels in urine compared with SLE patients without active nephritis and with controls [12-14]. Furthermore, levels of antibodies to HMGB1 are higher in patients with active SLE than in patients with quiescent disease and in controls [13]. In AAV, a cross-sectional study showed increased serum levels of HMGB1 in patients with active GPA [15]. In addition, one study found an association with granulomatous manifestations and another with biopsy-proven renal involvement [16,17].

Until now, HMGB1 levels have not been evaluated longitudinally as a biomarker of disease activity or as a predictor of ensuing relapses in patients with AAV. The aims of this study were to evaluate whether serial levels of HMGB1 reflect changes in disease activity and/or predict the occurrence of relapses, and to analyze whether AAV patients have antibodies to HMGB1.

Materials and methods

Patients

Patients on follow-up at the University Medical Center Groningen with a diagnosis of AAV, including GPA, MPA, and RLV, were eligible for the study. Patients had a clinical diagnosis of GPA or MPA according to the European Medicines Agency algorithm [18]. Patients with isolated renal involvement, ANCA positivity and biopsy-proven pauci immune necrotizing glomerulonephritis were classified as RLV. ANCA tests were performed in all patients by indirect immunofluorescence using ethanol-fixed neutrophils, while ANCA specificity for PR3 or myeloperoxidase was assessed by enzyme linked immunosorbent assay (ELISA). To assess whether HMGB1 levels are

increased in active disease, 52 AAV patients were included at presentation; characteristics are presented in Table 1. Additionally, 49 out of 52 AAV patients with sufficient follow-up data were included in a longitudinal analysis and were evaluated during a mean period of 54.4 ± 17.6 months. Thirty-five age-matched and sex-matched healthy controls (HC) were evaluated for HMGB1 levels in the study as well. All patients and HC gave informed consent. The study was conducted according to the ethical guidelines of the University Medical Center Groningen, approved by the ethical committee of the University Medical Center Groningen, and in accord with the Declaration of Helsinki.

AAV patients and HC were matched for age (58.8 ± 14.0 vs. 55.7 ± 11.7 years; $p = 0.277$) and gender (44.2% vs. 51.4% females; $p = 0.510$). Throughout the study, AAV patients were evaluated for HMGB1 levels, disease activity using the third version of the Birmingham Vasculitis Activity Score (BVAS) [19], ANCA status, C-reactive protein (CRP) levels, and therapy. Complete remission was defined as a BVAS of 0 in combination with a normal serum CRP level (<10mg/l). Relapse was defined as the need to restart or intensify immunosuppressive therapy due to biopsy-proven or clinically suspected vasculitic disease activity. Anti-HMGB1 antibodies were tested in a randomly selected sample of AAV patients with active disease and data were compared with those in HC. In the longitudinal analysis, AAV patients were evaluated for HMGB1 serum levels at presentation, and twice during the remission period, namely at 3 months (interquartile range 3 to 6) and 11 months (interquartile range 6 to 12) after presentation. For relapsing AAV patients, HMGB1 levels were measured 2 months (interquartile range 1 to 3) prior to each relapse and at the moment of relapse. Relapsing and nonrelapsing AAV patients were compared regarding HMGB1 levels at baseline and during the remission period.

Table 1. Baseline features at presentation and therapy in 52 patients with antineutrophil cytoplasmic antibodies-associated vasculitis.

Variable	Result
Diagnosis	
Granulomatosis with polyangiitis	33 (63.5)
Microscopic polyangiitis	11 (21.2)
Renal limited vasculitis	8 (15.4)
ANCA	
Proteinase 3 ANCA	30 (57.7)
Myeloperoxidase ANCA	22 (42.3)
Disease activity	
Median BVAS	15.0 (12.0 to 23.5)
Median C-reactive protein level (mg/l)	37.0 (11.5 to 81.5)
Disease manifestations	
Renal involvement	39 (75.0)
Systemic manifestations	32 (61.5)
Ear, nose and throat involvement	28 (53.8)
Pulmonary involvement	22 (42.3)
Arthritis/joint pain	18 (34.6)
Peripheral neuropathy	15 (28.8)
Eye involvement	13 (25.0)
Cutaneous vasculitis	12 (23.1)
Pulmonary involvement	
Pulmonary nodules and/or infiltrates	12 (23.0)
Alveolar hemorrhage	6 (11.5)
Pleural effusion	2 (3.8)
Endobronchial lesion	1 (1.9)
Renal-related variables	
Median 24-hour proteinuria (g)	0.90 (0.55 to 1.60)
Hematuria (>10 RBC/HPF)	39 (75.0)
Median creatinine (µmol/l)	137.0 (80.0 to 350.0)
Mean creatinine clearance (ml/minute/1.73 m^2)	65.7 ± 41.7
Dialysis dependent	8 (15.4)
Actual therapy	
Patients without treatment	27 (51.9)
Prednisolone and cyclophosphamide	13 (25.0)
Prednisolone only	7 (13.5)
Plasmapheresis	6 (11.5)
Mean number of plasmapheresis sessions	9.33 ± 1.50
Methotrexate	1 (1.9)

Data presented as *n* (%), median (interquartile range) or mean ± standard deviation. ANCA, antineutrophil cytoplasmic antibodies; BVAS, Birmingham Vasculitis Activity Score; HPF, high-power field; RBC, red blood cells.

ELISA for serum HMGB1

HMGB1 levels were assessed in AAV patients and HC using a commercial ELISA kit according to the manufacturer's instructions (Shino Test; Sagamihara, Kanagawa, Japan). Results of serum HMGB1 levels are expressed in nanograms per milliliter.

ELISA for anti-HMGB1 antibodies

Anti-HMGB1 antibodies were tested in 24 AAV patients with active disease and 18 HC using an in house-developed ELISA described previously [13]. Sera from two patients with active SLE and high titers of anti-HMGB1 antibodies were used as positive controls. Briefly, Maxisorp polystyrene 96-well plates were coated with 50 µl/well recombinant HMGB1 (R&D Systems, Abingdon, UK) at 1 µg/ml in phosphate-buffered saline and incubated overnight at 4°C. Plates were then blocked with 5% bovine serum albumin in phosphate-buffered saline for 2 hours. Serum samples, diluted 1:50 in incubation buffer, were added to the plate (100 µl/well) and incubated for 2 hours at room temperature. After five washes, 100 µl horseradish peroxidase-conjugated goat anti-human IgG (SouthernBiotech, Birmingham, AL, USA) diluted 1:3,000 was added to each well and incubated for 1 hour at room temperature. After washing, bound antibodies were detected using 3,3',5,5'-tetramethylbenzidine dihydrochloride. The reaction was stopped with 2 M sulfuric acid and the absorbance was measured at 450 nm using a microplate spectrophotometer (Vmax; Molecular Devices, Sunnyvale, CA, USA). Results are expressed as optical density (OD) and anti-HMGB1 antibodies were considered positive if OD values were above the cutoff level of 0.435 obtained from the mean plus twice the standard deviation in 18 HC.

Statistical analysis

Statistical analysis was performed using SPSS software version 20.0 and graphs were built using Graph Pad Prism version 3.02. Categorical

variables were presented as the total number and percentage whereas continuous data were presented as the mean ± standard deviation when variables were normally distributed or as the median and interquartile range in case of non-normal distribution. Comparison between groups was performed using the chi-square test or Fisher's exact test for categorical variables and Student's t test or the Mann-Whitney U test for continuous data. One-way analysis of variance test was used for comparisons between three or more groups for numerical variables, and post-hoc analysis was performed with Dunnet's or Tukey's tests. Correlation between numerical data was calculated using Spearman's or Pearson's correlation coefficient when appropriate. Fluctuations in HMGB1 levels during follow-up in AAV patients were evaluated by Friedman's test and in cases of significance the Wilcoxon rank-sum test was used. Comparison between relapsing and nonrelapsing AAV patients regarding HMGB1 levels during follow-up was performed by generalized estimating equations. Cox proportional hazard models were built to analyze whether changes in HMGB1 levels were related to time of first relapse in AAV patients. Results are expressed as the hazard ratio and 95% confidence interval. The significant level accepted was 5% ($p < 0.05$).

Results

Baseline features of AAV patients

Characteristics of the 52 patients included in the study are presented in Table 1. All AAV patients were positive for anti-PR3 ANCA (n = 30) or anti-myeloperoxidase ANCA (n = 22) at presentation. Eight GPA patients (15%) had localized disease restricted to the upper and/or lower respiratory tract, eyes and/or ears, whereas active renal involvement was the most frequent disease manifestation at baseline. Most AAV patients (52%) were not on treatment at baseline evaluation, while among treated patients the combination of prednisolone and oral cyclophosphamide was most commonly prescribed (Table 1). In the patients already on immunosuppressive therapy,

the median duration prior to baseline evaluation was 3.0 weeks (interquartile range 1.0 to 4.0). In this subgroup the median daily prednisolone dose was 60 mg while the median daily dose of oral cyclophosphamide was 150 mg. Although those patients were already under therapy at baseline evaluation, no difference regarding median BVAS could be found between treated and untreated patients [14.0 (12.0 to 23.0) vs. 15.0 (12.0 to 26.0); $p = 0.491$].

HMGB1 levels at baseline

Mean HMGB1 levels were similar between AAV patients and HC (2.64 ± 1.80 ng/ml vs. 2.39 ± 1.09 ng/ml; $p = 0.422$) and no significant differences were found regarding mean HMGB1 levels among AAV disease subsets (GPA: 2.66 ± 1.83 ng/ml vs. MPA: 3.11 ± 1.91 ng/ml vs. RLV: 1.92 ± 1.48 ng/ml; $p = 0.369$). Although not significant, HMGB1 levels were higher in GPA patients with localized disease in comparison with those presenting generalized disease [3.14 (1.87 to 3.78) ng/ml vs. 1.84 (1.30 to 3.36) ng/ml; $p = 0.240$] as well as in AAV patients with pulmonary nodules and/or infiltrates compared with those presenting alveolar hemorrhage (3.60 ± 1.99 ng/ml vs. 2.09 ± 1.18 ng/ml; $p = 0.107$), but these differences did not reach statistical significance. Patients who were already under treatment for AAV at baseline had similar mean HMGB1 levels in comparison with those patients without immunosuppressive therapy (2.52 ± 1.58 ng/ml vs. 2.75 ± 2.01 ng/ml; $p = 0.651$) while lower HMGB1 levels were found in AAV patients who underwent sessions of plasmapheresis and/or dialysis prior to baseline evaluation of HMGB1 than in AAV patients without these therapies [1.84 ng/ml (1.55 to 2.79) vs. 2.63 ng/ml (1.22 to 3.88); $p = 0.388$], but this difference was not significant. A positive but weak correlation was found between serum HMGB1 and CRP levels ($\rho = 0.341$; $p = 0.039$) (Figure 1), while no correlation was found between serum HMGB1 levels and BVAS ($\rho = -0.019$; $p = 0.896$), cytoplasmic ANCA titers ($\rho = -0.208$; $p = 0.271$) or perinuclear ANCA titers ($\rho = 0.054$; $p = 0.813$) at presentation.

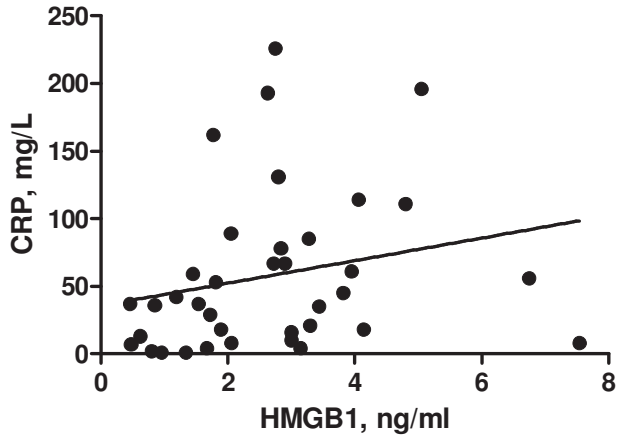

Figure 1. Correlation between serum HMGB1 and C-reactive protein levels at presentation in AAV patients. A significant positive correlation was found between serum high-mobility group box 1 (HMGB1) and C-reactive protein (CRP) levels in antineutrophil cytoplasmic antibodies-associated vasculitis (AAV) patients at presentation ($\rho = 0.341$; $p = 0.039$).

Patients without renal involvement (n = 13) had increased levels of HMGB1 compared with HC ($p = 0.023$). In contrast, HMBG1 levels in patients with renal involvement (n = 39) were no different from those in HC ($p = 0.733$) (Figure 2). No significant difference in mean HMGB1 levels was found between patients with renal involvement who presented granulomatous manifestations in comparison with those without associated granulomatous manifestations [2.34 ± 1.53 ng/ml (n = 22) vs. 2.37 ± 1.46 ng/ml (n = 17); $p = 0.826$]. A negative correlation was observed between serum HMGB1 levels and 24-hour proteinuria ($\rho = -0.361$, $p = 0.028$) whereas no correlation was found between serum HMGB1 levels and creatinine clearance in AAV patients with renal involvement ($r = 0.330$; $p = 0.144$).

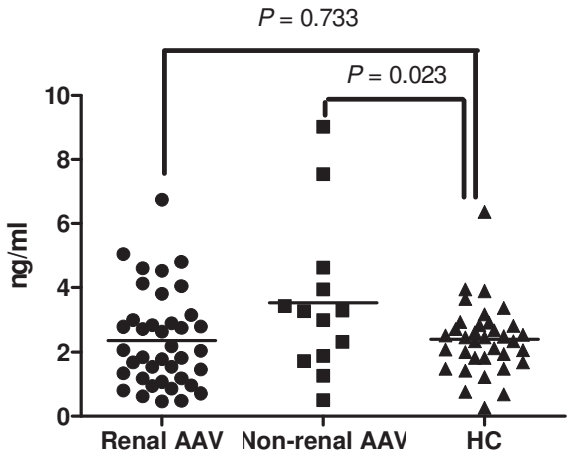

Figure 2. Baseline HMGB1 levels in AAV patients with renal involvement, without renal involvement and controls. High mobility group box 1 (HMGB1) levels at presentation in antineutrophil cytoplasmic antibodies-associated vasculitis (AAV) patients with renal involvement and without renal involvement and in healthy controls (HC) (2.35 ± 1.48 ng/ml vs. 3.52 ± 2.41 ng/ml vs. 2.39 ± 1.09 ng/ml; $p = 0.046$). Using Dunnett's post-hoc test, nonrenal AAV patients had higher HMGB1 levels when compared with HC ($p = 0.023$) whereas no difference was found between patients with renal involvement and HC ($p = 0.733$).

Longitudinal analysis of HMGB1 levels and relapses

To verify whether HMGB1 levels follow disease activity in AAV, HMGB1 levels were measured at presentation and during the remission period. A significant decrease in median HMGB1 levels [2.35 ng/ml (1.48 to 3.15) vs. 1.69 ng/ml (0.88 to 2.73); $p = 0.006$] was observed at the moment remission had been induced in comparison with baseline but then levels increased significantly again during ongoing remission [1.69 ng/ml (0.88 to 2.73) vs. 2.21 ng/ml (1.42 to 3.68); $p = 0.004$] (Figure 3 and Table 2). During follow-up, at least one disease relapse was observed in 17 AAV patients (34.7%), of whom six patients suffered from two relapses. GPA was the most frequent AAV subset among relapsing patients (82%). No significant differences regarding HMGB1 levels were observed between relapsing and nonrelapsing AAV patients during follow-up [relapsing: 2.18 ng/ml (1.49 to 3.15), 1.67 ng/ml (0.90 to 3.29) and 2.36 (1.33 to 2.75) vs. nonrelapsing: 1.97

ng/ml (1.10 to 3.72), 1.72 ng/ml (0.84 to 2.46) and 2.17 (1.44 to 3.97) at presentation and at remission 3 and 11 months after presentation, respectively; $p = 0.350$] (Figure 4).

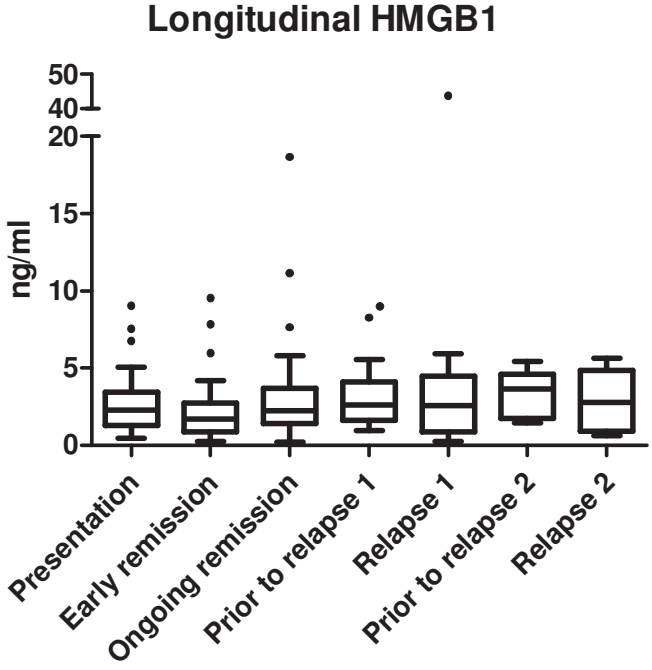

Figure 3. Longitudinal fluctuation of HMGB1 levels in different phases of the disease in AAV patients. High-mobility group box 1 (HMGB1) levels decrease significantly from presentation to when remission was induced ($p = 0.006$) but returned to levels similar to baseline at ongoing remission. No significant changes in HMGB1 levels were observed prior to or during disease relapses in antineutrophil cytoplasmic antibodies-associated vasculitis (AAV) patients. Box and whisker plots indicate the 25 to 75% range (boxes), the 5 to 95% range (error bars), and the median value (horizontal lines); dots represent outliers.

Aiming to evaluate whether an increase in HMGB1 levels during the remission period could indicate an increased risk of relapse, AAV patients were also evaluated prior to a relapse. However, no significant increase in serum HMGB1 levels could be observed prior to a relapse in comparison with HMGB1 levels during the remission period. Moreover, serum HMGB1 levels

prior to a relapse were similar in comparison with HMGB1 levels during the relapses (Figure 3 and Table 2). Serum HMGB1 levels at different time points were not associated with an increased risk of relapse of AAV while fluctuations of serum HMGB1 levels between remission and prior to the first relapse were not associated with an increased risk for relapses (Table 3).

Table 2. Longitudinal analysis of HMGB1 levels in patients with antineutrophil cytoplasmic antibodies-associated vasculitis.

Variables	Onset	Early remission[a]	Ongoing remission	Prior to relapse 1	Relapse 1	Prior to relapse 2	Relapse 2
HMGB1 (ng/ml)	2.35 (1.48-3.15)	1.69* (0.88-2.73)	2.21 (1.42-3.68)	2.59 (1.61-4.09)	2.56 (0.87-4.48)	3.65 (1.71-4.56)	2.77 (0.91-4.86)
Follow-up period (months)	0	3.0[b] (3.0-6.0)	11.0[b] (6.0-12.0)	2.0[c] (2.0-3.0)	20.0[b] (13.0-41.5)	2.0[c] (1.0-2.0)	44.0[b] (35.2-68.5)
BVAS	15.0 (12.0-23.5)	0	0	0	12.0 (5.5-13.5)	0	11.0 (5.7-15.7)
Number of patients	52	49	49	15	17	5	6

Data presented as median (interquartile range). BVAS, Birmingham Vasculitis Activity Score; HMGB1, high mobility group box 1. [a]Early remission indicates when remission was achieved following induction treatment. [b]months after presentation. [c]months prior to the relapse. *Significant *p* value for fluctuation in HMGB1 levels between presentation and early remission.

Table 3. HMGB1 levels as a biomarker of relapse risk in antineutrophil cytoplasmic antibodies-associated vasculitis patients.

HMGB1 levels	Hazard rate	95% confidence interval	*p* value
At baseline	0.812	0.529 to 1.248	0.327
At early remission[a]	0.963	0.641 to 1.446	0.854
At ongoing remission	1.344	0.746 to 2.420	0.325
Prior to relapse 1	0.967	0.776 to 1.205	0.762
Delta between early remission and prior to relapse 1	0.959	0.751 to 1.226	0.739
Delta between ongoing remission and prior to relapse 1	0.919	0.728 to 1.161	0.478

Cox hazard proportional model to evaluate the role of HMGB1 levels as a biomarker of relapse risk in antineutrophil cytoplasmic antibodies-associated vasculitis patients. HMGB1, high-mobility group box 1. [a]Early remission indicates when remission was achieved.

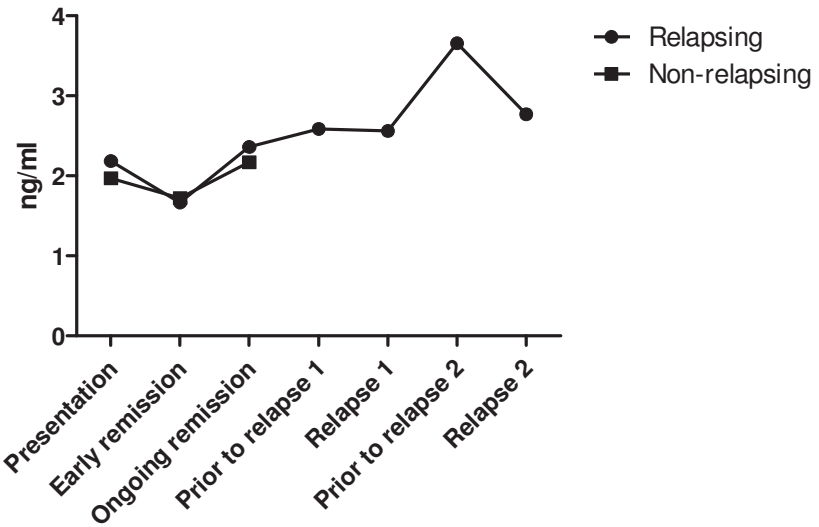

Figure 4. Comparison of HMGB1 levels during follow-up in relapsing and nonrelapsing AAV patients. Using a generalized estimating equation model, no significant differences were found between relapsing (n = 17) and nonrelapsing (n = 32) antineutrophil cytoplasmic antibodies-associated vasculitis (AAV) patients regarding longitudinal levels of high-mobility group box 1 (HMGB1) (p = 0.350).

