CHAPTER 9

# Summary and general discussion

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## 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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