CHAPTER 10

SUMMARY, DISCUSSION, AND FUTURE PERSPECTIVES
SUMMARY AND DISCUSSION
Systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and Wegener’s granulomatosis (WG) are associated with a significantly increased prevalence of cardiovascular disease (CVD) compared to age- and sex-matched controls. Many risk factors are involved in the pathogenesis of atherosclerosis, the major underlying cause of CVD. In this thesis, prevalence of premature atherosclerosis and the contribution of traditional and non-traditional risk factors, as reviewed in chapter 1, have been investigated. Different methods have been used to assess premature atherosclerosis, including measurement of intima-media thickness (IMT) and assessment of endothelial dysfunction. Furthermore, factors which might be involved in the development of atherosclerosis are described, such as accumulation of advanced glycation endproducts and the dysfunction of circulating endothelial progenitor cells.

INTIMA-MEDIA THICKNESS
IMT measured by ultrasound is an adequate marker for subclinical atherosclerosis, as it reflects atherosclerotic changes of the arterial wall. Carotid ultrasononography has been described as a non-invasive, validated and reliable technique, in which IMT of the carotid artery is determined, usually in the common carotid artery (CCA), as this segment can be assessed with high reproducibility. In this thesis, IMT of this segment was studied (chapters 2, 3, 7 and 8). However, the prevalence of atherosclerosis might be underestimated, since atherosclerotic lesions appear later in the CCA than in other segments, including internal carotid artery and bulb.

Systemic lupus erythematosus
In chapters 2 and 3, we showed that SLE patients have an increased IMT compared to controls, indicating accelerated atherosclerosis. In chapter 2 a cross-sectional study design was used, while chapter 3 describes a longitudinal study. The question remains which particular factors contribute most to this accelerated development of atherosclerosis. To answer this question, several studies as described in chapter 2 and 3 have been performed, showing that next to traditional also non-traditional, disease-related risk factors are involved, including disease duration, inflammation and damage.
Indeed, chronic low-grade systemic inflammation has been demonstrated to be associated with atherosclerosis. In autoimmune diseases increased levels of C-reactive protein (CRP) were found even during quiescent disease, reflecting presence of inflammation (chapters 2 and 3). Therefore, systemic inflammation could be a direct link between autoimmune disease and increased risk for CVD.

Other disease-related factors which might play a role in the development of atherosclerosis are the presence of autoantibodies and immunosuppressive treatment. Autoantibodies, directed to phospholipids or double stranded DNA, as present in SLE, have been hypothesized to directly activate the endothelium and, therefore, contribute to atherosclerosis. However, our studies as presented in chapters 2 and 3, as well as those of others, found no association between atherosclerosis and the presence of antiphospholipid antibodies or levels of antibodies against double stranded DNA.

Treatment, especially the use of steroids, might also influence the process of atherosclerosis. Steroid treatment is often believed to be atherogenic, because its adverse effects, including hypertension, diabetes mellitus and dyslipidemia, are all risk factors for CVD. However, steroid treatment could actually prevent atherosclerosis as well, because it suppresses inflammation, which is implicated in atherosclerosis. The results of studies about the effect of steroid treatment on atherosclerosis are conflicting, demonstrating a negative association, a positive association or no association between long-term treatment with steroids and atherosclerosis. In this thesis, no association between use of prednisolone and atherosclerosis was found in SLE patients. Clearly, the role of prednisolone treatment in development of CVD in SLE deserves further study.

In chapter 3, we describe that after follow-up of approximately three years mean progression of IMT was 0.012 ± 0.04 mm/year. This is increased compared to healthy controls in which a progression of mean IMT of the CCA was found of 0.001 mm/year. Thus, it appears that SLE patients have a much more accelerated progression than controls. The cause of this accelerated progression remains uncertain, because, except for age, no other predictors of progression of IMT could be determined over this relatively short interval. Others found that IMT was progressed in patients who had at baseline renal disease and active disease. Roman et al demonstrated that plaque progression was independently associated with older age at diagnosis, longer duration of SLE and higher homocysteine concentrations.
Wegener’s granulomatosis

In chapters 7 and 8 prevalence of subclinical atherosclerosis in WG patients is described and the involvement of traditional and non-traditional risk factors in this process is evaluated cross-sectionally (chapter 7) and longitudinally (chapter 8). IMT measurements were repeated after a follow-up of approximately six years in patients and controls.