Antibodies against HMGB1

Median OD values for anti-HMGB1 antibodies were similar between AAV patients with active disease and HC (0.175 (0.110 to 0.293) vs. 0.254 (0.177 to 0.297); P = 0.151). Anti-HMGB1 antibodies were positive in only three out of 24 patients (12.5%) with active AAV, in a median titer (OD: 0.465; range: 0.442 to 0.556) lower than in the two SLE patients included as positive controls (0.592 and 0.659, respectively). Albeit not significant, the AAV patients positive for anti-HMGB1 antibodies had a higher BVAS in comparison with those without anti-HMGB1 antibodies [26.0 (21.5 to 30.5) vs. 12.0 (6.0 to 19.0); p = 0.060].

Discussion

In this study, we evaluated serum HMGB1 levels as a biomarker of disease activity in AAV and investigated anti-HMGB1 antibodies in AAV

patients with active disease. We observed that even though a significant correlation was found between HMGB1 and CRP levels at presentation, only AAV patients without active renal disease had significantly higher serum HMGB1 levels than HC. Serum HMGB1 levels decreased significantly in a median of 3 months after presentation but then returned to levels similar to those found at baseline and no significant fluctuation was seen over time in AAV patients, not prior to or during disease relapses. Only a minority of AAV patients with active disease develop anti-HMGB1 antibodies.

Anti-HMGB1 antibodies have been described in sera of patients with septic shock, polymyositis, dermatomyositis and in active SLE [13,20]. In critically ill patients with septic shock, anti-HMGB1 antibodies were associated with a better prognosis and increased survival [21]. In patients with SLE, anti-HMGB1 antibodies were positively correlated with disease activity and anti-double-stranded DNA titers and were negatively correlated with serum complement levels [13,20]. In this study, only a small minority of AAV patients were positive for anti-HMGB1 antibodies. Moreover, no significant association between anti-HMGB1 antibodies and BVAS score could be observed. A previous study also failed to demonstrate anti-HMGB1 antibodies in 22 patients with AAV and active renal involvement [22].

The technique used to measure serum HMGB1 levels is a relevant issue because serum HMGB1 levels are usually five times higher when a western blot technique is used in comparison with ELISA, although both assays correlate well [23]. The binding of HMGB1 to different serum/plasma molecules, especially IgG1, interferes with its detection by ELISA systems, which is considered the main reason for this discrepancy [24]. Moreover, the depletion of IgG from the sera of SLE patients lowered serum HMGB1 levels detected by ELISA, indicating that this interference might in part be due to anti-HMGB1 antibodies in SLE [13]. We therefore firstly tested anti-HMGB1 antibodies in AAV patients before choosing the technique to measure serum HMGB1. Since only a few AAV patients presented anti-HMGB1 antibodies,

the ELISA technique was used to measure serum HMGB1 in the present study. Nevertheless, current ELISA methods seem to have limitations in assessing HMGB1 levels as a surrogate marker of active disease in different scenarios due to potential interference in its detection by serum factors, including anti-HMGB1 antibodies. Perhaps only free HMGB1 could be detected by current ELISA systems instead of total HMGB1 [24,25]. Furthermore, it is now known that functionality of HMGB1 is affected by the redox state of its three cysteine residues (C23, C45 and C106) and future methods to detect HMGB1 should take this functional nuance into account. The all-thiol form of HMGB1 has only chemotactic activity while disulfide-bonded HMGB1 (between C23 and C45) induces cytokine release through binding of TLR-4. No cytokine-stimulating or chemotactic activity is found in the fully oxidized HMGB1 [26,27].

We observed that AAV patients with renal involvement presented similar HMGB1 levels in comparison with HC while AAV patients without active nephritis had significantly higher HMGB1 levels than HC. We therefore speculate that the reason for finding serum HMGB1 levels in AAV patients at presentation similar to HC, in contrast to other studies evaluating HMGB1 in AAV [15,17], could be the high number of patients with active renal involvement evaluated in the present study (75.0%). Although Bruchfeld and colleagues have previously described higher serum HMGB1 levels in AAV patients with biopsy-proven active nephritis in comparison with patients without active renal inflammation, no systematic comparison was made with AAV patients presenting active disease in other organs or systems [16]. In line with our results, Henes and colleagues described lower serum HMGB1 levels in GPA patients with predominantly vasculitic manifestations when compared with those with predominantly granulomatous manifestations. Active nephritis was the most frequent feature observed among GPA patients with vasculitic manifestations. Granulomatous inflammation may have contributed to higher HMGB1 levels in GPA patients with granulomatous

manifestations [17]. In our study, patients with GPA and localized disease presented higher levels of HMGB1 at baseline than patients with generalized disease. Also, AAV patients with pulmonary nodules and/or lung infiltrates had higher levels of HMGB1 than those with alveolar hemorrhage. Differences, however, were not significant in both situations. Nonetheless, the presence of granulomatous manifestations in AAV patients did not seem to influence HMGB1 levels in AAV patients with simultaneously active nephritis, indicating that HMGB1 levels were mostly influenced by renal involvement. The increased expression of HMGB1 in renal tissue [16] and the necrotizing nature of glomerulonephritis in AAV indicate that both active release by activated cells and passive release by dying necrotic cells could be the source of extracellular HMGB1 in renal involvement of AAV besides systemic inflammation. However, whether leakage of HMGB1 into urine due to renal damage contributes to lower serum HMGB1 levels in parallel with increased urinary levels of HMGB1 in active glomerulonephritis in AAV is still unknown.

Serum HMGB1 levels have been correlated with disease activity in AAV in cross-sectional studies. Nevertheless, longitudinal evaluation of serum HMGB1 levels demonstrated only a significant decrease in HMGB1 levels approximately 3 months after presentation. Thereafter, with ongoing remission, serum HMGB1 returned to levels similar to those found at presentation. Serum HMGB1 levels prior to relapses and during relapses were somewhat higher than baseline levels but this difference was not significant. Achievement of disease remission in response to sustained immunosuppressive therapy may be the reason for lower HMGB1 levels within 3 months after disease presentation. Although approximately one-half of AAV patients at presentation were already under immunosuppressive therapy for a median 3 weeks and no difference in serum HMGB1 levels could be found between patients with and without therapy, both treated patients and untreated patients had a similar median BVAS at baseline evaluation. After achieving remission most AAV patients were still on oral

prednisolone and cyclophosphamide, whereas during ongoing remission immunosuppressive therapy was tapered and cyclophosphamide was changed to azathioprine. Prior to relapses in the majority of AAV patients, immunosuppressive therapy was withdrawn (data not shown). This reduction in immunosuppressive therapy may therefore account for the significant increase in serum HMGB1 levels from early remission to ongoing remission.

In this study, no difference regarding serum HMGB1 levels could be found during follow-up between relapsing and nonrelapsing AAV patients and fluctuations of serum HMGB1 levels during the remission period or prior to a relapse were not associated with an increased risk of relapses in AAV. Hence, fluctuations of serum HMGB1 levels cannot be used as a surrogate marker for disease activity in AAV.

Conclusions

Nonrenal AAV is associated with higher serum HMGB1 levels at presentation and serum HMGB1 levels decrease significantly within 3 months after presentation, possibly due to immunosuppressive treatment, but during ongoing remission HMGB1 levels return to levels similar to those observed at presentation. A slight nonsignificant increase in HMGB1 levels is observed prior to and during relapses in comparison with baseline levels. No association between fluctuations of serum HMGB1 levels and risk of relapse could be found. Circulating HMGB1 measured by ELISA therefore does not seem to be a useful biomarker of disease activity in AAV. Patients with AAV did not develop significant anti-HMGB1 antibodies during active disease.

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CHAPTER 5

Impact of serum high mobility group box 1 and soluble receptor for advanced glycation end-products on subclinical atherosclerosis in patients with granulomatosis with polyangiitis

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Abstract

The objective of this study was to evaluate whether levels of high mobility group box 1 (HMGB1) in granulomatosis with polyangiitis (GPA) patients are associated with carotid atherosclerosis, related to levels of soluble receptor for advanced glycation end-products (sRAGE) and influenced by immunosuppressive or lipid-lowering therapy. Twenty-three GPA patients and 20 controls were evaluated for HMGB1- and sRAGE levels and for carotid atherosclerosis using ultrasound to determine intima-media thickness (IMT). In vitro the effect of atorvastatin on the production of HMGB1 by lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVEC) was assessed. Serum HMGB1 and sRAGE levels did not differ between patients and controls. A negative correlation was found between sRAGE and maximum IMT but HMGB1 and carotid IMT were not related. HMGB1 levels were reduced in GPA patients on statins and prednisolone. In vitro, atorvastatin reduced HMGB1 levels in supernatants of activated HUVEC. In conclusion, carotid IMT is inversely correlated with sRAGE levels but not with HMGB1 levels. Statins and prednisolone are associated with reduced serum HMGB1 levels and atorvastatin decreases HMGB1 release by activated HUVEC in vitro, indicating an additional anti-inflammatory effect of statins.

Introduction

High mobility group box 1 (HMGB1) is a nuclear non-histone DNA binding protein that upon cellular death or activation is released into the extracellular milieu and acts as an alarmin. Extracellular HMGB1 stimulates cytokine production, cell proliferation, chemotaxis, angiogenesis, and cell differentiation through binding to its receptors that include the receptor for advanced glycation end-products (RAGE) and Toll-like receptors (TLR)-2, TLR4 and TLR9 [1]. Increased levels of HMGB1 have been found in patients with granulomatosis with polyangiitis (GPA) with active disease, especially in

patients with predominantly granulomatous manifestations and in patients without renal involvement at disease onset [2–4]. Individuals with subclinical atherosclerosis and cardiovascular (CV) events present high HMGB1 levels whereas atorvastatin decreases serum HMGB1 in hyperlipidemia [5,6].

RAGE is a multi-ligand surface molecule involved in the pathogenesis of vascular diseases. Soluble RAGE (sRAGE) is a decoy receptor for RAGE ligands and decreased sRAGE levels are associated with CV events [7]. Patients with GPA present increased advanced glycation end-products (AGEs) accumulation compared to controls and this accumulation is negatively correlated with sRAGE levels [8]. We hypothesized that serum HMGB1 levels contribute to subclinical atherosclerosis in GPA and that this process is influenced by sRAGE but also by treatment with prednisolone and statins.

Materials and methods

Patients and controls

A cross-sectional study was performed on 23 GPA patients and 20 age- and gender-matched controls (Table 1) enrolled in a previous study to evaluate carotid atherosclerosis in GPA [9]. The study was approved by the Medical Ethical Committee (METC) of the University Medical Center Groningen (UMCG) and written informed consent was obtained from patients and controls. GPA was classified according to the European Medicines Agency algorithm [10]. The mean disease duration was 131.2 ± 60.2 months. Birmingham Vasculitis Activity Score (BVAS) was recorded for all patients during admissions and out-patient visits allowing assessment of cumulative BVAS scores from the charts. All GPA patients were evaluated during remission (BVAS= 0) to exclude influence of disease activity. Prednisolone was used by 8 patients (34.8%) at a median of 5 mg/day (3.7–10.0 mg). Statins were prescribed for 5 (21.7%) GPA patients. Healthy controls did not use any medication at the time of the study. Carotid ultrasound was

performed to assess intima-media thickness (IMT) and carotid plaques as described previously [9,11]. Overall mean IMT (mean of IMT measurements performed on carotid bulbus, common and internal carotid arteries) and overall maximum IMT (average of highest IMT values found in above mentioned segments) were used for analysis. Traditional CV risk factors were evaluated according to established guidelines [12]. Serum HMGB1 (Shino Test, Kanagawa, Japan) and sRAGE levels (R&D Systems, Minneapolis, USA) were measured by enzyme-linked immunosorbent assay (ELISA).

Cell cultures

HUVEC (Lonza, Breda, The Netherlands) were cultured in EBM-2 medium supplemented with EGM-2 MV Single Quot Kit Supplements & Growth Factors (cat No. CC-3202, Lonza) and used when confluent. Three groups were evaluated: (1) HUVEC pre-incubated for 2 hours with 5 µM atorvastatin (Sigma Aldrich, Saint Louis, USA) and treated with LPS (100 ng/ml) (Sigma Aldrich, Saint Louis, USA), (2) HUVEC treated only with LPS, and (3) unstimulated HUVEC. Supernatants were collected for measuring HMGB1 and interleukin (IL)-8 at baseline, 4 hours and 24 hours. All in vitro experiments were performed twice and in duplicate. Cell viability of HUVEC was checked with 0.2% trypan blue dye (Invitrogen, Carlsbad, USA) on HUVEC treated with 0 µM, 0.1 µM, 1.0 µM, 5.0 µM and 10.0 µM atorvastatin; percentages of living cells were 94%, 95%, 95%, 92% and 91% at 36 hours, respectively. Western blot was used to measure HMGB1 in supernatants as previously described [13] while IL-8 levels were measured by ELISA (R&D Systems, Minneapolis, USA).

Statistical analysis

Statistical analysis was performed with SPSS 18.0 software and graphs were built using GraphPad Prism 5. Continuous variables are presented as mean 6 SD or as median and interquartile range. Categorical

variables are presented as total number and percentage. Comparisons between groups were performed using chi-square test or Fisher's exact test and Student's t-test or Mann-Whitney U test as appropriate. Analysis of longitudinal data from in vitro experiments was performed by two-way ANOVA and Bonferroni's test. Correlations were evaluated with Spearman's rank correlation coefficient. Differences were considered significant when $p<0.05$.

Results

HMGB1, sRAGE and subclinical atherosclerosis

Figure 1 shows similar serum HMGB1 levels in patients and controls whereas Figures 2A and 2B depicts similar overall mean IMT and maximum IMT in carotid arteries in GPA and controls. Furthermore, sRAGE levels and the prevalence of carotid plaques did not differ between patients and controls (Table 1).

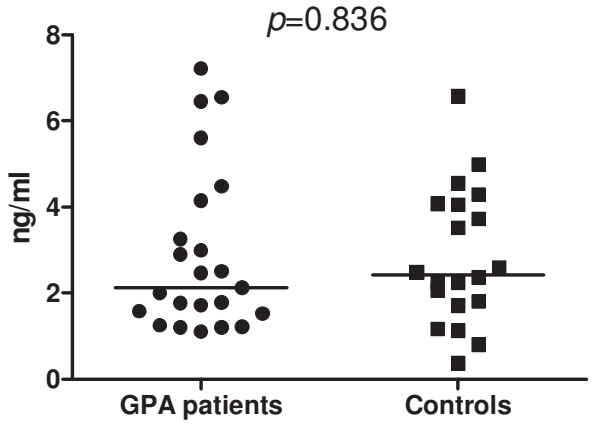

Figure 1. Serum HMGB1 levels in GPA patients and controls. GPA patients in remission present similar median HMGB1 levels compared to controls.

In GPA patients, no correlation was found between HMGB1 and overall maximum IMT in carotid arteries (rho = 0.062; p = 0.820). However, sRAGE levels were negatively correlated with overall maximum IMT in carotid arteries (rho = -0.565; p = 0.035). No correlation was present between

HMGB1 and sRAGE (rho = 0.068; p = 0.777), between overall mean carotid IMT and cumulative BVAS (rho = -0.070; p = 0.805) or between overall mean carotid IMT and time in remission since last relapse/presentation (rho = 0.337; p = 0.201). Moreover, no correlation was found between overall maximum carotid IMT and cumulative BVAS (rho = -0.050; p = 0.859) and between overall maximum IMT and time in remission since last relapse/presentation (rho = 0.338; p = 0.200). Also, no significant difference was found between patients with and without carotid plaques regarding HMGB1 [1.52 (1.20–2.89) ng/ml vs. 2.48 (1.74–4.22) ng/ml; p = 0.300] and sRAGE levels (1144.66±817.33 pg/ml vs. 1303.99±438.86 pg/ml; p = 0.574).

Table 1. Risk factors for cardiovascular disease, carotid ultrasound, HMGB1 and sRAGE levels in GPA patients and controls.

Variables	GPA patients (N = 23)	Controls (N = 20)	*p*
Median age at study, years	55.2 (45.7-62.4)	49.8 (43.0-57.4)	0.173
Females, n (%)	9 (39.1)	9 (45.0)	0.697
Age > 45 years for men and 55 years for women, n (%)	16 (69.6)	12 (60.0)	0.512
Mean total cholesterol, mmol/L	4.99 ± 0.78	4.98 ± 0.82	0.978
Mean HDL, mmol/L	1.41 ± 0.37	1.51 ± 0.33	0.359
Mean LDL, mmol/L	3.01 ± 0.79	3.29 ± 0.82	0.267
Median TGL, mmol/L	1.50 (1.00-2.00)	1.00 (1.00-2.00)	0.404
Mean systolic BP, mmHg	123.65 ± 14.55	119.24 ± 10.94	0.301
Mean diastolic BP, mmHg	70.61 ± 9.38	75.12 ± 8.07	0.120
Smoking, n(%)	2 (8.7)	1 (5.0)	0.635
Family history of premature CVD, n(%)	9 (39.1)	8 (40.0)	0.954
Median BMI, kg/m^2	26.0 (24.0-28.0)	23.5 (22.0-26.5)	0.256
Previous CVD, n(%)	3 (13.0)	0 (0.0)	0.236
Carotid plaques, n (%)	7 (30.4)	3 (15.0)	0.203
Overall mean IMT, mm	0.833 ± 0.256	0.765 ± 0.133	0.357
Median overall maximum IMT, mm	0.875 (0.810-1.215)	0.880 (0.830-0.990)	0.953
HMGB1, ng/ml	2.13 (1.53-4.15)	2.42 (1.73-4.07)	0.827
sRAGE, pg/mL	1256.1 ± 559.6	1483.3 ± 399.8	0.155

Numerical data are presented as mean ± standard deviation or as median and interquartile range; BMI: body mass index; BP: blood pressure; CVD: cardiovascular disease; HDL: high-density lipoprotein; HMGB1: high mobility group box-1; IMT: intima-media thickness; LDL: low-density lipoprotein; n: number of individuals; sRAGE: soluble receptor for advanced glycation end-products; TGL: triglycerides.

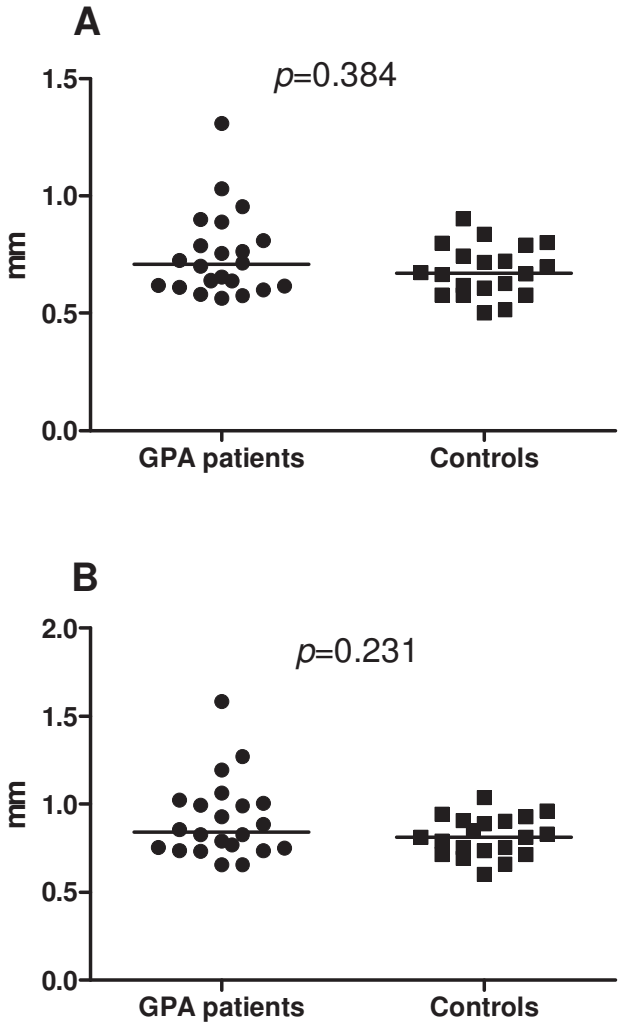

Figure 2. Intima media thickness in common carotid arteries in GPA and controls. GPA patients present mean IMT (2A) and maximum IMT (2B) in common carotid arteries similar to controls.

Impact of treatment on HMGB1 and sRAGE

Since HMGB1 and sRAGE levels did not differ between GPA patients in remission and controls, we evaluated whether therapy would have any influence on both biomarkers. Serum HMGB1 levels were lower in patients on

statins [1.26 (1.16–1.68) ng/ml vs. 2.70 (1.75–4.76) ng/ml; $p = 0.014$] and on prednisolone [1.49 (1.21–2.61) ng/ml vs. 2.51 (1.79–5.61) ng/ml; $p = 0.017$] compared to patients without these drugs, respectively (Figure 3A and 3B). Although not significant, we observed higher serum HMGB1 levels in 12 GPA patients without statins or prednisolone compared with 20 controls (4.03 ± 2.00 ng/ml vs. 2.84 ± 1.58 ng/ml, $p = 0.073$). sRAGE levels did not differ between patients with and without statins (976.20 ± 345.64 pg/ml vs.1326.19 ± 588.79 pg/ml; $p = 0.275$) or prednisolone (1366.9 ± 6450.80 pg/ml vs. 1196.55 ± 619.08 pg/ml; $p = 0.531$).

