Systemic lupus erythematosus

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The studies presented in this thesis describe two major aspects of SLE: pathophysiology and long-term outcome. SLE is a chronic, heterogeneous, and unpredictable disease, which typically affects individuals during their early adult life. As such, SLE has a major impact and burden on the patients suffering from it.

By studying pathophysiology, a better understanding of the vectors that drive this disease is pursued, which in turn could lead to the identification of subgroups of patients with increased risk on certain outcomes, or who might benefit from a certain treatment (strategy). By studying clinical outcome, the effects of therapeutic strategies can be evaluated and risk factors for adverse outcomes can be identified.

Part I of the thesis examines the genetic polymorphisms of the genes encoding complement component C4 and the low-affinity FcγRs as a genetic risk factor. The inflammatory effects of EBER1 loaded extracellular vesicles, secreted from EBV infected B cells, on kidney tissue are studied as an environmental factor. In Part II of the thesis, we present our investigations on long-term data obtained from our cohort with respect to remission, damage accrual, and health-related quality of life (HRQoL). In the final chapter, the use of antimalarials, which are the anchor drugs of SLE, is studied, with an emphasis on reasons for non-use.

Genetic risk factors

Genetic polymorphisms have a substantial contribution to SLE susceptibility. The emergence of whole genome sequencing has accelerated our understanding of the genetic background. These studies have identified many candidate genes that need further evaluation in subsequent studies.

Previous studies had identified low gene copy number (GCN) of C4 as a risk factor for SLE. The leading hypothesis is that C4 deficiency leads to a defective clearance of immune complexes and apoptotic cells. C4 consists of two isoforms: C4A and C4B. C4A is thought to be primarily involved in clearance of immune complexes through its preferential binding of free amino groups, while C4B is thought to be primarily involved in lysis of bacteria through its preferential binding of free hydroxyl groups. In **chapter 2**, our aim was to examine complement component C4 in relation to SLE and its clinical manifestations on a genetic, protein, and functional level in one comprehensive study.

We found that SLE was associated with low C4 GCN. This association was mainly explained by a difference in GCN variation of C4A genes and not by C4B genes. Secondly, we studied the association between C4 polymorphisms and clinical manifestations to evaluate whether C4 polymorphisms are associated with a distinct clinical syndrome. We observed an association between low C4A GCN and serositis. Two previous studies also examined clinical manifestations of SLE in relationship to C4 polymorphisms, but no common clinical manifestation was found. We therefore suspect that low C4A GCN is not associated with a distinct clinical syndrome. Thirdly, we reported a novel mutation leading to non-expression of C4A. The affected C4A gene carried one basepair insertion (A) after A1112, which caused a frameshift generating a premature stop codon in exon 11. Finally, using a new C4 substrate binding assay, that measures functional activity of C4 components, rather than the blood group antigens Chido and Rogers, we show that the function of available C4 to bind to their targets seems unaffected in SLE. This test also detected crossing over of Rodgers and Chido antigens, which will allow for more accurate measurement of C4 in future studies.

Another important genetic risk factor for SLE are polymorphisms of the genes encoding the low-affinity FcγRs. FcγRs play an important role in regulating the immune response. All FcγRs trigger a pro-inflammatory response, except for FcγRIIb, which triggers inhibitory signaling pathways. The region that encodes the low-affinity FcγRs is subject to single nucleotide polymorphisms (SNPs), copy number variation (CNV), and linkage disequilibrium. The frequency of these polymorphisms differ considerably between ethnic groups. Previous studies and meta-analyses focused on a single SNP or CNV in relationship to SLE and/or LN susceptibility. In **chapter 3** we studied the most relevant polymorphisms, including the novel FCGR2C-ORF, in a Caucasian patient population in relationship to disease susceptibility and occurrence of LN (LN). We demonstrated an independent and significant association with susceptibility to SLE of FCGR2A-131R, copy number of copy number region 1 (including FCGR3B), and the 2B.4 haplotype in the promoter region of FCGR2B, which encodes the inhibitory FcgRIIb. Two previous meta-analyses had also demonstrated FCGR2A-131R to be a susceptible genotype in patients of European descent. Low copy number of FCGR3B was a susceptible genotype for SLE in another meta-analysis. We have now shown that CNV of FCGR3B

solely occurs as a combined deletion of different genes, which are collectively termed copy number region 1 (CNR1). CNR1 contains 3 genes (FCGR2C, HSPA7 and FCGR3B) and additionally leads to ectopic expression of FcgRIIb on NK cells. Flow cytometry showed a gene dosage effect of copy number of FCGR3B on FcgRIIIb expression, which therefore seems the most logical explanation for the association with disease susceptibility, as it may lead to impaired clearance of apoptotic material. FcγRIIb has a unique function within the family of FcγRs as it is the only receptor that triggers inhibitory signaling. We found that the 2B.4 haplotype of FcγRIIb was associated with increased susceptibility to SLE. With flow cytometry we were able to demonstrate that the presence of the 2B.4 haplotype leads to increased expression of FcγRIIb on myeloid cells, but not B cells, which was previously suggested. Interestingly, a negative association between the 2B.4 haplotype and LN was found. Thus, while the presence of the 2B.4 haplotype is associated with an increased susceptibility to SLE, it was also associated with a decreased risk of LN.