In chapter 7, we showed that increased IMT was present in WG patients. As in SLE patients, this increased IMT could not be explained by the presence of traditional risk factors alone, suggesting that the disease itself contributes to the development of atherosclerosis.

To reveal the pathogenic mechanisms of this accelerated atherosclerosis, several non-traditional, disease-related risk factors were investigated, including inflammation and endothelial activation. In chapters 7 and 8, increased levels of CRP as a marker for systemic inflammation were found in WG patients with quiescent disease. Furthermore, endothelial activation markers were higher in WG patients compared to controls (chapter 8).

We also focused on vascular remodeling as reflected by levels of metalloproteinases (MMPs). MMPs have been demonstrated to be altered during atherosclerotic lesion progression and plaque disruption.\textsuperscript{15,16} In chapter 7, we found increased levels of MMP-3 and MMP-9 and their inhibitor TIMP-1 in WG patients. Whether this is a reflection of the increased prevalence of atherosclerotic changes of the vessel wall or of smouldering disease activity in these patients, as MMP levels might also be used as markers for disease activity, have to be clarified.\textsuperscript{17}

In chapter 8, IMT was longitudinally measured in WG. After follow-up of approximately six years, maximum IMT remained increased in patients compared to controls, as found at study entry. However, no increased progression of IMT was found in patients. Several explanations might be proposed. First, disease-related factors might be less prominent during follow-up. Indeed, parameters of disease activity, including cumulative BVAS, cumulative dose of prednisolone and mean CRP levels, were lower during follow-up compared to the period from diagnosis until study entry. Secondly, traditional risk factors for CVD were more aggressively treated during follow-up, as prevalence of dyslipidemia and high blood pressure decreased in patients during that period. Treatment and awareness of traditional risk factors of CVD in WG is comparable to the trend seen in other systemic autoimmune diseases. Furthermore, as
only the CCA was examined at entry, progression of IMT was determined only of this segment. This might result in underestimation of IMT progression, since atherosclerotic lesions appear earlier in other segments.

In conclusion, increased IMT (chapters 2 and 3) and enhanced progression of IMT (chapter 3) was found in SLE patients compared to controls, which could be only partly explained by the presence of traditional risk factors. Also, disease-related factors were revealed to play an important role in the development of accelerated atherosclerosis.

In WG patients, an increase in maximum IMT was found compared to controls (chapter 7 and 8). However, after follow-up no increased progression of IMT was found in WG patients (chapter 8). This might be explained by the fact that during follow-up disease activity was low and traditional risk factors were reduced.

**ENDOTHELIAL FUNCTION**

Endothelial dysfunction is seen as one of the earliest steps in the development of atherosclerosis. Therefore, it can be hypothesized that by assessing endothelial dysfunction patients with an increased risk to develop atherosclerosis can be selected in an early, still reversible phase.

The technique often used to determine endothelial dysfunction is the assessment of flow-mediated dilatation (FMD) in the brachial artery. However, this technique has a high intra-individual variability and demands major operator expertise. Furthermore, as SLE and WG are characterized by vasculitis of medium- to small-sized vessels, we expected that also smaller vessels are involved. In chapters 4 and 9, endothelial function of the microcirculation of the fingers was assessed using laser Doppler fluxmetry (LDF) in combination with iontophoresis of acetylcholine and sodium nitroprusside, resulting in the possibility to determine local vasodilator responses via endothelium-dependent and endothelium-independent pathways.