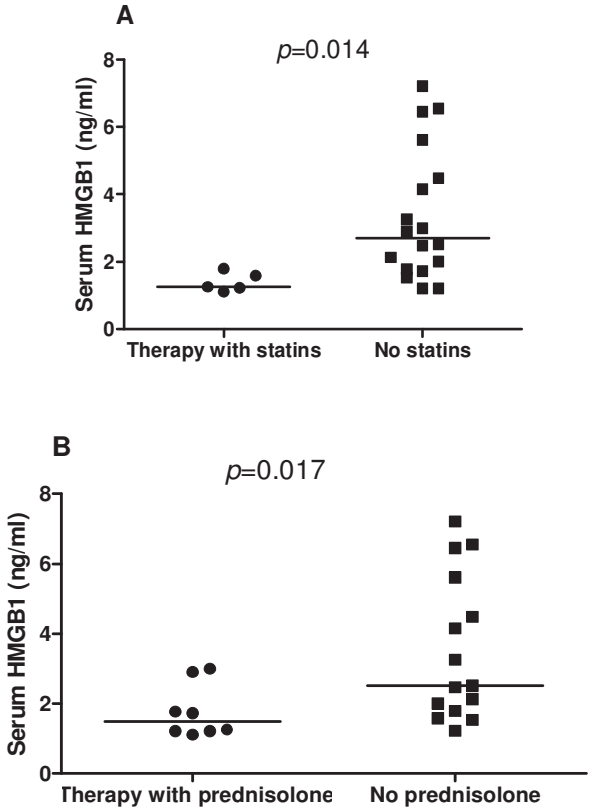

Figure 3. HMGB1 levels in GPA patients treated with statins or prednisolone. median serum levels of HMGB1 were significantly lower in GPA patients treated with statins (3A) and prednisolone (3B) when compared to patients without these drugs.

HMGB1 and atorvastatin in HUVEC

To investigate the relation between statins and HMGB1, we tested the effect of atorvastatin on HMGB1-release from LPS-stimulated HUVEC. HMGB1 in supernatants gradually increased in time with a peak after 24 hours of LPS stimulation and a decrease at 48 hours (Figure 4A).

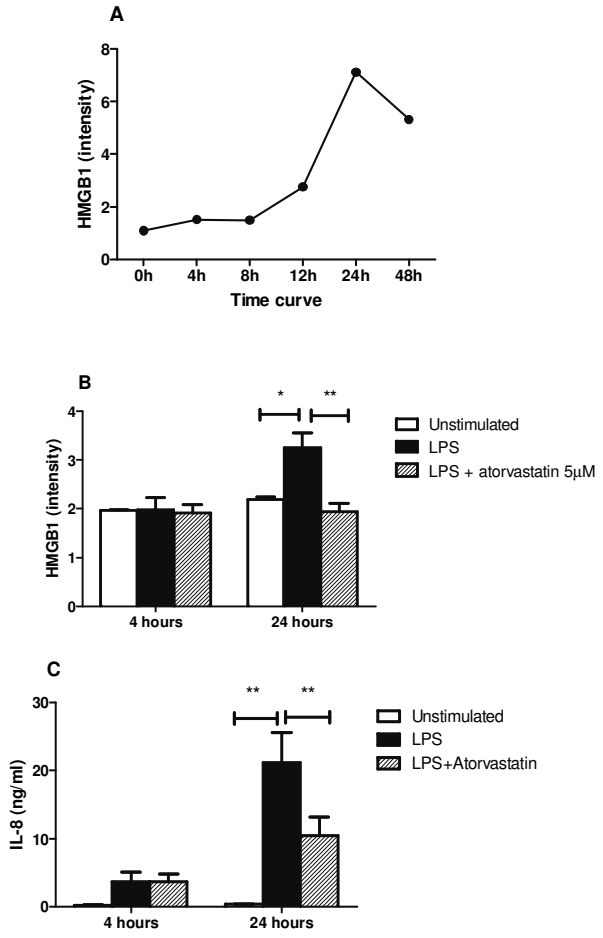

Figure 4. Effect of atorvastatin on HMGB1 and IL-8 levels in HUVEC supernatants. 4A – A time curve was built to evaluate HMGB1 release from HUVEC upon LPS stimulation. HMGB1 intensity in supernatants increases 8 hours after stimulation and a peak is observed at 24 hours with a decrease at 48 hours. 2B and C – HMGB1 and IL-8 levels are significantly higher at 24 hours in supernatants from HUVEC treated with LPS in comparison to unstimulated HUVEC while pre-incubation with 5 µM atorvastatin followed by LPS stimulation lowered HMGB1 and IL-8 levels significantly at 24 hours in HUVEC's supernatants. Experiments were performed twice and in duplicate. Data are presented as median and range, *p<0.01 and **p<0.001.

Thus, we compared HMGB1 and IL-8 levels in supernatants of HUVEC pre-incubated with atorvastatin followed by 24 hours of LPS stimulation. HUVEC stimulated with LPS showed a significantly higher production of HMGB1 and IL-8 than unstimulated cells. Pre-incubation with 5 mM atorvastatin prior to LPS stimulation led to significantly lower levels of both HMGB1 and IL-8 in supernatants after 24 hours in comparison to HUVEC stimulated by LPS only (Figures 4B and 4C).

Discussion

In this study, in GPA patients HMGB1 levels were not correlated with overall carotid maximum IMT, whereas sRAGE levels were negatively correlated with IMT. Furthermore, statins or prednisolone use was associated with lower HMGB1 levels.

In previous studies, GPA patients presented higher IMT levels in carotid arteries and an increased number of CV events compared to controls [11,14]. Accelerated atherosclerosis in GPA was not associated with traditional CV risk factors, rather enhanced levels of markers of vascular inflammation and remodeling were associated with atherosclerotic disease [11]. In this study, we evaluated HMGB1 and sRAGE levels in GPA patients who had been enrolled in a follow-up study to evaluate progression of atherosclerosis [9]. Patients were in remission and a reduction of traditional CV risk factors was achieved during follow-up of these patients that possibly resulted in similar IMT and similar prevalence of carotid plaques compared to controls [9]. Also, cumulative BVAS scores and time in remission were not related to IMT values. Moreover, proteinase 3 antineutrophil cytoplasmic antibody (PR3-ANCA) status has been shown to have a protective role on the risk of CV events in patients with ANCAassociated vasculitis and most of our patients were PR3-ANCA positive [15].

HMGB1 and sRAGE have been implicated in the pathogenesis of atherosclerotic disease. Higher serum HMGB1 levels are a potential marker

of subclinical atherosclerosis and CV events while lower serum sRAGE levels are associated with risk factors for CV disease and CV events [5,7]. Thus, we evaluated the association between both biomarkers and atherosclerotic disease in GPA. sRAGE was associated with subclinical carotid atherosclerosis whereas HMGB1 was not. We hypothesized that due to its multifaceted nature, HMGB1 is possibly more influenced by other factors such as therapy while sRAGE levels are more stable. Indeed, prednisolone or statins use were associated with lower serum HMGB1 levels in our study.

Previous studies had also shown reduction of HMGB1 levels upon statins in an experimental model of atherosclerosis and in humans with hyperlipidemia [6,16]. In this study, we demonstrated that statins are associated with lower serum HMGB1 levels in GPA patients in remission while a tendency for higher serum HMGB1 levels was observed in GPA patients in remission without statins compared with controls. Moreover, the addition of 5 μM atorvastatin which is within the range of atorvastatin concentration used in other in vitro studies [17–21] led to a decrease in extracellular HMGB1 levels in activated HUVEC. These findings indicate that endothelial cells are a possible source of extracellular HMGB1 that could be inhibited by atorvastatin. The decrease in IL-8 levels in supernatants of activated HUVEC by atorvastatin was used as a comparator since it is already known that statins inhibit mRNA expression of IL-8 in activated HUVEC [22]. Inhibition of HMGB1 release by activated HUVEC points to another potential anti-inflammatory effect of statins on vascular endothelium. The actual mechanism of inhibition of HMGB1 release by HUVEC (i.e. inhibition of mRNA expression or cytoplasmic translocation of HMGB1) still needs further elucidation.

HMGB1 levels were also lower in GPA patients on prednisolone therapy. Previous studies have demonstrated that corticosteroids can reduce extracellular release of HMGB1 by monocytes *in vitro* and they can also

reduce HMGB1 expression and circulating levels in vivo [23,24]. However, these findings were not confirmed in patients with rheumatoid arthritis [25].

In conclusion, no association was observed between subclinical carotid atherosclerosis and HMGB1 while sRAGE was negatively associated with carotid IMT in GPA. Statin use was associated with lower HMGB1 levels suggesting an additional anti-inflammatory property of statins. As subclinical atherosclerosis was similar between patients and controls, we suggest that development of premature atherosclerosis in GPA patients might be postponed by sRAGE and use of statins or prednisolone. Since our study had a relatively low number of patients and had a crosssectional design, further longitudinal studies are needed to evaluate if reduction of serum HMGB1 levels might be important in CV risk management in GPA.

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CHAPTER 6

Are urinary levels of high mobility group box 1 markers of active nephritis in anti-neutrophil cytoplasmic antibody-associated vasculitis?

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Summary

The objective of this study is to evaluate urinary high mobility group box 1 (HMGB1) levels as markers for active nephritis in patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) in comparison with urinary CD4+ effector memory T cells and urinary monocyte chemoattractant protein-1 (MCP-1). Twenty-four AAV patients with active nephritis and 12 healthy controls (HC) were evaluated. In nine patients, samples were also obtained during remission. Urinary levels of HMGB1 were measured by Western blot. $CD4^+$ T cells and $CD4^+$ effector memory T cells ($CD4^+CD45RO^+CCR7^-$) were determined in urine and whole blood by flow cytometry. Measurement of urinary levels of MCP-1 and serum HMGB1 levels were performed by enzyme-linked immunosorbent assay (ELISA). AAV patients with active nephritis had higher median intensity of HMGB1 in urine than HC [10.3 (7.05–18.50) *versus* 5.8 (4.48–7.01); $p = 0.004$]. Both urinary HMGB1 and MCP-1 levels decreased significantly from active nephritis to remission. The urinary MCP-1/creatinine ratio correlated with Birmingham Vasculitis Activity Score (BVAS) ($p = 0.042$). No correlation was found between the HMGB1/creatinine ratio and 24-h proteinuria, estimated glomerular filtration rate (eGFR), MCP-1/creatinine ratio, BVAS and serum HMGB1. A positive correlation was found between urinary HMGB1/creatinine ratio and $CD4^+$ T cells/creatinine ratio ($p = 0.028$) and effector memory T cells/creatinine ratio ($p = 0.039$) in urine. Urinary HMGB1 levels are increased in AAV patients with active nephritis when compared with HC and patients in remission, and urinary HMGB1 levels are associated with $CD4^+$ T cells and $CD4^+$ effector memory T cells in urine. Measurement of urinary HMGB1 may be of additional value in identifying active glomerulonephritis in AAV patients.

Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are pauci-immune necrotizing systemic vasculitides that affect

predominantly small-sized vessels, and are associated with ANCA against proteinase 3 (PR3) and myeloperoxidase (MPO). AAV include granulomatosis with polyangiitis (GPA) (formerly Wegener's), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg–Strauss) and renal limited vasculitis (RLV) [1,2]. Kidney involvement is observed in 70–88% of patients with GPA and MPA during follow-up [3–5], and is associated with an increased risk of mortality [5].

High mobility group box 1 (HMGB1) is a nuclear protein that can be released passively by necrotic cells or secreted actively by activated cells. Once in the extracellular milieu, HMGB1 triggers proinflammatory reactions activating both innate and adaptive immunity [6,7]. HMGB1 has been shown to induce the proliferation and survival of T cells [8,9]. However, the influence of HMGB1 on T helper cell polarization is still controversial, as it has been demonstrated that HMGB1 is involved in stimulating a T helper type 1 (Th1) response by dendritic cells [9,10] while directly inducing a Th17 response on $CD4^+$ T cells *in vitro* and *in vivo* in experimental autoimmune myocarditis [11,12]. Furthermore, inhibition of regulatory T cell activity with a decreased expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) and forkhead box protein 3 (FoxP3), and a decreased secretion of interleukin (IL)-10 after exposure to HMGB1 have also been reported [13,14].

In systemic lupus erythematosus (SLE), HMGB1 has been shown to be a good biomarker for active lupus nephritis as both serum and urinary HMGB1 levels are increased in patients with active nephritis compared to patients without nephritis and healthy controls (HC). Moreover, both serum and urinary HMGB1 levels were correlated positively with SLE disease activity index (SLEDAI) and negatively with serum complement levels [15,16]. Extracellular HMGB1 expression was increased in renal tissue from patients with active lupus nephritis [16,17].

In patients with GPA, an association between serum HMGB1 levels and active disease has been observed with either granulomatous

manifestations or with active nephritis [18-20]. Furthermore, HMGB1 expression is stronger in kidney tissue from AAV patients with active nephritis than in those with a normal biopsy [20]. However, in 52 AAV patients at disease presentation, no differences could be found in HMGB1 levels when compared to HC [21].

There is increasing evidence that T cells play an important role in the pathogenesis of AAV [22]. Infiltrating $CD4^+$ T cells are found within granulomatous lesions, and a persistent activation of $CD4^+$ T cells from peripheral blood is observed in AAV even during remission [23,24]. The persistent expansion of T cells in AAV patients is associated with a particular subtype of memory $CD4^+$ T cells referred to as effector memory T cells ($CD3^+CD4^+CD45RO^+CCR7^-$) [25], which are the main cells found in glomerular infiltrates from active AAV patients [26]. The number of $CD4^+$ T cells is increased in urine samples from AAV patients with active glomerulonephritis compared to AAV patients in remission and to AAV patients with disease activity in other organs and systems. $CD4^+$ effector memory T cells are the main T cell subtype found in urine from AAV patients with renal involvement [27].

Monocyte chemoattractant protein-1 (MCP-1), also designated as CCL2, is a member of the CC chemokine family that acts as a potent monocyte/macrophage attractant to sites of tissue injury and infection [28]. The expression of MCP-1 is increased in renal tissue, and high urinary MCP-1 levels have been observed in different renal diseases [29]. In AAV, urinary MCP-1 levels are significantly higher in patients with active nephritis than in those without renal involvement, a decrease in urinary MCP-1 levels is observed following therapy and a significant correlation is found between urinary MCP-1 and glomerular macrophage infiltration [30]. Moreover, MCP-1 has been shown to be the best urinary marker to discriminate active renal involvement and remission in AAV [31].

This study aims to evaluate whether urinary HMGB1 levels are increased in AAV patients with active renal involvement in comparison to HC and to analyse associations of urinary HMGB1 levels with parameters of renal disease activity, $CD4^+$ T cell– and $CD4^+$ effector memory T cell counts in urine and urinary MCP-1 levels.

Materials and methods

Patients and controls

Twenty-four patients with AAV and 12 HC were enrolled. Patients and HC had similar mean age (55.63 ± 13.35 years *versus* 49.83 ± 7.46 years; p = 0.105) and frequency of females (37.5% *versus* 58·3%, p = 0.236). In nine patients samples were also obtained during remission, with a mean interval of 36.2 ± 10.5 months from the time of active disease. A diagnosis of GPA and MPA was established according to the European Medicines Agency algorithm [32], while the diagnosis of RLV was based on the presence of isolated renal involvement, ANCA positivity and/or biopsy-proven pauci-immune necrotizing glomerulonephritis. All AAV patients had active renal involvement and were included either at diagnosis (n = 10) or at the time of relapse (n = 14). Only seven (29.2%) AAV patients were under immunosuppressive therapy when samples were collected (Table 1). Active nephritis was characterized by active urinary sediment with glomerular erythrocyturia and/or red blood cell casts associated with abnormalities in serum creatinine or decreased estimated glomerular filtration rate (eGFR) and/or with a renal biopsy showing active pauciimmune necrotizing glomerulonephritis. Disease activity was measured by the third version of the Birmingham Vasculitis Activity Score (BVAS) [33]. Remission was defined as a BVAS = 0, including normal urinary sediment, and stable creatinine/eGFR. Table 1 depicts features of AAV patients. None of the AAV patients or HC presented active infection when evaluated. The study was approved by the local Ethical Committee and informed consent (according to the Declaration of Helsinki) was obtained.

Antibodies

The following antibodies were used for flow cytometry analysis: phycoerythrin (PE)-conjugated CCR7, fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO, peridin chlorophyll protein (PerCP)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD3, multiTEST fourcolour antibodies (FITC-conjugated CD3, PE-conjugated CD8, PerCP-conjugated CD45 and APC-conjugated CD4) and isotype-matched control antibodies of irrelevant specificity. All antibodies were purchased from Becton Dickinson (Amsterdam, the Netherlands).

Table 1. Disease features, renal involvement and therapy of AAV patients.

Variables	AAV patients (N=24)
Diagnosis/relapse,	10/14
GPA, *n* (%)	15 (62.5)
MPA, *n* (%)	4 (16.7)
RLV, *n* (%)	5 (20.8)
ANCA positivity, *n* (%)	23 (95.8)
PR3-ANCA, *n* (%)	13 (54.2)
MPO-ANCA, *n* (%)	10 (41.7)
Median BVAS,	13.00 (10.0-18.7)
24-hour proteinuria, g	0.8 (0.5-3.5)
Hematuria (>10RBC/HPF), *n* (%)	24 (100.0)
Urinary MCP-1/creatinine ratio ng/mmol	18.7 (7.5-63.3)
Serum creatinine, µmol/l	147.5 (91.5-313.0)
Creatinine in spot urine, µmol/l	6.22 ± 2.78
eGFR, ml/minute/1.73m^2	38.0 (18.0-77.0)
Red cell casts, *n* (%)	10 (41.7)
Current therapy in patients with active disease, *n* (%)	7 (29.2)
Oral prednisolone	7
Oral cyclophosphamide	3
Azathioprine	2
Mycophenolate mofetil	2
Current therapy in patients in remission, *n* (%)	5 (55.5)
Oral prednisone	1
Azathioprine	3
Mycophenolate mofetil	1

Continuous data are presented as median and interquartile range (IQR) or as mean and standard deviation. GPA: granulomatosis with polyangiitis; HPF = high power field; MCP-1 = monocyte chemoattractant protein-1; MPA = microscopic polyangiitis; MPO = myeloperoxidase; PR3 = proteinase 3; RBC = red blood cells; RLV = renal limited vasculitis.

Sample preparation and flow cytometry

Ethylenediamine tetraacetic acid (EDTA) blood and urine samples were collected from AAV patients and analysed immediately by flow cytometry for total $CD4^+$ T cells and $CD4^+$ effector memory T cells ($CD3^+CD4^+CD45RO^+$ $CCR7^-$). After voiding, 100 ml of urine was diluted 1:1 with cold phosphate-buffered saline (PBS) and centrifuged at 558g for 15 minutes. The supernatant was collected for HMGB1 measurement, whereas the sediment was resuspended with 10 ml of PBS. We took 50 µl from the cell suspension for quantitative measurement using a TrueCount tube and mononuclear cells were isolated from the remaining cell suspension with densitygradient centrifugation by Lymphoprep (Axis-Shield, Oslo, Norway). Washing buffer (1% bovine serum albumin in PBS) was added to mononuclear cells isolated from urine and mixed with appropriate concentrations of anti-CD45RO-FITC, anti-CCR7-PE, anti-CD4-PerCP and anti-CD3-APC, and incubated for 15 min at room temperature in the dark. Whole blood samples were labelled using the same protocol. All samples were treated with 2 ml diluted fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson) for 10 min and samples were washed twice in washing buffer and analysed by flow cytometry. Four-colour staining was analysed by FACS Calibur (Becton Dickinson), and data were collected for 10^5 events in every sample and plotted with Win-List software (Verity Software House, Topsham, ME, USA). Positively and negatively stained populations were calculated by quadrant dot-plot analysis, based on isotype controls (Fig. 1).

Quantification of effector memory T cells

TrueCount tubes (Becton Dickinson) were used to quantify $CD4^+$ T cells in peripheral blood and urine. Briefly, 20 µl of MultiTEST four-colour antibodies (CD3-FITC, CCR7-PE, CD45RO-PerCP and CD4-APC) and 50 µl of sample (urine or blood) were put into bead-containing TrueCount tubes.

The cell suspension was processed and analysed and the absolute number of $CD4^+$ T cells and $CD4^+$ effector memory T cells was determined using a previously described protocol [27]. Results were displayed as cells/ml in urine and as $x10^6$ cells/ml in peripheral blood. In order to correct for variations in urine dilution, urine $CD4^+$ T cell and $CD4^+$ effector memory T cell counts were also expressed as ratios to urinary creatinine.

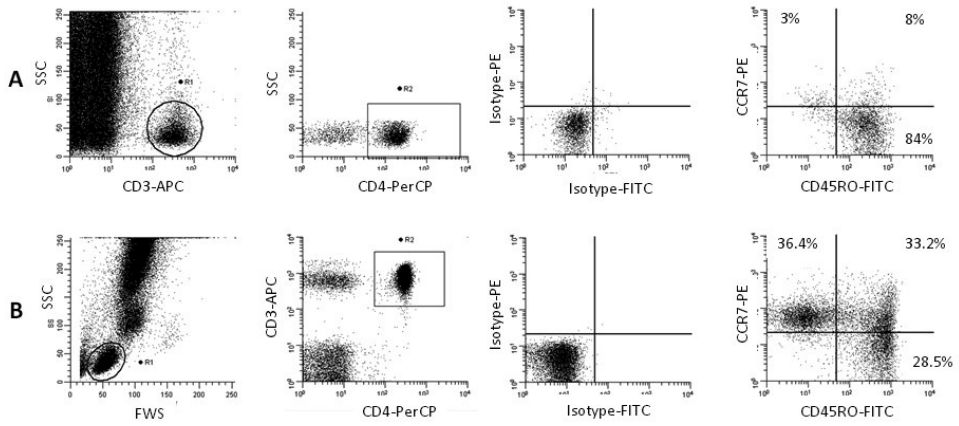

Fig. 1. Representative flow cytometry analysis of $CD4^+$ effector memory T cells in urine and whole blood. Samples were stained with allophycocyanin (APC)-conjugated anti-CD3, peridin chlorophyll protein (PerCP)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CCR7 and fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO. The encircled areas show lymphocytes and the quantification of T cells was performed by quadrant dot-plot analysis. $CD4^+$ effector memory T cells in urine (a) and in peripheral blood (b).

HMGB1 measurement by Western blot in urine

Three ml of urine supernatants, removed after centrifugation, were concentrated between 30 and 300 times with Vivaspin 6® tubes (Sartorius Stedim Biotech, Gottingen, Germany). Sodium dodecyl sulphate (SDS) buffer

was added to concentrated urine, and the volume of urine loaded to the gel was corrected for concentration factor. Western blot was performed as described previously [20]. In brief, proteins were resolved by 12.5% SDS-polyacrylamide gel electrophoresis (Criterion gel Bio-Rad, Veenendaal, the Netherlands) and transferred to polyvinylidene fluoride membrane (Millipore, Amsterdam, the Netherlands) followed by blocking with Odyssey buffer (LI-COR Biotechnology, Lincoln, NE, USA). Membranes were then incubated with anti-HMGB1 mouse monoclonal antibody 1:1000 (R&D Systems, Abingdon, UK) overnight at 4℃ and with goat anti-mouse IgG antibodies labelled with IRDye800 (1:10000; LI-COR Biotechnology). Blots were scanned with Odyssey infrared Imaging System (LI-COR Biotechnology). A lysate of Jurkat cells was prepared and this lysate was included twice in each blot as standard. HMGB1 levels were presented as the fluorescence intensity against the standard. To perform correlation studies, creatinine levels were measured in unconcentrated urine and urinary levels of HMGB1 were expressed as HMGB1 intensity/creatinine ratio in intensity/mmol.

ELISA for serum HMGB1

Serum HMGB1 levels in AAV patients were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Shino Test, Sagamihara, Kanagawa, Japan). Levels were expressed in ng/ml.

Urinary MCP-1

Urinary levels of MCP-1 in patients and controls were measured by ELISA. Briefly, the ELISA plate was coated with monoclonal anti-MCP-1 antibodies diluted 1:250 (R&D Systems). After blocking the plate for 1 h, urine samples and standards were incubated for 2 h. The standard curve was built with sequential dilutions of recombinant human MCP-1 (R&D Systems) and biotinylated polyclonal goat anti-human MCP-1 antibodies (R&D Systems)

were used as detection antibodies. Results were corrected for urinary dilution with spot urine creatinine and expressed as MCP-1/creatinine ratio in pg/mmol.

Statistical analysis

Statistical analysis was carried out with spss software version 20.0 and graphs were built using Graph Pad Prism version 3.02. Categorical variables were presented as total number and percentage while continuous variables were presented as mean ± standard deviation (s.d.) or as median and interquartile range (IQR) as appropriate. Comparison between groups was performed using χ^2 test or Fisher's exact test for categorical variables and Student's *t*-test or Mann–Whitney *U*-test for continuous variables. Correlations between continuous variables were performed with the Spearman's rank correlation coefficient. Wilcoxon's signed-rank test was used to analyse paired urinary HMGB1 and MCP-1 samples. The significant level accepted was 5% (p-value < 0.05).

Results

Serum HMGB1 levels

No significant differences were found in serum HMGB1 levels among AAV subsets [GPA: 2.86 (0.56–4.08) ng/ml *versus* MPA: 1.25 (0.56–7.80) ng/ml *versus* RLV: 2.36 (1.34–10.61) ng/ml, p = 0.760] (Fig. 2a) or onset/relapse patients [2·36 (0·56–3·42) ng/ml *versus* 2.48 (0.88–5.20) ng/ml, p = 0.502] (Fig. 2b). No significant correlations were found between serum HMGB1 levels and BVAS (rho = 0.073; p = 0.741), proteinuria (rho = 0.102; p = 0.669), urinary MCP1/creatinine ratio (rho = 0.086; p = 0.728) or eGFR (rho = 0.303; p = 0.195).

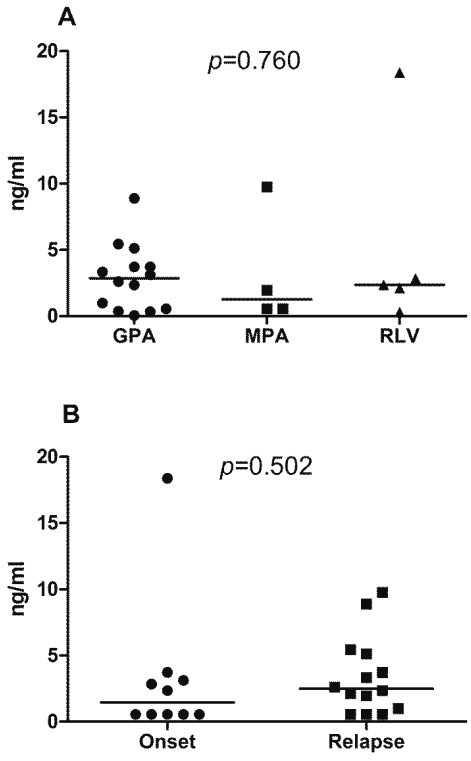

Fig. 2. Serum high mobility group box 1 (HMGB1) levels in subgroups of anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients. Serum HMGB1 levels are similar in different AAV subsets (a) and in patients at onset or with relapsing disease (b).

Urinary HMGB1 levels

AAV patients with active nephritis presented significantly higher median HMGB1 levels in urine in comparison to HC [10.3 (7.05–18.50) *versus* 5.8 (4.48–7.01); p = 0.004] (Fig. 3a). A representative blot used for urinary measurement of HMGB1 is shown in Fig. 3b with samples from patients with active nephritis and HC. In AAV patients, in order to correct for differences in urinary concentrations, analyses were performed with urinary HMGB1/urinary creatinine ratios. No difference could be found in median HMGB1/creatinine ratio in urine between AAV patients under immunosuppressive therapy and

those without [1.80 (1.43–2.24) *versus* 1.94 (0.67–3.62); p = 0.688] or between AAV patients with and without use of prednisolone [1.84 (1.43–2.24) *versus* 1.94 (0.67–3.62); p = 0.738]. In nine AAV patients urinary samples were collected again during remission (36.2 ± 10.5 months later). Urinary HMGB1/creatinine ratio decreased significantly when these samples were compared with those obtained during active nephritis [0.031 (0.017–0.135) *versus* 0.740 (0.360–2.110) p = 0.0078] (Fig. 4).

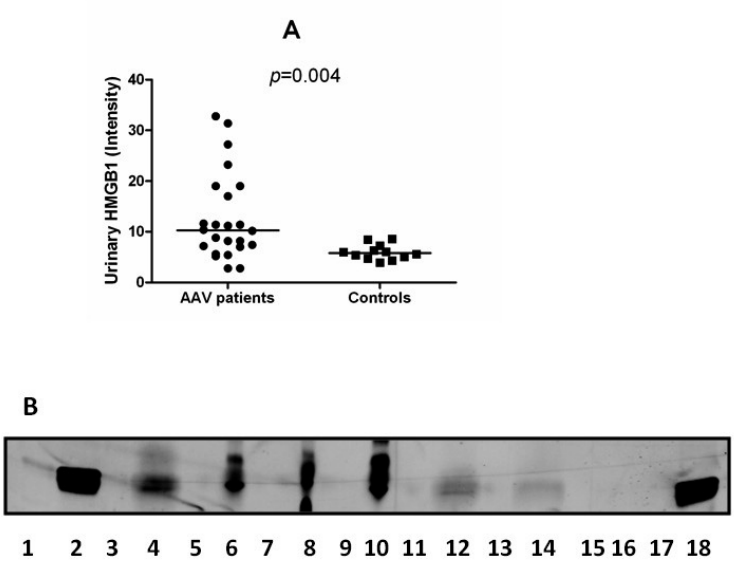

Fig. 3. Detection of urinary high mobility group box 1 (HMGB1) in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active glomerulonephritis and in healthy controls (HC). (a) AAV patients with active nephritis present significantly higher urinary HMGB1 levels than HC (P = 0·004). (b) A representative blot for measuring urinary HMGB1 levels. Lane 1: molecular weight marker; lanes 2 and 18: positive control; lanes 4, 6, 8, 10 and 12: urine samples from AAV patients; lanes 14 and 16: urine sample from HC.

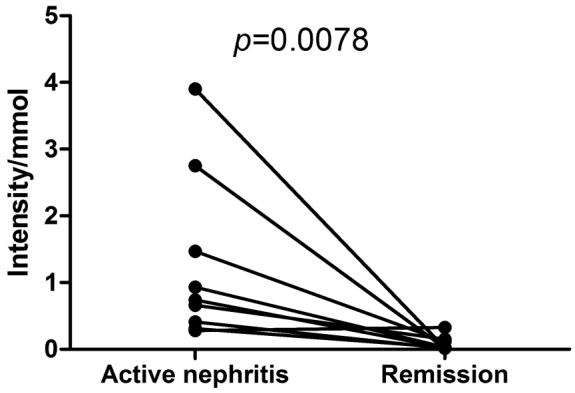

Fig. 4. Urinary high mobility group box 1 (HMGB1)/creatinine ratio in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active nephritis *versus* remission. A significant decrease in urinary HMGB1 levels is observed from active renal disease to remission at a mean 36·2 ± 10·5 months later.

Associations of urinary HMGB1 levels with clinical and laboratory parameters in AAV

No differences could be found in urinary HMGB1/creatinine ratios among different AAV subsets [GPA: 1.86 (1.32–3.12) *versus* MPA: 2.58 (1.91–3.57) *versus* RLV: 1.08 (0.30–2.35); $p = 0.186$], onset/relapse patients [1.96 (1.21–2.86) *versus* 1.84 (1.07–3.02); $p = 0.852$] or MPO-ANCA positivity *versus* PR3-ANCA positivity [2.24 (1.08–3.36) *versus* 1.86 (1.12–2.59); $p = 0.841$]. In order to evaluate whether urinary HMGB1/creatinine ratio was associated with parameters of renal involvement, systemic disease activity or with serum HMGB1 levels in AAV patients, we calculated the correlation coefficient between urinary HMGB1/creatinine ratio and 24-h proteinuria (rho = –0.151; $p = 0.515$), eGFR (rho = –0.178; $p = 0.452$), BVAS (rho = 0.018; $p = 0.934$) and serum HMGB1 (rho = –0.241; $p = 0.279$). None of these comparisons led to significant correlations.

Urinary HMGB1 levels and CD4⁺ T cells

As the presence of CD4⁺ effector memory T cells in urine has been found to reflect renal disease activity in AAV [26], we evaluated whether urinary HMGB1 could be associated with CD4⁺ T cell– and CD4⁺ effector memory T cell counts in urine. A positive correlation was found between urinary HMGB1/creatinine ratio and CD4⁺ T cells/creatinine ratio (rho = 0.431; p = 0.028) and effector memory T cells/creatinine ratio (rho = 0.403; p = 0.039) (Fig. 5a,b). The urinary HMGB1/creatinine ratio did not correlate with CD4⁺ T cells (rho = –0.153; p = 0.498) or with CD4⁺ effector memory T cells in peripheral blood (rho = –0.222; p = 0.320). Furthermore, urinary CD4⁺ T cell– and effector memory T cell counts were not correlated with BVAS (rho = –0.108; p = 0.652 and rho = –0.180; p = 0.449, respectively), proteinuria (rho = 0.105; p = 0.680 and rho = 0.091; p = 0.791, respectively) and eGFR (rho = 0.154; p = 0.542 and rho = 0.152; p = 0.548, respectively).

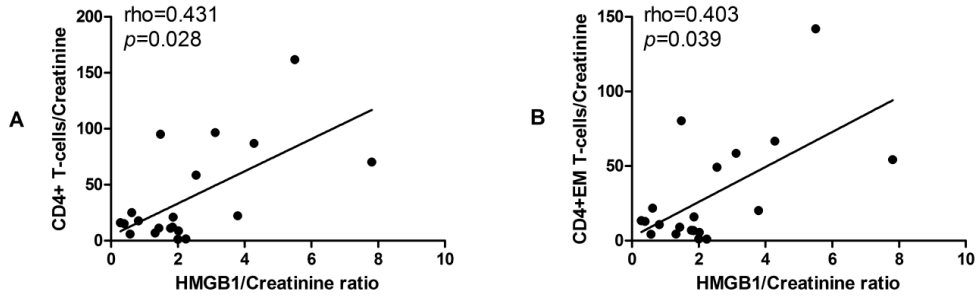

Fig. 5. Urinary high mobility group box 1 (HMGB1), CD4+T cell and CD4+ effector memory T cell counts in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active glomerulonephritis. A significant positive correlation was found between urinary HMGB1/creatinine ratio (a) and CD4+ T cells and CD4+ effector memory T cell counts in urine (b).

Urinary MCP-1 levels

AAV patients with active nephritis presented higher urinary MCP-1 levels than HC [99.19 (20.31–247.20) pg/ml *versus* 26.96 (5.74–59.58) pg/ml;

p = 0.035]. With regard to urinary MCP-1/creatinine ratios in AAV patients, levels decreased significantly when AAV patients with active nephritis achieved remission [6.79 (3.50–48.89) pg/mmol *versus* 0.11 (0.06–2.72) pg/mmol; p = 0.0039] (Fig. 6a). A positive correlation was found between urinary MCP-1/creatinine ratio with BVAS (rho = 0.447; p = 0.042) (Fig. 6b) but not with 24-h proteinuria (rho = 0.426; p = 0.069) or with eGFR (rho = –0.152; p = 0.545). In addition, no correlation could be found between urinary HMGB1/creatinine and urinary MCP-1/creatinine levels (rho = –0.164; p = 0.478) or between urinary MCP-1/creatinine ratio and $CD4^+$ T cells/creatinine ratio in urine (rho = 0.300; p = 0·226), $CD4^+$ effector memory T cells/creatinine ratio in urine (rho = 0.243; p = 0.332), $CD4^+$ T cell counts in peripheral blood (rho = –0.060; p = 0.801) and with $CD4^+$ effector memory T cell counts in peripheral blood (rho = –0.147; p = 0.537).

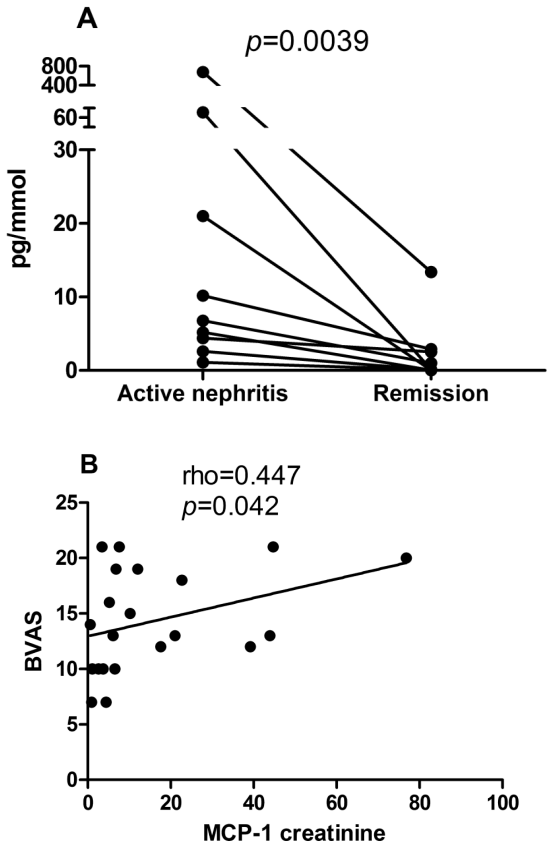

Fig. 6. Urinary monocyte chemoattractant protein-1 (MCP-1)/creatine ratio in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active disease and remission and correlation with Birmingham Vasculitis Activity Score (BVAS). Urinary MCP-1/creatinine ratio decreased significantly when patients with active nephritis achieved remission (a) and urinary MCP-1 creatinine ratio was correlated positively with BVAS (b).

Discussion

In this study, we observed that AAV patients with active glomerulonephritis present higher urinary HMGB1 levels than HC and patients in remission, and urinary HMGB1 correlates with the number of $CD4^+$ T cells and $CD4^+$ effector memory T cells in urine. However, urinary HMGB1 levels, $CD4^+$ T cells and $CD4^+$ effector memory T cells were not associated with

systemic disease activity (i.e. BVAS) or other parameters of renal involvement in AAV (i.e. 24-h proteinuria, eGFR and urinary MCP-1). In contrast, urinary MCP-1 levels were correlated positively with BVAS in AAV patients with active nephritis.

Non-invasive parameters are generally used to monitor disease activity and response to therapy in patients with renal involvement in AAV such as haematuria with dysmorphic red cells, proteinuria and renal function [33]. However, these parameters may not be sufficiently sensitive and specific to differentiate active glomerular vasculitis from permanent damage. Thus, investigation of novel urinary biomarkers to assess active glomerulonephritis in AAV is worthwhile. Levels of urinary MCP-1 have been associated with active renal vasculitis, response to therapy and prognosis in AAV [30,31]. Higher urinary MCP-1 levels in active renal vasculitis in AAV rather than changes in circulating MCP-1 seem to reflect increased renal production [30]. Therefore, we compared urinary MCP-1 as a biomarker for active renal involvement with urinary HMGB1. Indeed, in accordance with a previous study [30], we found a positive correlation between urinary MCP-1 levels and BVAS. However, urinary HMGB1 levels were not correlated with urinary MCP-1 or BVAS. Thus, increased urinary HMGB1 levels in AAV active nephritis seem to be a reflection of the underlying pathological inflammatory process in the kidney rather than a biomarker for systemic disease activity of AAV in clinical practice.

This is the first report demonstrating increased urinary HMGB1 levels in AAV patients with active renal involvement. To date, only serum HMGB1 levels have been evaluated in AAV patients presenting active glomerulonephritis, but associations with parameters of renal involvement have not been analysed [19,20]. Serum HMGB1 levels are lower in AAV patients with renal involvement at presentation in comparison to non-renal patients [21] and in GPA patients with predominantly vasculitic manifestations than in GPA patients with granulomatous manifestations [19]. Bruchfeld *et al.*,

however, observed higher serum HMGB1 levels in AAV patients with active glomerulonephritis than in AAV patients with inactive renal disease [20]. Serum HMGB1 levels decreased 6–9 months after baseline when a new biopsy showed improvement in renal histopathology and the expression of HMGB1 in renal tissue decreased from active disease to remission [20]. Similarly to serum levels and tissue expression of HMGB1, urinary HMGB1/creatinine ratio decreased significantly when patients with active nephritis achieved remission in the current study.

Differently from AAV, the association between HMGB1 and renal involvement is well established in SLE. Both serum and urinary HMGB1 levels are higher in SLE patients with active lupus nephritis in comparison to patients without renal involvement and HC [15,16]. Furthermore, both serum and urinary levels of HMGB1 correlated with SLEDAI and complement levels while serum HMGB1 was also associated significantly with proteinuria and anti-dsDNA levels [15,16]. Of note, urinary HMGB1 levels have been measured by Western blot, as HMGB1 ELISAs are not validated for measurement in urine. Also serum levels of HMGB1 in SLE patients are detected by Western blot, because of the presence of anti-HMGB1 antibodies in lupus serum [15,16]. These antibodies are hardly present in AAV patients, so HMGB1 levels can be measured by ELISA in these sera [21]. In lupus nephritis, the cytoplasmic and extracellular expression of HMGB1 in renal tissue was higher than in control renal tissue and did not decrease with follow-up [16,17].

It remains speculative whether urinary HMGB1 in renal AAV results from release by local renal inflammation and infiltrating cells or necrosis in the kidney. The association between urinary HMGB1 and urinary T cells as well as the lack of correlation between urinary HMGB1 and urinary MCP-1 levels, a well-known inflammatory urinary marker [34], suggests passive release of HMGB1 by necrotic cells from the kidney. HMGB1 released from necrotic cells is in a reduced form and has chemotactic properties, whereas HMGB1

released from activated cells has a disulphide bond between C23 and C45 and C106 in the thiol form. This form of HMGB1 acts differently, as it can induce cytokine production by signalling through Toll-like receptor (TLR)-4 [35]. Clearly, the source of urinary HMGB1 in renal AAV needs further evaluation.

The presence of $CD4^+$ T cells, mainly effector memory T cells, in urine reflects renal involvement in AAV and a reduction in the number of these cells in urine is observed following treatment [27]. Indeed, the number of $CD4^+$ effector memory T cells was increased in urine in AAV patients with active nephritis, but no correlation could be found with BVAS, 24-hour proteinuria and eGFR in this study. However, both $CD4^+$ T cell– and $CD4^+$ effector memory T cell counts in urine were associated with urinary HMGB1. These correlations could indicate a possible interplay between HMGB1 and the adaptive immune response in active nephritis in AAV [8–12]. Otherwise, the lack of association between urinary MCP-1 levels and $CD4^+$ T cell– and $CD4^+$ effector memory T cell counts in urine could be explained by the fact that the chemoattractant effect of MCP-1 is predominantly exerted on monocytes and macrophages rather than on T cells [28].

Limitations of this study include the relatively low number of AAV patients evaluated and the lack of comparison with AAV patients with active disease but without renal involvement.

Urinary HMGB1 levels are increased in AAV patients with active nephritis in comparison to HC and decrease when remission is achieved. Urinary HMGB1 levels correlate with urinary $CD4^+$ T cell– and urinary $CD4^+$ effector memory T cell counts. However, this analysis suggests that urinary HMGB1 reflect renal involvement in AAV less well than urinary MCP-1.

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CHAPTER 7

High mobility group box 1 levels in large vessel vasculitis are not associated with disease activity but are influenced by age and statins

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Abstract

Introduction: Takayasu arteritis (TA) and giant cell arteritis (GCA) are large vessel vasculitides (LVV) which usually present as granulomatous inflammation in arterial walls. High mobility group box-1 (HMGB1) is a nuclear protein that acts as an alarmin when released by dying or activated cells. This study aims to evaluate whether serum HMGB1 can be used as a biomarker in LVV.