**Systemic lupus erythematosus**

In chapter 4, 42 SLE patients with quiescent disease were included to evaluate microvascular responses in the fingers using LDF. Twelve out of these patients had secondary Raynaud’s phenomenon, and in these patients both endothelium-dependent and endothelium-independent responses were significantly decreased, indicating
endothelial dysfunction. In patients without Raynaud’s phenomenon no differences in vascular responses were found compared to controls. These results are in contrast with studies using FMD, that showed reduced dilatation in SLE patients. These discrepancies in LDF compared to FMD might be related to methodological differences. First, different vascular beds are investigated in both methods. Whereas FMD measures the response to reactive hyperemia in the brachial artery, LDF measures the responses of the microcirculation. Secondly, acetylcholine-mediated vasodilatation involves nitric oxide, prostanoids and endothelium-derived hyperpolarizing factor, and FMD results from shear stress-induced nitric oxide production alone. Therefore, it might be hypothesized that endothelial dysfunction is expressed differently in resistance or microvascular vessels than in large vessels. Thus, no altered endothelial function of the microcirculation was found in SLE patients using LDF. This might indicate that the microcirculation is not affected and, therefore, LDF seems not to be the appropriate method to identify SLE patients with an increased risk to develop CVD.

Wegener’s granulomatosis

In chapter 9, we performed LDF measurements in quiescent WG patients in relation to IMT. We observed that the microvascular vasodilator response was increased in patients compared to controls. After exclusion of patients with manifest atherosclerosis or with an increased IMT, these differences were even more pronounced, indicating abnormal endothelial function of the microcirculation in WG. The increased response to acetylcholine in our patients is consistent with the results of a small study on 10 patients (7 with ANCA-associated systemic vasculitis) in which an enhanced vasodilator response to acetylcholine in resistance vessels using forearm plethysmography was demonstrated. Such an increased microvascular vasodilatory response has been reported in other conditions associated with an increased risk of atherosclerosis, such as preeclampsia. We propose that low-grade inflammation, which is present in quiescent disease and is characterized by a relative overproduction of endothelial-derived vasodilatory substances, results in an enhanced vasodilator response to acetylcholine. Longitudinal studies should be performed to elucidate whether this increased response to acetylcholine is a predictor of CVD in WG.
ADVANCED GLYCATION ENDPRODUCTS
Recently, the accumulation of advanced glycation endproducts (AGEs) has been recognized as a contributing factor in the progression of atherosclerosis. AGEs are involved in the development of atherosclerosis via different pathways. Through interaction with their major cellular receptor (RAGE), AGEs may prime monocytes and endothelial cells, thereby amplifying pro-inflammatory mechanisms in atherosclerotic plaque formation. Although the primary causal factor leading to the formation of AGEs in diabetes is chronic exposure to high levels of glucose, it has been shown that AGEs can be formed in other circumstances, such as in inflammation and renal failure. In systemic autoimmune diseases inflammation of blood vessels is common and increased AGE formation at these sites can be expected.

In chapter 5, accumulation of AGEs was cross-sectionally measured in SLE patients and controls. Tissue AGEs accumulation can be assessed as skin autofluorescence, following the principles of the AGE-reader, which is a validated and non-invasive technique. As expected, we found increased AGEs in SLE compared to controls, as described in chapter 5. Comparable with previous reports, AGE accumulation was correlated to age and levels of creatinine. Also, a positive correlation was found between accumulation of AGEs and IMT, indicating that these AGEs may constitute a link between inflammation and development of atherosclerosis in SLE. Interestingly, we found a moderate, but significant correlation between AGEs and disease duration, which remained significant in multivariate analysis. This might suggest that the disease itself may play an important role in the increased accumulation of AGEs. This is supported by the fact that AGEs also were positively correlated to damage due to SLE.

CIRCULATING PROGENITOR CELLS
A healthy endothelium is important to prevent atherogenesis. Therefore, disturbance in the equilibrium between vascular injury and vascular repair might be followed by atherosclerosis. Recent insights suggest that injured endothelium can be repaired by circulating bone marrow-derived progenitor cells. In chapter 6, we describe our findings of a reduced number and a partly impaired function of circulating progenitor cells (CPCs) in SLE patients.