Methods: 29 consecutive TA patients with 29 age- and sex-matched healthy controls (HC) were evaluated in a cross-sectional study. Eighteen consecutive GCA patients with 16 age and sex-matched HC were evaluated at the onset of their disease and in part of them during follow-up. Serum HMGB1 levels were measured by enzyme-linked immunosorbent assay.

Results: In GCA patients at disease onset mean serum HMGB1 levels did not differ from HC (5.74 ± 4.19ng/ml vs. 4.17 ± 3.14ng/ml; $p = 0.230$). No differences in HMGB1 levels were found between GCA patients with and without polymyalgia rheumatica ($p = 0.167$), ischemic manifestations ($p = 0.873$), systemic manifestations ($p = 0.474$) or relapsing disease ($p = 0.608$). During follow-up, no significant fluctuations on serum HMGB1 levels were observed from baseline to 3 months (n=13) ($p = 0.075$), 12 months (n=6) ($p = 0.093$) and at the first relapse (n=4) ($p = 0.202$). Serum HMGB1 levels did not differ between TA patients and HC [1.19 (0.45-2.10)ng/ml vs. 1.46 (0.89-3.34)ng/ml; $p = 0.181$] and no difference was found between TA patients with active disease and in remission [1.31 (0.63-2.16)ng/ml vs. 0.75 (0.39-2.05)ng/ml; $p = 0.281$]. HMGB1 levels were significantly lower in 16 TA patients on statins compared with 13 patients without statins [0.59 (0.29-1.46)ng/ml vs. 1.93 (0.88-3.34)ng/ml; $p = 0.019$]. GCA patients at disease onset had higher serum HMGB1 levels than TA patients with active disease [4.70 (2.55-8.92)ng/ml vs. 1.31 (0.63-2.16)ng/ml; $p = 0.0075$] and age was independently associated with higher HMGB1 levels.

Conclusion: Patients with TA and GCA present similar serum HMGB1 levels compared with HC. Serum HMGB1 is not useful to discriminate between active disease and remission. In TA, use of statins was associated with lower HMGB1 levels. HMGB1 is not a biomarker for LVV.

Introduction

Takayasu arteritis (TA) and giant cell arteritis (GCA) are large vessel vasculitides (LVV) characterized by granulomatous inflammation of the vessel wall [1]. Although both diseases present significant overlap in features and some similarities in the distribution of angiographic lesions [2,3], TA predominantly affects young females and involves the aorta and its main branches whereas GCA affects predominantly branches of carotid and vertebral arteries in individuals older than 50 years [1].

Despite clinical symptoms, acute phase reactants and vascular imaging help to assess disease activity in LVV, there is a need for novel biomarkers for diagnosis, prognosis and to distinguish active disease from damage or infection. In TA, active disease is associated with higher serum levels of pentraxin-3, MMP-9, interleukin (IL)-6, IL-8, IL-18, B-cell activating factor (BAFF), monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T-cell expressed and secreted (RANTES) [4-10]. In GCA, high serum levels of tumor necrosis factor α (TNFα), IL-6, and BAFF are associated with disease activity and relapses [11-14]. Moreover, adaptive immunity is triggered during GCA pathogenesis manifested by Th1 and Th17 responses with the production of interferon (IFN)γ and IL-17A which enhance arterial inflammation [15,16].

High mobility group box 1 (HMGB1) is a nuclear non-histone protein that acts as an alarmin when released into the extra-cellular milieu either by cellular death or upon activation of inflammatory cells such as macrophages

by LPS or IFNγ [17,18]. High serum HMGB1 levels have been observed in infectious diseases, atherosclerosis, mechanical trauma, cancer, and in systemic autoimmune diseases such as systemic lupus erythematosus (SLE) [19-23]. In systemic vasculitis, high serum HMGB1 levels were observed in Kawasaki disease, IgA vasculitis, and in patients with ANCA-associated vasculitis, especially in granulomatosis with polyangiitis (GPA) with granulomatous manifestations [24-27]. Serum HMGB1 levels have not been evaluated in patients with LVV. This study aims to evaluate serum HMGB1 levels as a surrogate marker of disease activity in patients with LVV and associations between serum HMGB1 and acute phase reactants, disease manifestations and therapy in patients with TA and GCA. Due to epidemiological differences in the prevalence of both diseases, patients with TA were recruited from Brazil whereas GCA patients were recruited from The Netherlands.

Patients and methods

Study population

The study comprised 18 GCA patients with 16 healthy controls (HC), both from the *University Medical Center Groningen* (UMCG), The Netherlands (Table 1), and 29 consecutive TA patients from *Universidade Federal de São Paulo* (Unifesp), Brazil with 29 HC from the same region (Table 1). Inclusion criterion for TA patients was the fulfillment of the 1990 American College of Rheumatology (ACR) classification criteria [28] while the exclusion criteria were current chronic infectious disease, malignancy, and pregnancy. GCA patients were included if they fulfilled the 1990 ACR criteria [29] or when presenting compatible manifestations associated with an enhanced 18^{F}-fluorodeoxyglucose uptake in large vessels by positron emission computed tomography (18FDG-PET/CT). Exclusion criteria for GCA included current

chronic infectious disease and malignancy. The study was approved by the institutional ethics committees from both university hospitals and complied with the Declaration of Helsinki.

Active disease in GCA was considered if patients presented manifestations of active disease (e.g. temporal headache, optic neuritis, jaw claudication) not attributable to other causes and/or polymyalgia rheumatica (PMR) symptoms with an increase in ESR > 30mm/hour whereas remission was considered in the absence of GCA manifestations with normal ESR [30]. Kerr's criteria and the Indian Takayasu activity score 2010 (ITAS2010) with ITAS.A using ESR or CRP were employed to ascertain disease activity in TA [31-33].

In the 18 GCA patients, blood samples were collected at disease onset prior to glucocorticoid therapy and follow-up samples were obtained from 13 patients at 3 months and from 6 patients at 12 months. Blood samples were collected from 29 TA patients as a cross-sectional evaluation.

Serum HMGB1

Serum HMGB1 levels were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Shino Test, Sagamihara, Kanagawa, Japan) according to manufacturer's instructions. Results were expressed in nanograms per milliliter.

Statistical analysis

Statistical analysis was performed using SPSS software version 20.0 and graphs were created with Graph Pad Prism version 3.02. Mean ± standard deviation or median and interquartile range were used to present

normally distributed and non-normally distributed continuous variables, respectively. Categorical variables were presented as total number and percentage. Comparisons between groups were performed using Student's *t* test or Mann-Whitney U test for continuous data or using Chi-square test or Fisher's exact test for categorical variables. Correlations between numerical data were performed with Spearman's correlation coefficient. A linear regression model was built to analyze whether age and the diagnosis of LVV were independently associated with serum HMGB1 levels. Receiver operating characteristics (ROC) analysis was performed to find out the HMGB1 cut-off with the best sensitivity and specificity to differentiate GCA from TA. The cut-off value was chosen from the maximized sum of sensitivity and specificity. Paired *t*-test or Wilcoxon's test were used to analyze longitudinal data. The significance level accepted was 5% ($p < 0.05$).

Results

Disease features and therapy of GCA and TA patients

Disease features and therapy of GCA and TA patients are described in Table 1. After the first evaluation, all GCA patients were treated with high-dose prednisolone (60mg/day) with slow tapering after improvement of disease symptoms and laboratory abnormalities. Disease relapse was observed in 4 (22.2%) GCA patients and the median time to the first relapse after diagnosis was 6.0 months (6.0-15.0). Methotrexate 10-15mg per week was added to two patients (11.1%) after the first relapse during steroid tapering. Five GCA patients (27.8%) were on statins at disease onset.

Previous ischemic events in TA included unstable angina (4 patients), stroke (3 patients), acute myocardial infarction (2 patients), transient ischemic attacks and mesenteric ischemia in one patient each. Two TA patients were treated only with prednisone whereas the remainder used either an

immunosuppressive drug or a biologic agent. ESR, ITAS.A ESR and ITAS.A CRP values were significantly higher in TA patients with active disease than in those in remission, whereas there was a trend for higher serum CRP levels in patients with active disease. No significant differences could be found between patients with active disease and remission regarding therapy (Table 2).

Table 1 – Demographic, disease features and therapy of patients with giant cell arteritis at disease onset and Takayasu arteritis.

Variables	GCA (n=18)	HC (n=16)	*p*	Variables	TA (n=29)	HC (n=29)	*p*
Demographic features							
Age, years	72.0 (63.7-75.0)	68.5 (63.0-72.0)	0.643	Age, years	38.0 (34.5-48.5)	38.0 (27.5-48.5)	0.392
Females, n (%)	14 (77.8)	11 (68.8)	0.551	Females, n (%)	28 (96.6)	27 (93.1)	0.553
Disease features and therapy							
GCA	**Results**			**TA**	**Results**		
Headache, n (%)	12 (66.7)			Disease duration, months	108 (60-186)		
Constitutional symptoms, n (%)	8 (44.4)			Angiographic type V, n (%)	16 (55.2)		
Cranial ischemic manifestations, n (%)	8 (44.4)			Previous ischemic events, n (%)	11 (37.9)		
Jaw claudication, n (%)	6 (33.3)			Active disease, n (%)	11 (37.9)		
Visual symptoms, n (%)	4 (22.2)			Remission, n (%)	18 (62.1)		
Polymyalgia rheumatica, n (%)	4 (22.2)			Statins, n (%)	16 (55.2)		
Headache, n (%)	12 (66.7)			Prednisone, n (%)	16 (55.2)		
ESR, mm/ 1st hour	69.6 ± 28.7			Prednisone daily dose, mg	8.7 (5.0-28.7)		
CRP, mg/l	40.0 (20.2-84.2)			Immunosuppressive agents, n (%)	19 (65.5)		
Positive TAB, n/total	8/11			Biological agents, n (%)	9 (31.0)		
Positive PET-CT scan, n/total	13/15						

Continuous variables are presented as mean ± standard deviation or as median and interquartile range; CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; GCA – giant cell arteritis; n – number of patients; PET-CT scan – positron emission computed tomography; TA – Takayasu arteritis; TAB – temporal artery biopsy.

Table 2 – Comparison between patients with Takayasu arteritis with active disease and in remission.

Variables	Active disease (N=11)	Remission (N=18)	*p*
HMGB1, ng/ml	1.31 (0.63-2.16)	0.75 (0.39-2.05)	0.281
ESR, mm/ 1st hour	39.0 (25.0-68.0)	17.5 (8.0-25.5)	0.017
CRP, mg/l	6.0 (4.4-24.9)	2.0 (0.1-10.7)	0.053
ITAS2010	3.0 (2.2-5.2)	--	--
ITAS.A ESR	3.5 (2.0-6.2)	1.0 (1.0-1.7)	0.001
ITAS.A CRP	5.1 ± 2.5	2.1 ± 0.9	0.012
Statins, n (%)	7 (63.6)	9 (50.0)	0.702
Prednisone, n (%)	6 (54.5)	10 (55.6)	0.958
Prednisone daily dose, mg	20.0 (7.5-45.0)	5.0 (2.5-13.7)	0.055
Immunosuppressive agents, n (%)	7 (63.6)	12 (66.7)	0.868
Biological agents, n (%)	3 (27.3)	6 (33.3)	0.732

Continuous variables are presented as median and interquartile range or as mean ± standard deviation; CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; ITAS – Indian Takayasu activity score; ITAS.A - Indian Takayasu activity score with acute phase response; n – number of patients.

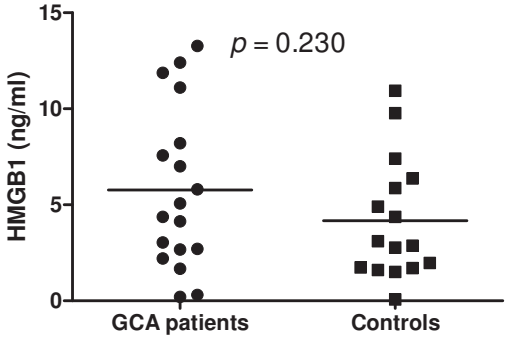
HMGB1 levels in giant cell arteritis

In GCA patients with active disease at onset and prior to therapy mean serum HMGB1 levels did not differ between patients and HC (5.74 ± 4.19ng/ml vs. 4.17 ± 3.14ng/ml; $p = 0.230$) (Figure 1). Furthermore, among GCA patients mean serum HMGB1 levels at onset were not higher in patients with or without PMR [1.25 (0.21-10.50)ng/ml vs. 5.42 (2.94-8.92)ng/ml; $p = 0.167$], cranial ischemic manifestations (5.56 ± 3.31ng/ml vs. 5.89 ± 4.95ng/ml; $p = 0.873$), constitutional symptoms (4.92 ± 3.90ng/ml vs. 6.40 ± 4.50ng/ml; $p = 0.474$) or relapsing disease (4.75 ± 3.31ng/ml vs. 6.02 ± 4.47ng/ml; $p = 0.608$), respectively.

Mean serum HMGB1 levels in GCA patients were 5.74 ± 4.19ng/ml at baseline, 5.18 ± 3.98ng/ml at 3 months, 8.19 ± 6.80ng/ml at 12 months, and 6.23 ± 2.48ng/ml at the first relapse. During follow-up, no significant fluctuations on serum HMGB1 levels were observed from baseline levels to 3

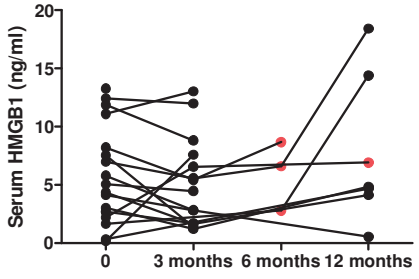
and 12 months (Figure 2). Moreover, serum HMGB1 levels in relapsing patients were not different from their levels at disease onset (p = 0.825), at 3 months (p = 0.629), at 12 months (p = 0.601) and from HC (p = 0.170) (Table 3). In GCA patients no correlation was present between HMGB1 and ESR (rho = -0.220; p = 0.380) or between HMGB1 and CRP levels (rho = -0.258; p = 0.301).

Figure 1 – Serum HMGB1 levels in patients with giant cell arteritis and controls.



GCA patients at disease onset present similar serum HMGB1 levels compared to healthy controls.

Figure 2 – Longitudinal levels of serum HMGB1 in patients with giant cell arteritis.



Serum HMGB1 in individual GCA patients along follow-up and during relapses (red dots).

Table 3 – Longitudinal data on disease activity and serum HMGB1 levels in patients with giant cell arteritis.

Variables	Baseline (n=18)	3 months (n=13)	12 months (n=6)	Relapse (n=4)
HMGB1, ng/ml	5.74 ± 4.19	5.18 ± 3.98	8.19 ± 6.80	6.23 ± 2.48
ESR, mm/ 1st hour	69.6 ± 28.7	15.1 ± 6.6	21.0 ± 4.9	57.5 ± 24.2
CRP, mg/l	40.0 (20.2-84.2)	2.5 (2.5-7.0)	8.0 (5.1-14.7)	38.5 (12.0-82.2)
Prednisolone, mg/day	--	20.0 (18.7-27.5)	18.7 (3.7-30.0)	6.2 (1.2-9.3)

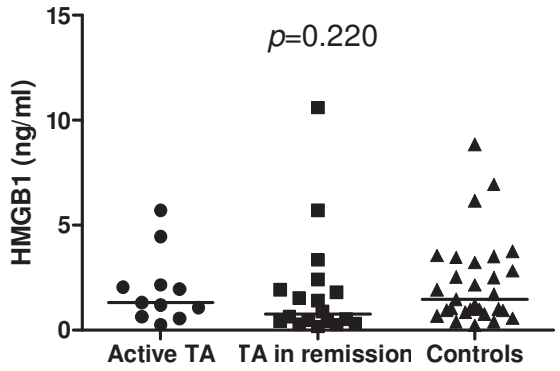
Continuous variables are presented as median and interquartile range or as mean ± standard deviation; CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; HMGB1 – High mobility group box 1.

Serum HMGB1 in Takayasu arteritis

As depicted in Figure 3, serum HMGB1 levels did not differ between TA patients with active disease [1.31 (0.63-2.16)ng/ml], patients in remission [0.75 (0.39-2.05)ng/ml] and HC [1.46 (0.89-3.34)ng/ml] (p = 0.220). Similar median serum HMGB1 levels were found in TA patients with and without previous ischemic events [1.53 (0.42-3.34)ng/ml vs. 0.97 (0.50-1.93)ng/ml; p = 0.486]. There was no difference in serum HMGB1 levels in TA patients under prednisone therapy compared with those not receiving prednisone [1.13 (0.45-2.34)ng/ml vs. 1.31(0.36-1.94)ng/ml; p = 0.676] or between TA patients receiving immunosuppressive agents compared with those on biological agents [1.59 (0.43-2.45)ng/ml vs. 0.59 (0.42-0.96); p = 0.140]. However, serum HMGB1 levels were significantly lower in TA patients on statins compared with patients not receiving these agents [0.59 (0.29-1.46)ng/ml vs. 1.93 (0.88-3.34)ng/ml; p = 0.019] (Figure 4).

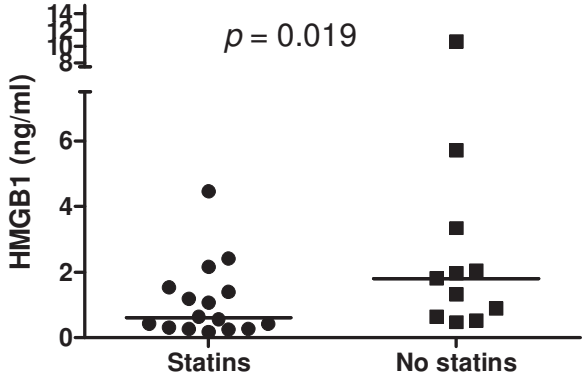
No correlation could be observed between serum HMGB1 levels and ESR (rho = 0.104; p = 0.590), CRP (rho = 0.090; p = 0.642), ITAS2010 (rho = 0.230; p = 0.231), ITAS.A ESR (rho = 0.216; p = 0.261) or ITAS.A CRP (rho = 0.070; p = 0.720).

Figure 3 – Serum HMGB1 levels in patients with Takayasu arteritis and controls.



TA patients and HC present similar serum HMGB1 levels.

Figure 4 – Influence of stains use on serum HMGB1 levels in patients with Takayasu arteritis.



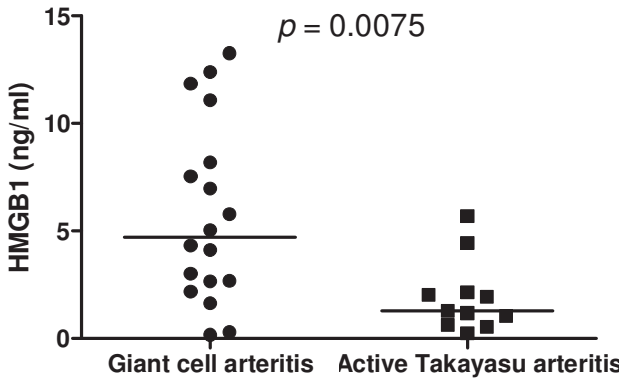
Statins use was associated with significantly lower serum HMGB1 levels in TA patients.

Comparison between Takayasu arteritis and giant cell arteritis regarding serum HMGB1 levels

GCA patients at disease onset presented significantly higher median serum HMGB1 levels compared with TA patients with active disease [4.70 (2.55-8.92)ng/ml vs. 1.31 (0.63-2.16)ng/ml; $p = 0.0075$] (Figure 5). Even when GCA and TA patients without statins were analyzed separately, serum HMGB1 levels were significantly higher in GCA patients compared to TA patients [5.06 (2.86-10.0)ng/ml vs. 1.80 (0.63-3.34); $p = 0.015$].

Higher serum HMGB1 levels observed in GCA compared with TA seems to be an effect of aging, since serum HMGB1 levels were also higher in GCA controls than in TA controls [2.98 (1.70-6.23)ng/ml vs. 1.46 (0.89-3.34)ng/ml; $p = 0.019$]. A weak correlation was found between serum HMGB1 levels and age in all study participants (rho = 0.244; $p = 0.019$) while in a linear regression model, age was independently associated with serum HMGB1 levels ($\beta = 0.056$; $p = 0.003$; $R^2 = 0.099$), regardless of the diagnosis of LVV or control status. ROC analysis of GCA and TA patients showed that the best HMGB1 cut-off value for differentiating GCA from TA is 2.17ng/ml with 83.3% sensitivity and 79.3% specificity.

Figure 5 – Serum HMGB1 levels in patients with giant cell arteritis and Takayasu arteritis with active disease.



GCA patients at disease onset and prior to any therapy present higher serum HMGB1 levels than TA patients with active disease but already on treatment with prednisone and immunosuppressive or biological agents.

Discussion

In this study, we observed that patients with active LVV present similar serum HMGB1 levels compared with patients in remission and HC. TA patients in remission and those with relapsing disease were already under therapy and the use of statins was associated with lower serum HMGB1 levels. Furthermore, in GCA patients with active disease prior to therapy, serum HMGB1 levels were not different from HC but were higher than HMGB1 levels found in TA patients with active disease.

The need for reliable biomarkers for disease activity is an issue of utmost importance in TA. The evaluation of disease activity is a challenge, since the disease course is protracted and silent relapses are common, occurring in up to 96% of patients who attained remission [34,35]. It is not easy to define when the disease is actually in remission and most patients develop new angiographic lesions over time usually without clear

manifestations of disease activity. In this context, a novel biomarker would help medical decisions for TA [34].

Granulomatous inflammation and vessel wall necrosis are well-known features of LVV. Either necrosis or infiltrating macrophages are important sources of HMGB1 release into the extra-cellular milieu that in turn activate innate and adaptive immunity [36,37]. Patients with GPA and predominant granulomatous inflammation present higher serum HMGB1 levels compared with GPA patients with predominantly vasculitic manifestations [25]. Thus, we evaluated associations between disease activity in LVV and serum HMGB1 levels. Unfortunately, no difference could be found between patients with active disease and remission or between patients with LVV and HC.

On the other hand, GCA patients at disease onset and prior to therapy presented serum HMGB1 levels that were similar to those of HC, and no association could be found between HMGB1 and acute phase reactants, disease manifestations or disease relapse. Moreover, during follow-up no significant fluctuations in serum HMGB1 levels were observed in GCA patients. Novel biomarkers in GCA would help to recognize active disease in patients with signs and symptoms of GCA but normal acute phase reactants. However, serum HMGB1 levels were not increased in patients with active disease.

Serum HMGB1 levels were significantly higher in GCA patients than in TA patients, and a cutoff value of 2.17ng/ml in HMGB1 levels was shown to be of some use in differentiating GCA from TA. Furthermore, GCA controls had higher serum HMGB1 than TA controls. These findings indicate that serum HMGB1 levels increases during aging and may be influenced by the burden of atherosclerosis in older individuals. In mice, the age-dependent DNA double-strand break is associated with a reduction of nuclear HMGB1 in neurons leading to an increased release of extracellular HMGB1 [38]. However, in a population study performed in Japan with 626 subjects, aging

did not seem to affect serum HMGB1 levels in healthy subjects [39]. In the present study, although only a weak correlation was found between age and serum HMGB1 levels, age was independently associated with serum HMGB1 regardless of the diagnosis of LVV or control status.

We found a strong association between statins and lower serum HMGB1 levels in 16 patients with TA (55.2%). Recently, lower HMGB1 levels were observed in hyperlipidemic patients and in GPA patients in remission both on statin therapy [40,41]. Moreover, atorvastatin was able to reduce *in vitro* the release of HMGB1 in stimulated HUVEC (human umbilical vein endothelial cells) cultures. This indicates that the inhibition of HMGB1 release by activated cells is one of the pleiotropic effects of statins [41]. Other drugs may also influence HMGB1 release from cells such as dexamethasone and metformin [42,43]. These findings may explain in part why TA patients already under treatment presented serum HMGB1 levels similar to HC.

The role of statins in GCA has still to be determined. No impact on relapse rate or on the prevention of severe ischemic events was observed in retrospective studies [44-46]. However, conflicting results were found regarding the influence of statins on acute phase reactants and daily glucocorticoid dose in GCA patients on statins [47,48]. In TA patients, a retrospective study could not find any difference in ischemic events in patients with and without statins but associations with disease activity were not analyzed [49]. In this study, more TA patients used statins than GCA patients at diagnosis although this difference was not statistically significant (data not shown). This could be due to the long disease course of our TA patients in comparison with the GCA patients who were evaluated at disease onset.

Limitations of this study are its mainly cross-sectional nature and the inclusion of patients already on therapy for TA, whereas the low number of patients and the short-term follow-up period are limitations for the GCA

patients. Nevertheless, the data seem robust enough to conclude that HMGB1 is not a suitable biomarker in LVV in contrast to SLE [24].

Conclusions

Serum HMGB1 levels were not different between patients with LVV and HC as well as between patients with active disease and those in remission. Therefore, serum HMGB1 levels were not a useful biomarker for LVV. Moreover, serum HMGB1 levels were not associated with any disease phenotypes in LVV. In long-standing TA, therapy with statins seems to lead to lower serum HMGB1 levels.

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CHAPTER 8

Increased serum high mobility group box 1 levels in Behçet's disease – No association with disease activity or specific organ involvement

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Abstract

Objectives. High mobility group box 1 (HMGB1) is a nuclear protein that acts as an alarmin when released into the extracellular milieu. HMGB1 is a biomarker of active disease in several systemic autoimmune diseases. Behçet's disease (BD) is a systemic inflammatory disorder with a waxing/waning course. The objective of this study is to evaluate serum HMGB1 levels as a possible biomarker for disease activity in BD.

Methods. A cross-sectional study was performed in 26 BD patients and 20 healthy controls measuring serum HMGB1 levels. The Brazilian version of the simplified Behçet's Disease Current Activity Form (BR-BDCAFs) was used to measure disease activity.

Results. Serum HMGB1 levels were higher in patients with active disease [3.82 (2.54-6.11)ng/ml], in patients with BD without active disease but still on therapy [2.76 (1.89-5.78)ng/ml] and in patients in remission without treatment [2.66 (1.86-4.70)ng/ml] than in healthy controls [0.96 (0.59-1.39)ng/ml], $p < 0.001$. Levels were comparable between BD patients with active disease, BD without active disease but still on therapy and those in remission without treatment ($p = 0.432$). There was no correlation between serum HMGB1 levels and BR-BDCAFs (Rho = 0.195; $p = 0.339$). No association could be found between serum HMGB1 levels and specific disease involvement or therapy. So, serum HMGB1 levels cannot be used as a biomarker in BD.

Conclusion. Serum HMGB1 levels are increased in patients with BD as compared to healthy controls. However, no association was found with disease activity, specific organ involvement or therapy in BD.

Introduction

Behçet's disease (BD) is a chronic multisystemic inflammatory disease of unknown etiology classified as a systemic vasculitis. It is characterized by recurrent attacks of oral ulcers, genital ulcers, cutaneous lesions, eye inflammation, and the occurrence of articular, neurological, vascular and intestinal manifestations [1]. Although BD is reported to occur all over the world, its prevalence is higher in countries along the ancient Silk Road. The etiology of BD is unknown and it is believed that environmental factors play a role in genetically predisposed individuals. HLA-B51 is the most important genetic marker associated with BD, especially in Turkish and Asian populations from the Middle East to the Far East whereas the association between BD and HLA-B51 is weaker in Caucasians [1,2].

Innate immunity plays an important role in the pathophysiology of BD with the participation of γδ T cells, NK cells, and neutrophils. γδ T cells are involved in the protection of mucosa and skin against invading organisms. Increased numbers of γδ T cells are found in mucosal lesions and in peripheral blood of BD patients. These cells express activation markers (e.g. CD29 and CD69) and produce inflammatory cytokines such as interferon (IFN)γ, tumor necrosis factor (TNF)α, and interleukin(IL)-8 [3,4]. Activated NK cells have been shown to be increased in peripheral blood of patients with active BD [7]. Neutrophils are hyperactive and contribute to tissue injury in BD with increased chemotaxis, phagocytosis, myeloperoxidase expression and production of reactive oxygen species (ROS) [3]. Cytokines and chemokines secreted by antigen presenting cells and T cells have been suggested to cause abnormal neutrophil activation in BD [4].

High mobility group box-1 (HMGB1) is a non-histone nuclear protein that contributes to chromatin architecture and regulates transcription. HMGB1 may be passively released by necrotic cells or actively secreted by activated

cells [5]. Once outside the cell, HMGB1 acts as an alarmin or a danger associated molecular pattern (DAMP) binding to receptors and sensors of innate immunity such as the receptor for advanced glycation end-products (RAGE) and Toll-like receptor (TLR)-2, TLR-4, and TLR-9. Extra-cellular HMGB1 triggers inflammatory reactions mainly by inducing cytokine secretion and chemotaxis [6].

An association between increased serum HMGB1 levels and disease activity has been described in several systemic autoimmune diseases such as systemic lupus erythematosus, juvenile idiopathic arthritis, rheumatoid arthritis, primary Sjögren's syndrome, systemic sclerosis and in ANCA-associated vasculitis [5-8]. Recently, a study performed in Korea found an association between higher serum HMGB1 levels and intestinal involvement in BD [9]. Gastrointestinal involvement of BD is commonly described in patients from the Far East, being observed in one third of BD patients from Japan [10]. However, intestinal involvement in BD is extremely rare in BD patients from other countries, including Brazil where this manifestation is found in only 3.3-6.6% of cases [10,11]. In this study we evaluated serum HMGB1 levels in patients with BD in order to assess associations with disease activity and specific disease manifestations in a Brazilian population of BD patients.

Patients and methods

Study population

Twenty-six consecutive patients with BD and 20 age (39.0 ± 11.5 years vs. 38.5 ± 13.5 years; $p = 0.888$) and sex (65.4% vs. 90.0% females; $p = 0.082$) matched healthy controls were included in this cross-sectional study. BD patients were under regular follow-up at the Vasculitis Outpatient Clinic at *Universidade Federal de São Paulo* (Unifesp). Inclusion criteria included age

above 18 years and fulfillment of the International Study Group diagnostic criteria for BD [12]. Disease activity of BD was evaluated with the Brazilian version of the simplified Behçet's Disease Current Activity Form (BR-BDCAFs) [13]. Active disease was considered if BR-BDCAF(s) was equal to or greater than 2, patients with a BR-BDCAF(s) score equal to zero were divided in two groups: BD without still on therapy and BD patients without treatment. Thirteen BD patients presented active disease, 10 BD patients were without active disease but still on therapy and 3 BD patients were in remission without treatment. All patients gave informed written consent according to the Declaration of Helsinki and the study was approved by the institutional ethics committee.

Information about previous and current BD manifestations and about current therapy was retrieved from medical charts. The BR-BDCAF(s) was recorded at each visit, and comprehended scoring for oral ulcers, genital ulcers, cutaneous lesions (i.e. erythema nodosum-like and acne-like lesions), joint complaints, eye involvement (i.e. uveitis and/or retinal vasculitis), neuro-BD, vasculo-BD (i.e. venous thrombosis and/or arterial aneurysms), and intestinal involvement. Eye disease, neuro-BD and vasculo-BD were considered major organ involvement of BD.

Serum HMGB1

Serum HMGB1 levels were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Shino Test, Sagamihara, Kanagawa, Japan) according to the manufacturer's instructions. Results were expressed in nanograms per milliliter (ng/ml).

Statistical analysis

Statistical analysis was performed using SPSS software version 20.0 and graphs were created with Graph Pad Prism version 3.02. Mean ± standard deviation or median and interquartile range were used to present continuous variables as appropriate. Categorical variables were presented as total number and percentage. Continuous parameters were analyzed using Student's *t* test, Mann-Whitney U test or by Kruskal-Wallis test and categorical variables were analyzed using Chi-square test or Fisher's exact test. Correlations between numerical data were performed with Spearman's correlation coefficient. The significance inference level was established at 5% ($p < 0.05$).

Results

Table 1 depicts clinical features and current therapy of BD patients. Half of the patients were using immunosuppressive therapy and only one patient was treated with anti-TNFα agents. Five BD patients (19.2%) had only mucocutaneous involvement and joint complaints, whereas the remaining 21 BD patients had at least one actual or previous major involvement of BD (i.e. ocular, neurological or vascular).

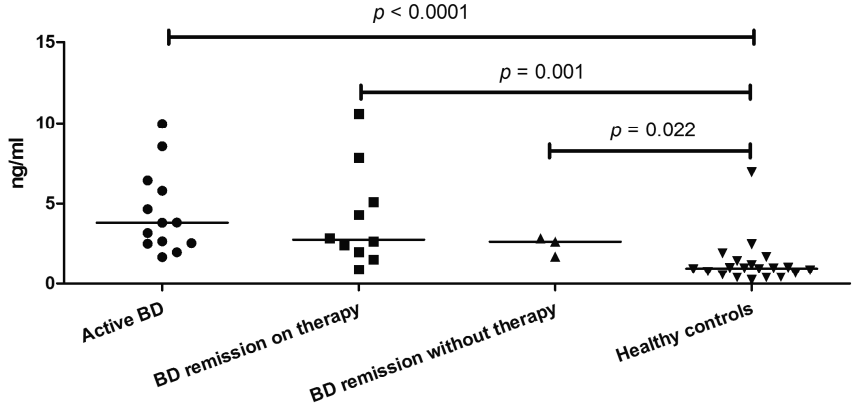
Table 1 – Disease features and therapy of 26 patients with Behçet's disease at the time of the study.

Variables	Results
Age at disease onset, years	30.5 ± 10.8
Disease duration, years	8.5 (2.7-12.0)
Active disease, n (%)	13 (50.0)
BR-BDCAFs	2.0 (2.0-2.5)
Disease manifestations at study	
Oral ulcers, n (%)	7 (26.9)
Genital ulcers, n (%)	6 (23.1)
Cutaneous involvement, n (%)	2 (7.7)
Eye involvement, n (%)	5 (19.2)
Neuro-BD, n (%)	3 (11.5)
Disease manifestations ever presented by BD patients	
Oral ulcers, n (%)	26 (100.0)
Genital ulcers, n (%)	23 (88.5)
Cutaneous manifestations, n (%)	22 (84.5)
Eye involvement, n (%)	18 (69.2)
Arthralgias or arthritis, n (%)	12 (46.2)
Neuro-BD, n (%)	6 (23.1)
Vasculo-BD, n (%)	4 (15.4)
Therapy	
Colchicine, n (%)	9 (34.6)
Colchicine + benzathine penicillin, n (%)	4 (15.4)
Thalidomide, n (%)	4 (15.4)
Prednisone, n (%)	10 (38.5)
Azathioprine, n (%)	11 (42.3)
Cyclosporine A, n (%)	2 (7.7)
Methotrexate, n (%)	1 (3.8)
Cyclophosphamide, n (%)	1 (3.8)
Infliximab, n (%)	1 (3.8)
No therapy, n (%)	3 (11.5)

Continuous data are presented as mean ± standard deviation or as median and interquartile range; BD – Behçet's disease; BR-BDCAFs – simplified Behçet's Disease Current Activity Form; n – number of patients.

Serum HMGB1 levels were significantly higher in BD patients as compared to healthy controls [2.86 (2.32-5.27)ng/ml vs. 0.96 (0.59-1.39)ng/ml; $p < 0.0001$]. Also, no significant differences were found in serum HMGB1 levels among BD patients with active disease, BD patients without active disease but still on therapy and those in remission without treatment [3.82 (2.54-6.11)ng/ml vs. 2.76 (1.89-5.78)ng/ml vs. 2.64 (1.72-2.86)ng/ml; $p = 0.432$]. Serum HMGB1 levels were significantly higher in all BD subgroups compared with healthy controls (Figure 1). There was no correlation between serum HMGB1 levels and BR-BDCAFs score (Rho = 0.195; $p = 0.339$) or disease duration (Rho = 0.010; $p = 0.962$).

Figure 1 – Serum HMGB1 levels in patients with Behçet's disease with active disease, BD in remission while on therapy or BD in remission without treatment and healthy controls.



Median serum HMGB1 levels are significantly higher in BD patients with active disease, BD in remission but still on therapy and BD in remission without treatment than in healthy controls ($p < 0.0001$). However, no difference is seen among BD subgroups.

Within the group of thirteen BD patients with active disease at the time of the study, no difference could be found in serum HMGB1 levels between the eight patients with major organ involvement (i.e. five presenting eye disease and three with neuro-BD) and the five patients with only mucocutaneous disease [3.82 (2.28-5.50)ng/ml vs. 2.67 (2.54-7.48)ng/ml; p = 0.909].

Finally, serum HMGB1 levels did not differ between BD patients with and without prednisone [2.86 (1.65-4.94)ng/mL vs. 2.92 (2.45-6.09)ng/mL; p = 0.598] or between BD patients with and without azathioprine [3.18 (2.57-5.10)ng/mL vs. 2.67 (2.01-5.79)ng/mL; p = 0.815]. Amongst BD patients on prednisone, serum HMGB1 levels were similar between patients with a high daily dose (i.e. >20mg/day) and those with a low daily dose (i.e. <10mg/day) [3.83 (2.28-5.22)ng/ml vs. 2.64 (1.22-6.41)ng/ml; p = 0.251].

Discussion

In this study, we observed higher serum HMGB1 levels in BD patients than in healthy controls. All BD subgroups including patients with active disease, patients without active disease but still on therapy and patients in remission without treatment, presented significantly higher serum HMGB1 levels than healthy controls. Although, serum HMGB1 levels were higher in patients with active disease than in patients without active disease (with or without therapy), the difference was not significant. No correlation could be found between serum HMGB1 levels and disease activity measured by BR-BDCAFs. No association could be found with individual BD manifestations or with major disease involvement such as neuro-BD, eye inflammation and vasculo-BD. Furthermore, there was no association between prednisone or immunosuppressant use and serum HMGB1 levels.

Neutrophils are important effector cells in the pathogenesis of BD contributing to tissue damage and disease manifestations [3,4]. There is evidence that extracellular HMGB1 exerts several effects on neutrophils. *In vitro* studies have shown that binding of HMGB1 to TLR4 activates neutrophils leading to nuclear translocation of NFκB. In addition, HMGB1 induces the production of cytokines such as TNFα and IL-8, and the activation of NADPH oxidase resulting in ROS production and increase of Mac-1-mediated adhesive and migratory functions of phagocytes [14-17]. Moreover, HMGB1 also induces the formation of neutrophil extracellular traps (NET) through interactions with TLR4 as well [18]. Thus, the observation of elevated serum HMGB1 levels in BD patients might indicate a possible role of extracellular HMGB1 as a mediator of neutrophil activation in BD.

A previous study evaluated HMGB1 levels in Korean patients with BD. Similarly to our study, BD patients had significantly higher serum HMGB1 levels than healthy controls and no difference could be found between patients with active disease and remission. Patients with gastrointestinal involvement presented the highest HMGB1 serum levels, whereas no association could be found with any other manifestation of BD [9]. The association between higher serum HMGB1 levels and intestinal involvement in BD seems reasonable, since gastrointestinal involvement may be extensive and is characterized by deep ulcerations involving any segment of the gastrointestinal tract [1,2].

The lack of association between serum HMGB1 levels and specific disease manifestations in the present study may be due to the relatively low disease activity in BD patients evaluated in this study and the absence of BD patients with active gastrointestinal involvement. Vascular and neurological involvements of BD are severe disease manifestations that have an impact on prognosis of BD patients [1,2]. Amongst the 13 BD patients with active disease enrolled in this study only three had active neuro-BD at the time of

evaluation while the remaining patients presented either ocular or mucocutaneous disease which may not be associated with intense systemic inflammatory reactions. Therefore, evaluation of a higher number of patients with severe BD manifestations would help to clarify this issue. Although neurological involvement may be found in up to one third of BD patients in Brazil, it might be difficult to enroll patients at the onset of neuro-BD [11]. We could not find any association between serum HMGB1 levels and specific disease manifestations in BD including severe disease involvement (i.e. neuro-BD, eye disease and vasculo-BD), since BD has a relapsing-remitting course and most of these patients were in remission at the time of the study.

We also could not find associations between therapy and serum HMGB1 levels. Patients on prednisone or on azathioprine presented similar HMGB1 levels compared to those without those agents. Reduced serum HMGB1 levels have been described in patients on statins or prednisone [19]. However, this effect has not been observed with immunosuppressive agents [20]. Limitations of the present study include the relatively small number of BD patients and the cross-sectional nature of this survey. The lack of longitudinal data makes it difficult to draw definite conclusions about associations between serum HMGB1 levels and disease activity as well as expression of particular manifestations of BD.

In conclusion, serum HMGB1 levels were shown to be increased in patients with BD. However, no association could be found between serum HMGB1 and disease activity or specific organ involvement in BD.

Key messages

- BD is associated with increased serum HMGB1 levels in comparison to healthy controls.
- No association was found between circulating HMGB1 and disease activity or specific manifestations in BD.
- No association was observed between prednisone or azathioprine use and serum HMGB1 levels.

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CHAPTER 9

Summary and general discussion

Alexandre Wagner Silva de Souza

Summary and discussion

Systemic vasculitides are a heterogeneous group of diseases that is characterized by inflammation of blood vessels of different types and sizes. The consequence of this process is the destruction of the vessel wall resulting in necrosis (fibrinoid necrosis) and eventually rupture of the vessel wall may occur with bleeding to surrounding tissues when small vessels are affected (i.e. capillaries and post-capillary venules) [1,2]. When the vasculitic process affects medium- and/or large-sized arteries, the inflammatory infiltrate and fibrinoid necrosis leads to changes of vessel walls such as stenosis and occlusion, usually in muscular arteries whereas dilation and aneurysm formation are often observed in elastic arteries. Moreover, extravascular inflammation, tissue necrosis and a strong systemic inflammatory response are also features of systemic vasculitides [3,4].

In this context, alarmins or danger associated molecular patterns (DAMPs) are candidates for biomarkers of the underlying inflammatory process or may be useful for diagnosis or for determining the prognosis in systemic vasculitis. Alarmins are multifunctional endogenous molecules with important intracellular roles that are passively released by necrotic cells or actively secreted by activated immune cells or epithelia. In the extracellular environment, alarmins usually activate the innate immune system through binding to pattern recognition receptors, such as Toll-like receptors (TLRs). The best characterized alarmins are high mobility group box 1 (HMGB1), S100 proteins and heat shock proteins (HSPs) [5].

HMGB1 has been widely evaluated in systemic inflammatory and autoimmune diseases, cancer, atherosclerotic disease and sepsis [6-8]. When starting this thesis, we wrote a literature review about the role of HMGB1 in vascular diseases (**chapter 2**) including systemic vasculitides and atherosclerotic disease. At that time, only a few cross-sectional studies had been performed to evaluate circulating HMGB1 in systemic vasculitides,

mainly in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) [9-11] and in Kawasaki disease (KD) [12,13]. In AAV, serum HMGB1 levels were higher in patients with granulomatosis with polyangiitis (GPA) with active disease than in patients with inactive GPA and healthy controls (HC). However, no significant differences regarding serum HMGB1 levels could be observed amongst patients with microscopic polyangiitis (MPA) with active disease, patients in remission and HC [9]. Another study from the same group observed higher serum HMGB1 levels in GPA patients with predominantly granulomatous manifestations compared to patients with predominantly vasculitic manifestations. A positive correlation was found between serum HMGB1 levels and the volume of pulmonary "granuloma" [10]. Both studies used a commercial ELISA kit to measure serum HMGB1 levels [9,10]. Bruchfeld *et al* used an in house Western blot technique to measure serum HMGB1 in AAV patients and observed that those with biopsy-proven glomerulonephritis presented higher serum HMGB1 levels when compared to those with a normal renal biopsy. Moreover, a significant decrease in serum HMGB1 levels was observed in some patients who had a repeated biopsy in remission. Although serum HMGB1 levels were higher in AAV patients than in HC, no significant differences could be found among AAV subsets including GPA, MPA and eosinophilic granulomatosis with polyangiitis (EGPA) [11].