The decrease of the number of CPCs, defined as CD34/CD133 double positive cells, in SLE patients is also seen in other diseases with vascular inflammation, such as
rheumatoid arthritis and chronic renal failure. Furthermore, function of CPCs was impaired. CPCs of SLE patients had a decreased capacity to form colony forming units and were less viable. However, after 14 days of culture no significant differences were observed in functionality of patient and control CPCs.

An explanation for the decreased quantity and quality of CPCs in SLE might be chronic exposure to inflammatory cytokines, resulting in reduced mobilization and increased apoptosis. Indeed, we found a reduced mRNA expression of caspase 8L, a ligand which protects against apoptosis, in the peripheral blood-derived mononuclear cells fractions of SLE patients. Interestingly, after culturing for 14 days, we observed an increase of caspase 8L expression in the cultured CPCs of SLE patients. Apparently, during culture, CPCs with better protection against apoptosis remained. Whether this indicates the existence of subsets of CPCs that intrinsically differ in susceptibility for apoptosis or that CPCs can adapt to culture conditions remains to be investigated.

In conclusion, culturing of CPCs in defined conditions for 14 days resulted in near to normal functionality of these cells. We, therefore, propose that CPCs of SLE patients are not intrinsically impaired in their functionality, but are impaired due to extrinsic factors.

CONCLUSION
The pathogenesis of accelerated atherosclerosis in systemic autoimmune diseases is very complex. In this thesis, several methods have been used to measure the extent of atherosclerosis in different phases of its development in patients with systemic autoimmune diseases. As a marker for subclinical atherosclerosis, IMT was measured, which was increased in SLE and WG patients compared to controls. Traditional risk factors play an important role in this process. However, these factors cannot fully explain the increased IMT, and non-traditional, disease-related risk factors, including endothelial activation, accumulation of AGEs, and decreased quantity and quality of CPCs, are contributing as well.

Measurement of endothelial dysfunction, as one of the earliest steps in this process, was performed using LDF in combination with iontophoresis. In WG patients, this technique showed abnormal vascular responses compared to controls, as endothelial-dependent vasodilatation was increased. However, LDF did not differ between SLE patients and controls. Furthermore, the presence of Raynaud’s phenomenon decreased both endothelial-dependent and -independent vasodilatory responses.
More promising is the accumulation of AGEs, which is increased in SLE. These glycated compounds were revealed to correlate to IMT, and are possibly reflecting enhanced vascular damage and increased risk to CVD. Furthermore, measurement of AGE accumulation in the skin is a non-invasive and simple method, which can easily be implicated in the clinic.

The results of this thesis have clinical implications concerning the prevention of CVD in systemic autoimmune diseases. Traditional risk factors for CVD should be identified, a total cardiovascular risk score should be calculated, and traditional risk factors should be corrected when indicated. This should imply that in translating the conventional risk scores into treatment guidelines, the additional risk of these diseases should be taken into account. For example, in recent guidelines on identification and treatment of increased CVD risk in patients with rheumatoid arthritis, it has been suggested to multiply the 10-year CVD risk score by two and then use the current treatment cut-off values to decide about starting or intensifying treatment of traditional risk factors. Clinically easily applicable methods such as IMT and measurement of AGEs may be used as additional risk qualifiers to identify those patients with risk scores in the “grey zone” (around 10% 10-year risk) who deserve treatment of risk factors.

Furthermore, the disease itself should be treated as adequately as possible, because disease activity results in ongoing systemic inflammation and endothelial activation, resulting in accelerated development of atherosclerosis.