In atherosclerotic disease, patients with acute ischemic events (e.g., coronary artery disease and stroke) present significantly higher circulating HMGB1 levels compared to HC. Furthermore, an association between high HMGB1 levels and poor outcomes was observed in patients with acute coronay syndromes. The expression of HMGB1 is increased in the nuclei and cytoplasm of macrophages and smooth muscle cells in the atherosclerotic lesion compared to normal arteries. Studies evaluating experimental models of atherosclerotic disease showed that HMGB1 is not only involved in the amplification of the inflammatory response during the acute ischemic injury but also in the healing process after ischemia [14].

In **chapter 4**, we performed a longitudinal study to evaluate serum HMGB1 levels in AAV at disease onset, early and late remission, prior to and during relapses. In the same study, we tested AAV patients with active disease for anti-HMGB1 antibodies. At disease onset, serum HMGB1 levels were not different between AAV patients and HC. However, only AAV patients without renal involvement presented higher serum HMGB1 levels than HC. A positive correlation was found between serum HMGB1 and C-reactive protein levels while a negative correlation was observed between serum HMGB1 and 24-h proteinuria. AAV patients with active disease had similar median OD value of anti-HMGB1 antibodies compared to HC, and only 12.5% of AAV had positive anti-HMGB1 antibodies [15]. In contrast, patients with systemic lupus erythematosus (SLE) present higher OD values of anti-HMGB1 antibodies than HC, especially patients with lupus nephritis [16]. Still in **chapter 4**, the longitudinal analysis of serum HMGB1 levels showed no significant increase prior to a relapse and fluctuations in HMGB1 levels were not associated with an increased risk of relapse in AAV. Therefore, we concluded that HMGB1 was not a useful biomarker in AAV and renal involvement was associated with lower serum HMGB1 levels in AAV [15].

In **chapter 5**, we evaluated serum HMGB1 levels and serum levels of the soluble receptor for advance glycation end-products (sRAGE) as predictors of subclinical atherosclerosis in carotid arteries in GPA patients. Due to the association between higher serum HMGB1 levels and subclinical atherosclerosis in coronary arteries of patients with and without diabetes compared to individuals without coronary artery disease [17], we hypothesized that serum HMGB1 and sRAGE levels would be associated with atherosclerosis in carotid arteries of GPA patients. In the study described in **chapter 5**, GPA patients and HC presented similar prevalence of atherosclerotic plaques and similar overall mean and maximum intima-media thickness (IMT) in carotid arteries as well as similar serum HMGB1 and sRAGE levels. All GPA patients were in remission when evaluated. sRAGE

levels were negatively correlated with overall maximum IMT in carotid arteries while no association could be found between serum HMGB1 levels and subclinical atherosclerosis in carotid arteries. GPA patients on statin or prednisolone use presented significantly lower serum HMGB1 levels than GPA patients without these drugs [18].

Statins have anti-inflammatory properties through inhibition of pro-inflammatory effects of cytokines on endothelial cells [19]. Furthermore, statins also lower circulating HMGB1 levels in experimental models of atherosclerosis and in patients with hyperlipidemia [20,21]. Therefore, still in **chapter 5** we decided to check whether atorvastatin could inhibit the release of HMGB1 by human umbilical vein endothelial cells (HUVEC) *in vitro* upon activation with lipopolysaccharide (LPS). LPS induced a slow release of HMGB1 by HUVEC with a peak at 24 hours. We also measured interleukin (IL)-8 levels in HUVEC's supernatants as a reference chemokine to ascertain that endothelial cells were activated by LPS. Incubation of HUVEC with 5µM atorvastatin prior to activation with LPS led to lower HMGB1 and IL-8 levels in supernatants compared to HUVEC activated with LPS. We concluded that even though no association could be found between serum HMGB1 levels and carotic atherosclerosis in GPA patients, use of statins had an impact on serum HMGB1 levels as well as on HMGB1 release by activated HUVEC. Those findings suggested an additional anti-inflammatory effect of statins [18].

Another issue related to the role of HMGB1 as a biomarker in AAV is the detection of HMGB1 in urine of AAV patients with active nephritis. In **chapter 4**, we described that serum HMGB1 levels in AAV patients with active nephritis at disease presentation were not different from HC while non-renal AAV patients had significantly higher serum HMGB1 compared to HC [15]. We raised the hypothesis of HMGB1 loss in urine due to active nephritis as a potential cause for the relatively lower serum HMGB1 levels in AAV patients with active nephritis in comparison to non-renal active AAV.

Furthermore, a recent work from our group had shown higher urinary HMGB1 levels in patients with active lupus nephritis than in SLE patients without active nephritis and HC [22]. Thus, we decided to evaluate urinary levels of HMGB1 in AAV patients with active nephritis in **chapter 6**. In addition, we decided also to evaluate $CD4^+$ T cells and $CD4^+$ effector memory T cells in peripheral blood and urine together with HMGB1 levels in AAV patients, since previous studies had shown effects of HMGB1 on T cell proliferation and polarization and a recent study from our group had shown increased numbers of $CD4^+$ effector memory T cells in urine of AAV patients with active nephritis compared with patients in remission and AAV patients with active disease without renal involvement [14,23].

Firstly, we wrote a review about the role of mononuclear cells, especially CD4+ effector memory T cells in the pathogenesis of AAV in **chapter 3** [24]. In GPA, the expansion of $CD4^+$ T cells occurs within the effector memory population and the majority of infiltrating T cells in lung lesions and glomeruli show a memory phenotype. However, in peripheral blood a decrease in the number of circulating CD4+ effector memory T cells is observed and that may be due to migration of these CD4+ T cells to organs affected by the disease [24].

In **chapter 6**, we evaluated AAV patients with active nephritis regarding HMGB1 levels in urine and serum, $CD4^+$ T cells and $CD4^+$ effector memory T cells, and urinary monocyte chemoattractant protein-1 (MCP-1) levels compared with HC and with some patients who achieved remission of disease activity at mean 36.2 ± 10.5 months after the first assessment. Urinary HMGB1 levels were higher in AAV patients with active nephritis than in HC. Moreover, urinary HMGB1 levels significantly decreased in AAV patients who achieved remission. No association could be found between urinary HMGB1/creatinine ratio and serum HMGB1, Birmingham Vasculitis Activity Score (BVAS), 24-hour proteinuria, and estimated glomerular filtration

rate (eGFR). Nonetheless, we observed a significant correlation between urinary HMGB1/creatinine ratio and urinary $CD4^+$ T cells/creatinine ratio and urinary $CD4^+$ effector memory T cells/creatinine ratio. Urinary MCP-1 levels were also higher in AAV patients with active nephritis compared with HC and those levels decreased significantly when patients achieved remission. In contrast with urinary HMGB1/creatinine ratio, urinary MCP-1/creatinine ratio was associated with BVAS, but not with urinary $CD4^+$ T cells/creatinine ratio or with urinary $CD4^+$ effector memory T cells/creatinine ratio [25].

Apart from AAV, we also evaluated serum HMGB1 levels in patients with large-vessel vasculitides (LVV) including Takayasu arteritis (TA) and giant cell arteritis (GCA) in **chapter 7**. This study was performed in Brazil and in the Netherlands due to epidemiological differences in the prevalence of both diseases. GCA patients were recruited in the Netherlands while TA patients were evaluated in Brazil. The assessment of GCA patients was performed at disease onset (i.e., prior to starting treatment with corticosteroids), 3 months and 12 months after onset, and during a disease relapse. Serum HMGB1 levels were not different between GCA patients at disease onset and age- and sex-matched HC and no significant fluctuations in serum HMGB1 levels could be observed during follow-up of GCA patients. No association could be observed between serum HMGB1 levels and acute phase reactants, presence of polymyalgia rheumatica, systemic manifestations and relapsing disease in GCA patients.

Differently from GCA, TA patients were evaluated only once in a cross-sectional way in this study. Similar serum HMGB1 levels were observed in TA patients with active disease, TA patients in remission and HC. TA patients on statins presented significantly lower serum HMGB1 levels compared to those without statins. In TA patients, no association could be found between serum HMGB1 levels and acute phase reactants, measures of disease activity, previous ischemic events or the use of other therapeutic

agents such as prednisone and biologics. Linear regression analysis showed that statin use was independently associated with lower serum HMGB1 levels. The effects of statins on HMGB1 levels had already been observed in GPA patients (**chapter 5**), in patients with hyperlipidemia and in an experimental model of atherosclerotic disease [18,20,21]. Regarding disease activity in TA, univariate logistic regression analysis showed association with the Indian Takayasu Clinical Activity Score (ITAS2010) and erythrocyte sedimentation rate (ESR) values, but not with serum C-reactive protein (CRP) or HMGB1 levels. In the multivariate logistic regression analysis, only ITAS2010 score was independently associated with active disease in TA patients.

Serum HMGB1 levels were significantly higher in GCA patients at onset than in TA patients with active disease. Even when GCA and TA patients both on statins were analyzed separately, serum HMGB1 levels were significantly higher in the former group. This difference between TA and GCA in HMGB1 levels might indicate an influence of aging on this biomarker.

In **chapter 8**, we evaluated serum HMGB1 levels in patients with Behçet's disease (BD) which is classified as a variable vessel vasculitis [26]. In this study, BD patients presented significantly higher serum HMGB1 levels than HC. However, no significant differences could be found between BD patients with active disease and in remission. There was no correlation between serum HMGB1 levels and the simplified Brazilian version of Behçet's disease Current Activity Form (BR-BDCAFs), a validated assessment tool used to evaluate disease activity in BD. Furthermore, no association could be found between serum HMGB1 levels and specific disease involvement or therapy.

During the development of this thesis, two studies that evaluated HMGB1 in vasculitis were published [27]. One study included AAV patients while the other study evaluated patients with IgA vasculitis. In the former

study, plasma HMGB1 levels were assessed in patients with GPA and MPA from China [27,28]. Plasma HMGB1 levels in AAV patients with active disease were higher than in patients in remission and HC. A significicant but weak correlation was found between plasma HMGB1 levels and CRP, BVAS, serum creatinine and estimated glomerular filtration rate. In this study, a potential evidence of association between circulating HMGB1 levels and granulomatous manifestations was found, since AAV patients with PR3-ANCA presented higher plasma HMGB1 levels than patients with MPO-ANCA independently from creatinine levels. After therapy with intravenous or oral cyclophosphamide, plasma HMGB1 levels decreased significantly [27].

Serum HMGB1 levels were reported to be higher in patients with IgA vasculitis, allergic vasculitis and urticarial vasculitis than in HC. Abundant cytoplasmic expression of HMGB1 could be observed in endothelial cells of the involved skin in patients with IgA vasculitis. When a human microdermal endothelial cell line was stimulated with recombinant HMGB1, an increase in the release of TNFα and IL-6 in supernatants was observed [28].

Conclusions

Circulating HMGB1 levels do not seem to be a reliable biomarker of disease activity in some systemic vasculitides, such AAV and LVV. In AAV patients, serum HMGB1 levels are influenced predominantly by granulomatous manifestations while AAV patients with active nephritis presented high urinary HMGB1 levels that correlated with CD4+ T cells and CD4+ effector memory T cells in the urine but not with urinary MCP-1 levels. Moreover, induction of remission in AAV nephritis leads to a significant decrease in urinary HMGB1 levels. In GPA patients in remission and in TA patients, serum HMGB1 levels are decreased by therapy, including statins. Atorvastatin inhibits HMGB1 release *in vitro* by HUVEC stimulated with LPS.

In BD, serum HMGB1 levels are higher than in HC regardless of disease activity.

Future investigations

HMGB1 has indeed an effector role in different pathological settings including sepsis and sterile inflammation [6]. However, there is still much left to know about the influence of several post-translational factors on its functions, including the redox state of HMGB1, acetylation and complexes with other molecules [7]. Different *in vitro* and *in vivo* studies have shown that the inhibition of HMGB1 may be a potential therapeutic target for the future [6-8].

The evidence that statin use may be a potential therapy to decrease HMGB1 levels in atherosclerotic disease in humans came initially from *in vitro* studies that showed fluvastatin decreasing the expression of intracellular HMGB1 in a monocyte lymphoma cell line (U937) stimulated with hyperlipidemic serum [20]. HMGB1 release into supernatants was also inhibited by atorvastatin in HUVEC activated by LPS. Those findings were replicated in an *in vivo* model of hyperlipidemia with fluvastatin decreasing serum HMGB1 levels in Syrian hamsters as well as decreasing serum HMGB1 in patients with hyperlipidemia and in GPA patients treated with statins [18,20]. However, to date no clinical trial have evaluated if decreasing serum HMGB1 levels may lead to a decrease in cardiovascular events.

Metformin is another agent that inhibited LPS-stimulated HMGB1 release *in vitro* from a macrophage cell line (RAW 264.7). Metformin inhibited HMGB1 cytosolic translocation from the nucleus and subsequently decreased extracellular levels of HMGB1 [29]. Moreover, metformin also inhibited hyperglycemia-induced HMGB1 expression in cardiomyocytes in a dose-dependent manner and protects against injury of these cells stimulated by

high glucose levels [30]. However, no studies have been performed in animal models or in humans to evaluate whether metformin decreases serum HMGB1 levels or if there might be any clinical benefit from this inhibition.

Therapy targeting HMGB1 has shown benefits in animal models of different conditions [6-8]. Anti-HMGB1 neutralizing antibodies and the recombinant A box domain of HMGB1 are the HMGB1 antagonists most commonly evaluated in animal models whereas a few studies tested anti-RAGE antibodies, and thrombomodulin (Table 1) [8]. However, no studies have evaluated efficacy and safety of these agents in human diseases.

Table 1 – Experimental models of therapy targeting HMGB1 [adapted from reference 8].

Model	Agents
Arthritis	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
	Thrombomodulin
Neuropathic pain	Anti-HMGB1 antibodies
Endotoxemia	Anti-HMGB1 antibodies
	Thrombomodulin
Sepsis	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
	Anti-RAGE antibodies
Pancreatitis	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
Colitis	Anti-HMGB1 antibodies
Hemorrhagic shock	Anti-HMGB1 antibodies
Stroke	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
Epilepsy	Recombinant HMGB1 A box
Ischemia-reperfusion injury	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
Atherosclerosis	Anti-HMGB1 antibodies
Myocardial infarction	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
Transplantation	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
Respiratory diseases	Anti-HMGB1 antibodies
	Recombinant HMGB1 A box
Acetaminophen-induced liver damage	Anti-HMGB1 antibodies

HMGB1 – high mobility group box-1; RAGE – receptor for advanced glycation end products.

In systemic vasculitides, the inhibition of HMGB1 might be helpful in the following situations: granulomatous inflammation of GPA, AAV nephritis, acute phase of KD, IgA vasculitis and in patients with BD. Firstly, pre-clinical

studies using HMGB1 antagonists (e.g. anti-HMGB1 neutralizing antibodies and the recombinant A box domain of HMGB1) should be tried in animal models of PR3-ANCA and MPO-ANCA-associated vasculitis in order to verify whether inhibition of extra-cellular HMGB1 would result in clinical benefit. Phase I studies would be worthwhile to ascertain clinical safety of those agents and then optimal dose-regimen and efficacy in controlling disease activity would have to be tested in further phase II to III clinical trials. The use of agents that inhibit HMGB1 release from cells such as statins or metformin in animal models of PR3-ANCA and MPO-ANCA-associated vasculitis could demonstrate whether these agents have any effect on disease activity. Finally, further longitudinal studies should be performed to demonstrate whether chronic use of either agent (e.g. statins or metfomin) would be of benefit in controlling disease activity in different forms of systemic vasculitis.

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CHAPTER 10

Nederlandse samenvatting en discussie

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Nederlandse samenvatting en discussie (voor leken)

Systemische vasculitiden vormen een heterogene groep van ziekten die gekenmerkt worden door ontsteking van bloedvaten van verschillende aard en grootte. Als gevolg van dit ontstekingsproces treedt beschadiging op van de wand van bloedvaten wat kan leiden tot afstervan van het bloedvat ("fibrinoide necrose") en soms tot doorbraak van de wand van het bloedvat wat bloeding in de omgevende weefsels tot gevolg heeft met name wanneer kleine bloedvaten zijn aangedaan (de haarvaten en de kleine aders) [1,2]. Wanneer het ontstekingsproces middelgrote en grote slagaders betreft dan leidt dit proces vaak tot vernauwing en afsluiting van het bloedvat. Deze vernauwing en afsluiting treedt veelal op in zgn musculaire slagaders terwijl in elastische slagaders de vorming van aneurysma's (plaatselijke verwijding van slagaders) kan optreden. Daarnaast kan bij systemische vasculitis ook ontsteking buiten bloedvaten optreden terwijl het ontstekingsproces ook kan leiden tot algemene symptomen van ontsteking zoals koorts en vermoeidheid [3,4].

Alarmines, ook wel "danger associated molecular patterns (DAMPs)" genoemd, zijn stofjes die kandidaat lijken te zijn om het onderliggende ontstekingsproces te weerspiegelen. Dergelijke stofjes worden dan biomerkers van het ontstekingsproces genoemd. Als zodanig zouden ze van betekenis kunnen zijn voor de diagnose van systemische vasculitis of om de prognose hiervan te bepalen. Alarmines zijn eiwitten met meerdere functies die een belangrijke rol spelen binnen in de cel. Ze kunnen echter ook vrijkomen uit cellen die afsterven of door bepaalde cellen, zoals cellen van het afweersysteem of slijmvliescellen, worden uitgescheiden. Eenmaal buiten de cel activeren deze alarmines cellen van het zgn aangeboren afweersysteem door binding aan receptor-moleculen op deze cellen, zoals de zgn Toll-like receptors (TLRs). De best gekaraktiseerde alarmines zijn de volgende

eiwitten: high mobility group box 1 (HMGB1), de S100 eiwitten en de heat shock proteins (HSPs) [5].

De rol van HMGB1 is uitvoerig onderzocht in systemische ontstekings- en autoimmuunziekten, in kanker, atherosclerose en in sepsis [6-8]. Toen we begonnen met het huidige onderzoek hebben we eerst een literatuuroverzicht geschreven over de rol van HMGB1 bij vaataandoeningen met inbegrip van vasculitis en atherosclerose (**hoofdstuk 2**). Op dat moment waren maar enkele studies bekend waarin transsectioneel de gehaltes van HMGB1 in het bloed waren bepaald, met name bij patiënten met ANCA-geassocieerde vasculitis (AAV) [9-11] en bij de ziekte van Kawasaki (KD) [12,13]. Bij AAV werd gevonden dat de gehaltes van HMGB1 in het bloed hoger waren bij patiënten met granulomatosis met polyangiitis (GPA) met aktieve ziekte dan bij patiënten bij wie de ziekte niet aktief was en bij gezonde controles. Bij patiënten met microscopische polyangiitis (MPA) werd echter geen verschil gevonden tussen patiënten met aktieve ziekte en patiënten bij wie de ziekte in remissie was en bij gezonde controles [9]. Een andere studie van dezelfde groep onderzoekers liet zien dat de gehaltes van HMGB1 hoger waren bij patiënten met GPA die voornamelijk granulomateuze ontstekingen hadden dan bij GPA patiënten die voornamelijk vasculitis hadden. Er werd een positieve correlatie gevonden tussen het gehalte aan HMGB1 in het bloed en de hoeveelheid granulomateus weefsel in de longen [10]. Beide studies maakten gebruik van een ELISA methode om het gehalte aan HMGB1 te meten. Bruchfeld en collega's gebruikten een eigen Western blot techniek om HMGB1 in serum te meten bij patiënten met AAV en zij toonden aan dat patiënten bij wie in het nierbiopt glomerulonefritis aanwezig was het gehalte van HMGB1 in het serum hoger was dan bij patiënten bij wie het nierbiopt geen afwijkingen vertooonde. Bovendien vonden zij dat het gehalte van HMGB1 significant daalde wanneer er een remissie van de ziekte optrad. Hoewel de gehaltes van HMGB1 bij patiënten met AAV hoger waren dan bij

gezonde controles, was er geen verschil in deze waarden tussen patiënten met GPA, MPA en eosinofiele granulomatosis met polyangiitis (EGPA) [11].

Bij patiënten met atherosclerotische ziekte is gevonden dat ten tijde van een acute ischemische gebeurtenis (zoals kan optreden in de kransslagaderen of in de hersenvaten) hogere waarden van HMGB1 in het bloed aanwezig zijn dan bij gezonde controles. Bovendien werd gevonden dat hogere waarden van HMGB1 gepaard gingen met een slechte afloop bij patiënten met een acuut coronair syndroom. In atherosclerotische lesies is de expressie van HMGB1 in de kernen en het cytoplasma van macrofagen en gladspiercellen toegenomen in vergelijking met normale slagaders. Bij studies met experimentele modellen van atherosclerose bleek dat HMGB1 niet alleen betrokken is bij de versterking van de ontstekingsreaktie maar ook bij de herstelfase na een periode van ischemie [14].