FUTURE PERSPECTIVES

Research of CVD in systemic autoimmune diseases has extensively emerged since Urowitz et al. reported in 1976 a bimodal pattern of mortality in SLE, consisting of disease-related mortality early and cardiovascular mortality late in the course of the disease. Since then, many studies have confirmed the increased prevalence of CVD in systemic autoimmune diseases and demonstrated that, besides traditional factors, disease-related factors play a crucial role in this process. However, which particular factors are the most important and would predict CVD remained unsolved. In this thesis, it appeared that although IMT values are slightly increased in patients and endothelial activation markers are elevated, the presence of CVD is not that increased as would have been expected based on the data of Urowitz et al. That study was published in 1976 and describes a cohort of patients in the seventies. We suggest that
due to improved treatment of the disease itself, resulting in lower cumulative disease activity, and awareness and treatment of traditional risk factors of CVD, the prevalence of CVD has been decreased. Longitudinal studies including a large cohort of patients are therefore required to determine the prevalence of CVD and to investigate underlying mechanisms.

Another challenge is the choice of endpoint for these studies. Although a cardiovascular event would be the ultimate endpoint, an extraordinary long period of follow-up would be needed to achieve enough statistical power to perform adequate analyses in the relatively small number of patients with systemic autoimmune diseases. Therefore, surrogate markers for CVD are needed to replace CVD events as primary endpoint. Carotid ultrasonography, measuring IMT, has been shown to be an adequate and non-invasive method. It is still unclear which segment of the carotid artery is the best indicator to predict CVD. Benedetto et al recently showed that only new plaque formation in the bulbar area, and not IMT, was independently correlated to cardiovascular events in patients with end-stage renal disease. Therefore, it might be suggested to measure progression of IMT in all segments and also include plaque score in further studies.

Nevertheless, IMT reflects atherosclerotic lesions of the vessel wall, which is a rather late and irreversible phase in the development of atherosclerosis. In clinical practice, it would be preferable to have the possibility to select patients with an increased risk for CVD in a more reversible phase, such as the occurrence of endothelial dysfunction. The method used should be non-invasive, easily clinically applicable and reliable. In this thesis, LDF measurements could not discriminate SLE patients from controls, and, thus, this is not the method which could be used for this purpose in SLE. In WG patients without manifest atherosclerosis or an increased IMT microvascular vasodilator response was increased compared to controls. Longitudinal studies should be performed to elucidate whether this altered endothelial function as measured by LDF is a predictor of CVD in WG. Another method to measure endothelial function might be the assessment of small artery elasticity using pulse-wave analysis. Impaired or low small artery elasticity has been shown to be associated with and predictive of vascular events in the general population. Pulse-wave analysis is more readily available and well-tolerated.

Measurement of accumulation of AGEs might also be a possible endpoint to predict CVD. These AGEs have been demonstrated to be involved in atherosclerosis and to
predict CVD in diabetic patients and patients with renal failure. Furthermore, AGEs can be measured using the AGE-reader, which is an easy, simple, non-invasive and rapid method. Therefore, longitudinal studies combining all these different techniques would be recommended.

A pathogenic mechanism of accelerated development of atherosclerosis in systemic autoimmune disease might be the reduced number and impaired function of circulating progenitor cells (CPCs) as demonstrated in SLE. We proposed that CPCs of SLE patients are not intrinsically impaired in their functionality, but are impaired due to extrinsic factors. This hypothesis deserves further research to consider the possibility for cell therapy purposes, in which CPCs are firstly cultured before they are re-administered to the patient. Also, it would be worthwhile to investigate whether amelioration of the *in vivo* environment would be necessary to improve the CPC biology of SLE patients and open the way to prevent the accelerated atherosclerosis found in these patients.

Systemic autoimmune diseases are characterised by a relapsing course. Hypothetically, endothelial activation and dysfunction occur even more during these episodes of active disease. Hence, it would be interesting to monitor endothelial activation markers, endothelial dysfunction, AGEs and CPCs during and after active disease.

Also, as it is no point of discussion that disease activity and inflammation in systemic autoimmune diseases are implicated in atherosclerosis, more *in vitro* studies would be needed to elucidate the pathogenic mechanisms of this accelerated atherosclerosis. For example, the effects of AGEs on endothelial cells or the presence of anti-endothelial cell antibodies in serum of patients with systemic autoimmune diseases might be interesting topics of future research.
REFERENCES