In **hoofdstuk 4** beschrijven wij een longitudinale studie waarbij gehaltes van HMGB1 in het bloed van patiënten met AAV gemeten zijn bij het begin van de ziekte,bij het begin van de remissie en wat later in deze fase,en direkt voor en tijdens een terugval van de ziekte. In deze zelfde studie hebben wij bij patiënten met AAV gekeken of er ook antistoffen tegen HMGB1 aanwezig waren ten tijde van aktieve ziekte. Bij het begin van de ziekte verschilden de waarden van HMGB1 niet tussen patiënten met AAV en gezonde controles. Echter,verhoogde waarden van HMGB1 werden wel gevonden bij patiënten zonder betrokkenheid van de nier bij het vasculitis proces. Tevens werd er een positieve correlatie gevonden tussen tussen de waarden van HMGB1 in het serum en de waarden van het C-reaktieve proteine (CRP) terwijl er een negatieve relatie bestond tussen deze HMGB1 waarden en de 24-uurs uitscheiding van eiwit in de urine. Slechts 12.5% van de patiënten met AAV hadden antistoffen tegen HMGB1 in het bloed; de gemiddelde hoogte van HMGB1 antistoffen verschilde echter niet tussen de totale groep van AAV patiënten en de gezonde controles [15]. In tegenstelling

hiermee hebben patiënten met SLE hogere gehaltes van HMGB1 antistoffen in het bloed dan gezonde controles,vooral patiënten met SLE die nierbetrokkenheid hebben [16]. Verder beschrijven wij in dit hoofdstuk dat dat de waarden van HMGB1 niet stijgen voorafgaande aan een opvlamming van de ziekte en dat veranderingen,met name toename van HMGB1 waarden, niet voorspellend zijn voor de ontwikkeling van een opvlamming. We concluderen daarom dat HMGB1 geen geschikte biomerker is voor de aktiviteit van het ziekteproces bij AAV en dat nierbetrokkenheid samen gaat met lagere waarden van HMGB1 [15].

In **hoofdstuk 5** onderzochten we of de gehaltes van HMGB1 en van de oplosbare receptor voor “advanced glycation end products” (sRAGE) in het serum voorspellende waarde hebben voor de aanwezigheid van subklinische atherosclerose in de halsslagaders bij patiënten met GPA. Vanwege de gevonden associatie tussen hogere HMGB1 gehaltes in het serum en subklinische atherosclerose in de kransslagaders bij patiënten met en zonder diabetes in vergelijking met personen zonder afwijkingen aan de kransslagaders [17] veronderstelden wij dat er een associatie zou kunnen zijn tussen de gehaltes van HMGB1 en sRAGE in het serum enerzijds en atherosclerose in de halsslagaders anderzijds bij patiënten met GPA. Wij vonden,zoals beschreven in **hoofdstuk 5**, dat er geen verschillen waren tussen patiënten met GPA en gezonde controles voor wat betreft de aanwezigheid van atherosclerotische plaques en de dikte van de intima-media laag (IMT) van de halsslagaders terwijl er ook geen verschillen waren in de gehaltes van HMGB1 en sRAGE in het serum. Ten tijde van dit onderzoek waren alle patiënten met GPA in remissie. De gehaltes van sRAGE in het serum waren negatief gecorreleerd met de IMT waarden terwijl er geen correlatie bestond tussen de gehaltes van HMGB1 in het serum en het aanwezig zijn van subklinische atherosclerose in de halsslagaders. Patienten met GPA die statines of prednisolon gebruikten hadden significant

lagere waarden van HMGB1 in het serum dan patiënten die deze geneesmiddelen niet gebruikten.

Statines hebben ontstekingsremmende eigenschappen aangezien zij de ontstekingsbevorderende effecten van cytokines op endotheelcellen tegen gaan [19]. Bovendien is gebleken dat het gebruik van statines leidt tot lagere gehaltes van HMGB1 in experimentele modellen van atherosclerose en bij patiënten met hyperlipidemie [20,21]. Daarom hebben wij in **hoofdstuk 5** tevens onderzocht of atorvastatine,een veel gebruikt statine, het vrijkomen van HMGB1 uit endotheelcellen van menselijke navelstrengaders (HUVEC) die geactiveerd worden met lipopolysaccharide (LPS) kan tegen gaan.LPS leidde tot een langzaam vrijkomen van HMGB1 uit HUVEC met een maximum na 24 uur.We bepaalden ook de waarden van interleukine (IL)-8 in de bovenstaande vloeistof van de endotheel kweken om er zeker van te zijn dat de endotheelcellen inderdaad geactiveerd zijn door LPS. Wanneer we de HUVEC voorafgaande aan de activatie met LPS incubeerden met atorvastatine dan waren de gehaltes van HMGB1 en IL-8 lager in de bovenstaande vloeistof. We concludeerden hieruit dat, hoewel er geen relatie werd gevonden tussen de gehaltes in het serum van HMGB1 en het aanwezig zijn van atherosclerose in de halsslagaders bij patiënten met GPA, het gebruik van statines invloed heeft op de gehaltes van HMGB1 in het serum en op het vrijkomen van HMGB1 uit geactiveerde endotheelcellen. Deze bevindingen doen vermoeden dat statines additionele ontstekingsremmende effecten hebben [18].

Een ander aspect van de rol van HMGB1 als biomerker in AAV is het aantonen van HMGB1 in de urine van patiënten met AAV die een aktieve nierontsteking hebben. In **hoofdstuk 4** beschrijven we dat de gehaltes van HMGB1 in het serum van patiënten met AAV en aktieve nierontsteking ten tijde van de presentatie van hun ziekte niet verschilden van de gehaltes bij gezonde controles terwijl de patiënten met AAV zonder aktiviteit van de ziekte

in de nieren hogere gehaltes van HMGB1 in hun serum hadden dan gezonde controles [15]. We veronderstelden dat dit mogelijk het resultaat is van verlies van HMGB1 in de urine bij patiënten met aktiviteit van de ziekte in de nieren wat zou leiden tot lagere gehaltes in het serum in vergelijking met patiënten zonder aktiviteit van de ziekte in de nieren. Een recent beschreven onderzoek van onze groep heeft laten zien dat patiënten met SLE die aktiviteit van de ziekte in de nieren hebben hogere gehaltes van HMGB1 in de urine hebben dan SLE patiënten zonder aktieve nierziekte en gezonde controles [22]. Daarom besloten we ook bij patiënten met AAV de gehaltes van HMGB1 in de urine te meten en de resultaten hiervan staan beschreven in **hoofdstuk 6**. Bovendien onderzochten we, naast de gehaltes van HMGB1 in de urine, de aantallen van CD4-positieve T-cellen en CD4-positieve effector geheugen T-cellen in het perifere bloed en in de urine bij deze patiënten. Dit deden we aangezien eerdere studies effecten hebben laten zien van HMGB1 op de proliferatie en polarisatie van T-cellen terwijl een recente studie van onze groep aantoonde dat het aantal CD4-positieve effector geheugen T-cellen toegenomen is in de urine van patiënten met AAV en aktieve ziekte in de nieren in vergelijking met patiënten bij wie de ziekte rustig was en patiënten bij wie de nieren niet mee deden in het ziekteproces [14,23].

Allereerst hebben we een overzichtsartikel geschreven over de rol van mononucleaire cellen, in het bijzonder CD4-positieve effector geheugen T-cellen, in de pathogenese van AAV wat weergegeven is in **hoofdstuk 3** [24]. Bij GPA wordt een expansie gezien van CD4-positieve T-cellen binnen de populatie van effector geheugen T-cellen en de meerderheid van de T-cellen die aanwezig zijn in de longen en de glomeruli van de nieren vertonen het fenotype van geheugen cellen. Echter, ten tijde van aktieve ziekte is er een afname van deze CD4-positieve effector geheugen cellen in het bloed wat het gevolg kan zijn van migratie van deze cellen naar de zieke weefsels [24].

In **hoofdstuk 6** beschrijven we de resultaten van onderzoek naar de gehaltes van HMGB1 en de aantallen CD4-positieve T-cellen en CD4-positieve effector geheugen T-cellen in bloed en urine alsmede het gehalte van monocyt-chemoattractant eiwit-1 (MCP-1) in de urine bij patiënten met AAV en aktieve ziekte in de nieren. We vergeleken deze waarden met de waarden bij gezonde controles en bij een aantal van deze patiënten ook ten tijde van rustige ziekte (gemiddeld 36.2 maanden na het moment van aktieve ziekte). De gehaltes van HMGB1 in de urine waren hoger bij patiënten met aktieve nierontsteking dan bij gezonde controles. Bovendien daalden de waarden wanneer de ziekte rustig werd. Er werden geen associaties gevonden tussen de voor de verdunning gecorrigeerde waarde van HMGB1 in de urine (ratio van HMGB1 en kreatinine in de urine) enerzijds en het gehalte van HMGB1 in het serum,de aktiviteit van de ziekte (gemeten als de zogenaamde Birmingham Vasculitis Activity Score (BVAS),de 24-uurs uitscheiding van eiwit in de urine en de glomerulaire filtratie snelheid (GFR) anderzijds. De waarden van MCP-1 in de urine waren ook hoger bij patiënten met aktieve nierziekte in vergelijking met gezonde controles en deze waarden daalden significant wanneer de ziekte rustig werd. In tegenstelling tot HMGB1 was de voor de verdunning gecorrigeerde waarde van MCP-1 in de urine (ratio van MCP-1 en kreatinine) wel geassocieerd met de aktiviteit van de ziekte (BVAS), maar niet met de voor de verdunning gecorrigeerde waarden van de aantallen CD4-positieve T-cellen en CD4-positieve effector geheugen T-cellen in de urine [25].

Ook bij patiënten met vasculitis van de grote vaten (LVV),met name bij patiënten met Takayasu arteritis (TA) en reuscel arteritis (GCA) onderzochten wij de gehaltes van HMGB1 in het serum zoals beschreven in **hoofdstuk 7**. Deze studie werd zowel in Brazilie als in Nederland verricht aangezien TA meer in Brazilie voorkomt en GCA meer in Nederland. De patiënten met GCA in deze studie kwamen uit Nederland en de patiënten met TA uit Brazilie. De

patiënten met GCA werden onderzocht bij het begin van hun ziekte (voordat begonnen was met behandeling met corticosteroiden), 3 en 12 maanden na het begin van de ziekte, en tijdens een terugval van de ziekte. De gehaltes van HMGB1 bij het begin van de ziekte verschilden niet van die bij gezonde controles (van dezelfde leeftijd en geslacht) en ook in het beloop van de ziekte werden geen verschuivingen in de waarden waargenomen. Er werd geen associatie gevonden tussen de waarden van HMGB1 en die van de acute fase eiwitten. Ook werd er geen associatie gevonden tussen de gehaltes van HMGB1 en de aanwezigheid van polymyalgia reumatica,systemische manifestaties van de ziekte en het al of niet terugvallen van de ziekte.

Bij patiënten met TA onderzochten we sera in een transsectionele studie. Er bestond geen verschil in de waarden van HMGB1 tussen patiënten met aktieve ziekte, patiënten in remissie en gezonde controles. Patienten met TA die statines gebruikten hadden significant lagere waarden van HMGB1 in hun serum in vergelijking met patiënten die geen statines gebruikten. Er bestonden geen associaties tussen de gehaltes van HMGB1 in het serum enerzijds en de gehaltes aan acute fase eiwitten, de mate van aktivitiet van de ziekte, de aanwezigheid van voorgaande ischemische gebeurtenissen, en het gebruik van geneesmiddelen zoals prednison en zgn biologics. Lineaire regressie analyse liet zien dat het gebruik van statines onafhankelijk geassocieerd was met lagere gehaltes van HMGB1 in het serum. Deze effecten van statines op de gehaltes van HMGB1 zijin eerder waargenomen bij patiënten met GPA (**hoofdstuk 5**), patiënten met hyperlipidemie en in een experimenteel model van atherosclerose [18,20,21]. Met betrekking tot de aktiviteit van de ziekte bij patiënten met TA bleek in een linaire regressie analyse dat de aktiviteit van de ziekte geassocieerd was met de zgn Indian Takayasu Clinical Activity Score (ITAS-2010) en met de waarden van de bezinkingssnelheid van het bloed (ESR), maar niet met de waarden van CRP

of die van HMGB1 in het serum. Bij multivariatie logistische regressie analyse bleek uitsluitend de ITAS-2010 score onafhankelijk geassocieerd te zijn met de aktiviteit van de ziekte bij patiënten met TA.

De waarden van HMGB1 in het serum waren significant hoger bij patiënten met GCA bij het begin van de ziekte dan bij patiënten met TA ten tijde van aktieve ziekte. Zelfs wanneer alleen patiënten met GCA en TA die statines gebruikten met elkaar vergeleken werden, bleven de waarden van HMGB1 in het serum hoger bij patiënten met GCA. Het zou kunnen zijn dat dit verschil wijst op een invloed van de leeftijd op het gehalte van HMGB1 aangezien de patiënten met GCA significant ouder zijn dan de patiënten met TA.

In **hoofdstuk 8** beschrijven we ons onderzoek naar de waarden van HMGB1 in het serum van patiënten met de ziekte van Behcet (BD), een vorm van vasculitis waarbij de grootte van de aangedane vaten varieert [26]. Patienten met BD bleken significant hogere waarden van HMGB1 in het serum te hebben dan gezonde controles. Er werd echter geen verschil gevonden tussen patiënten met aktieve ziekte en patiënten in remissie. Er was ook geen correlatie tussen de waarden van HMGB1 in het serum en de aktiviteit van de ziekte gemeten met de vereenvoudigde Braziliaanse versie van de zgn Behcet's disease current activity form (BR-BDCAFs), een gevalideerd instrument om de aktiviteit van de ziekte van Behcet te meten. Ook werd er geen associatie gevonden tussen de gehaltes van HMGB1 en specifieke manifestaties van BD of de wijze van behandeling.

Tijdens het onderzoek dat beschreven is in dit proefschrift zijn twee studies gepubliceerd waarin HMGB1 is gemeten bij patiënten met vasculitis [27,28]. In een studie betroffen dit patiënten AAV en in de andere studie patiënten met IgA-vasculitis. In de eerste studie werden de waarden van HMGB1 gerapporteerd bij patiënten met GPA en MPA uit China [27]. De

waarden van HMGB1 in plasma waren hoger bij patiënten met AAV en aktieve ziekte dan bij patiënten in remissie en bij gezonde controles. Een significante maar zwakke correlatie werd gevonden tussen de gehaltes van HMGB1 in het plasma enerzijds en de waarden van CRP, BVAS, het serum kreatinine gehalte en de geschatte glomerulaire filtratie snelheid anderzijds. In deze studie werden tevens aanwijzigingen gevonden dat de gehaltes van HMGB1 geassocieerd waren met de aanwezigheid van granulomateuze manifestaties van de ziekte aangezien patiënten met AAV en PR3-ANCA hogere gehaltes van HMGB1 hadden dan patiënten met AAV en MPO-ANCA; dit was onafhankelijk van de waarden van kreatinine in het serum. Na intraveneuze of orale behandeling met cyclofosfamide daalden de waarden van HMGB1 significant [27].

De gehaltes van HMGB1 in het serum bleken in de andere studie hoger bij patiënten met IgA-vasculitis, patiënten met allergische vasculitis en patiënten met urticariele vasculitis dan bij gezonde controles. Endotheelcellen in de aangedane huid bij patiënten met IgA-vasculitis lieten een overvloedige expressie van HMGB1 in het cytoplasma zien. Stimulatie van een humane microdermale endotheelcellijn met recombinant HMGB1 resulteerde in een toename van de uitscheiding van TNFα en IL-6 in de bovenstaande vloeistof [28].

Conclusies

Gehaltes van HMGB1 in het serum zijn geen betrouwbare biomerker om de aktivitiet van de ziekte vast te stellen bij patiënten met verschillende vormen van vasculitis zoals AAV en LVV. Bij patiënten met AAV worden de serum spiegels van HMGB1 in belangrijke mate beïnvloed door de aanwezigheid van granulomateuze ontsteking terwijl patiënten met aktieve

nierontsteking hogere waarden van HMGB1 in de urine laten zien die correleren met de aantallen CD4-positieve T-cellen en CD4-positieve effector geheugen T-cellen in de urine maar niet met de waarden van MCP-1 in de urine. Het bereiken van remissie leidt tot een significante daling van de waarden van HMGB1 in de urine. Bij patiënten met GPA in remissie en bij patiënten met TA dalen de waarden van HMGB1 in het serum onder therapie, waaronder het gebruik van statines. Atorvastatine verhindert het vrijkomen van HMGB1 uit HUVEC die gestimuleerd worden met LPS. Bij patiënten met BD zijn de waarden van HMGB1 in het serum hoger dan bij gezonde controles onafhankelijk van de mate van ziekte.

Toekomstig onderzoek

HMGB1 speelt inderdaad een rol bij verschillende pathologische processen met inbegrip van sepsis en steriele ontsteking [6]. Er ontbreekt echter nog veel kennis over de invloed van verschillende post-translationele factoren op zijn functies, met inbegrip van de redox-status van HMGB1, de mate van acetylering en het samengaan met andere moleculen [7]. Verschillende in vitro en in vivo experimentele studies hebben laten zien dat remming van HMGB1 een potentieel therapeutische benadering kan zijn in de toekomst [6-8].

Het bewijs dat het gebruik van statines een potentiele therapie kan zijn om het gehalte van HMGB1 bij patienten met atherosclerosis te doen dalen kwam aanvankelijk uit in vitro studies die lieten zien dat fluvastatine de expressie van intracellulair HMGB1 kon laten afnemen in een monocyten-lymfoom cellijn (U937) die gestimuleerd werd met een hyperlipidemisch serum [20]. Het vrijkomen van HMGB1 in het supernatant van humaan navelstreng endotheel (HUVEC) geactiveerd door LPS werd ook

tegengegaan door atorvastatine. Deze bevindingen werden bevestigd in een in vivo model van hyperlipidemie in Syrische hamsters waarbij fluvastatine resulteerde in een daling van het serum HMGB1 gehalte,terwijl ook bij patienten met hyperlipidemie en patienten met GPA behandeling met statines een daling van het serum HMGB1 gehalte liet zien [18,20]. Er zijn echter geen data beschikbaar vanuit klinische trials die bewijzen dat afname van het gehalte aan HMGB1 ook leidt tot een afname van cardiovasculaire accidenten.

Metformine is een ander middel dat het vrijkomen van HMGB1 uit een met LPS gestimuleerde macrofagen cellijn (RAW 264.7) in vitro doet tegengaan. Metformine verhindert de translocatie van HMGB1 vanuit de kern naar het cytoplasma en leidt daardoor ook tot een afname van de extracellulaire hoeveelheid HMGB1 [29]. Bovendien gaat metformine ook de door hyperglycemie geinduceerde expresie van HMGB1 in cardiomyocyten tegen op een dosis-afhankelijke wijze en beschermt zodoende tegen beschadiging van deze cellen tijdens verhoogde glucose spiegels [30]. Er zijn echter nog geen dierexperimentele of humane studies beschreven die laten zien dat metformine het serum gehalte van HMGB1 doet afnemen en dat deze afname klinisch van voordeel is.

Behandeling gericht op HMGB1 heeft echter wel therapeutische effecten laten zien in verschillende diermodellen [6-8]. Neutraliserende antilichamen tegen HMGB1 en het recombinante A box domein van HMGB1 zijn de meest gebruikte antagonisten van HMGB1 in diermodellen terwijl in enkele studies ook antistoffen tegen RAGE en thrombomoduline gebruikt zijn (tabel 1) [8]. Deze agentia zijn echter nog niet getest op effectiviteit en veiligheid in humane studies.

Tabel 1 - Experimentele modellen van een behandeling gericht op HMGB1 [overgenomen uit referentie 8].

Model	Agentia
Artritis	Anti-HMGB1 antistoffen
	Thrombomoduline
	Recombinant HMGB1 A box
Neuropathische pijn	Anti-HMGB1 antistoffen
Endotoxemie	Anti-HMGB1 antistoffen
	Thrombomoduline
Sepsis	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
	Anti-RAGE antistoffen
Pancreatitis	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
Colitis	Anti-HMGB1 antistoffen
Hemorrhagische shock	Anti-HMGB1 antistoffen
Stroke	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
Epilepsie	Recombinant HMGB1 A box
Ischemie-reperfusie schade	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
Atherosclerose	Anti-HMGB1 antistoffen
Myocard infarct	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
Transplantatie	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
Respiratoire ziektes	Anti-HMGB1 antistoffen
	Recombinant HMGB1 A box
Paracetamol geinduceerde leverschade	Anti-HMGB1 antistoffen

HMGB1-high mobility group box-1; RAGE-receptor for advanced glycation end products.

Bij systemische vaaculitiden zou remming van HMGB1 van belang kunnen zijn in de volgende situaties: granulomateuze ontsteking bij GPA, nierbetrokkenheid bij AAV, in de acute fase van KD, bij IgA vasculitis en bij patienten met BD. Allereerst moeten echter pre-klinische studies worden verricht met HMGB1 antagonisten (zoals neutraliserende antistoffen tegen HMGB1 en het recombinante A box domein van HMGB1) in diermodellen van PR3-ANCA en MPO-ANCA geassocieerde vasculitis teneinde na te gaan of remming van extracellulair HMGB1 inderdaad een klinisch effect heeft. Fase 1 studies zijn vervolgens nodig om de veilighied van deze middelen te waarborgen met daarna fase 2 en fase 3 studies om de optimale dosis nodig om de ziekte aktivitiet te controleren vast te stellen. Van agentia die het vrijkomen van HMGB1 uit cellen verhinderen zoals statines en metformine kan dan in diermodellen van PR3-ANCA en MPO-ANCA geassocieerxde vasculitis worden vastgesteld of deze middelen enig effect hebben op de ziekte aktiviteit. Tenslotte moet worden nagegaan of het chronisch gebruik van dergelijke middelen van nut is om verschillende vormen van vasculitis in remissie te houden.

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List of publications

Alexandre Wagner Silva de Souza

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Catoggio LJ, Soriano ER, Imamura PM, Wojdyla D, Jacobelli S, Massardo L, Chacón Díaz R, Guibert-Toledano M, Alvarellos A, Saurit V, Manni JA, Pascual-Ramos V, **Silva de Souza AW**, Bonfa E, Tavares Brenol JC, Ramirez LA, Barile-Fabris LA, De La Torre IG, Alarcón GS, Pons-Estel BA; on behalf of Grupo Latino Americano De Estudio del Lupus (GLADEL). Late-onset systemic lupus erythcematosus in Latin Americans: a distinct subgroup? Lupus. 2014 Dec 11. [Epub ahead of print]

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