Epstein-Barr virus
EBV has long been suspected as an important environmental factor in the pathophysiology of SLE. In **chapter 4** we investigated whether extracellular vesicles loaded with nuclear EBV RNAs (EBER1), which are secreted from EBV infected B cells, are able to potentiate a pro-inflammatory response in LN. First, we showed increased levels of EBER1 in LN biopsies, but absence of EBER2-RNA and EBV-DNA, suggesting absence of EBV infected cells. EBER in situ hybridization (EBER-ISH), the gold standard for detection of EBV infected cells, confirmed the absence of EBV infected cells in the biopsies. However, we did observe atypical cytoplasmatic staining in tubular epithelial cells (TECs). We hypothesized that EBER1 in LN was derived from an extra-renal source. Indeed, we demonstrated that SLE patients secrete EBER1 into the circulation via extracellular vesicles, presumably exosomes. Next, we showed that TECs that have high expression of phosphatidylserine (PS) receptor kidney injury molecule 1 (KIM1) endocytose exosomes more efficiently than TECs that have low expression of KIM1. The involvement of PS receptors in the internalization of extracellular vesicles was further supported with entry competition experiments. Then, we showed elevated cytokine levels of IL-6 and TNFα on an mRNA and protein level, after incubation of EBER1 with primary TECs. Thus, demonstrating that EBER1 RNA, upon entering TECs, triggers the release of pro-inflammatory cytokines that are implicated in LN pathogenesis. Finally, by blocking toll-like receptor 3 (TLR3), using hydroxychloroquine (HCQ) and a small molecule inhibitor of TLR3, we were able to show that TECs are responsive to EBER1 in a TLR3-dependent manner. All our data combined suggest that TECs express KIM1, which likely acts as an extracellular vesicle (exosome) entry receptor. Upon intracellular delivery of exosomal-EBER1, a pro-inflammatory response via TLR3 is initiated, with the production of pro-inflammatory cytokines IL-6 and TNFα.

Long-term oucome and treat-to-target
Part II presents the clinical-epidemiological studies on long-term outcome. The main treatment focus of SLE has shifted from survival to the prevention of damage accrual and improving quality of life. Treat-to-target strategies, which have been beneficial for hypertension, diabetes mellitus and rheumatoid arthritis, might provide an opportunity to further improve outcome in SLE as well. A group of international experts identified remission as a possible target in such an approach. This consortium defined remission as a durable state, characterized by the absence of clinical (but not serological) disease activity, supplemented with a physician's global assessment. Maintenance therapy with immunosuppressants and a maximum daily equivalent dose of 5 mg prednisone were allowed as medication. A group from the Pacific and South-East Asia region identified a low disease activity state (the permission of some disease activity) as another potential target. This low disease activity state was termed Lupus Low Disease Activity State (LLDAS). LLDAS was defined as a SLEDAI-2K $\leq$4, with no activity in major organ systems or gastrointestinal activity, no new feature of lupus disease activity, physician global assessment $\leq$1 (scale 0-3), equivalent prednisone dose $\leq$7.5 mg per day, and well tolerated standard maintenance doses of immunosuppressive drugs or approved biologic agents. In **chapter 5**, we analyzed 5 year follow-up data from our Amsterdam SLE cohort. Data of 183 patients were used, of whom 117 had at least 5 years of follow-up. The majority of patients were female and of Caucasian ethnicity. Mean age at baseline was 41 years, with a mean disease duration of 8.1 years. Arthritis was the most common clinical manifestation, followed by UV intolerance.

Nephrological manifestations were present in a third of patients. At baseline, over half of our patients already accrued organ damage as defined by a damage index score ≥1. Renal damage was the most frequently observed type. Prednisone was used by nearly half of patients, antimalarials by 85.2%, and other immunosuppressants by 50.8% of patients. Ten patients died during follow-up. We identified 3 predictors of organ damage: occurrence of ≥1 major flare, mean daily prednisone dose during follow-up, and nephrological manifestations according to ACR criteria at baseline. Next, we assessed prolonged remission and LLDAS at yearly study visits. Prolonged remission, defined as absence of clinical disease activity at all study visits, was present in 32.5% and LLDAS in ≥50% of observations in 64.5%. Both the presence of prolonged remission during 5 years and LLDAS in ≥50% of observations were associated with a reduced risk of damage accrual, without clear superiority of either set of criteria. We concluded that both prolonged remission and LLDAS are feasible targets and associated with reduced damage accrual.

Shortly after chapter 5 had been published, international consensus was achieved on criteria defining remission states in SLE. We then studied the construct validity of remission on HRQoL (**chapter 6**). Remission was defined as a clinical SLEDAI-2K of 0 and a PGA of ≤ 2 (on a 0 – 10 Likert scale). Furthermore, patients needed to be on stable maintenance immunosuppressants and/or a maximum daily equivalent dose of 5 mg prednisone. HRQoL was measured with short-form 36 (SF-36) and reported as physical component score (PCS) and mental component score (MCS). PCS and MCS were corrected for SF-36 scores in the Dutch general population and sex. A score higher than 50 indicate higher HRQoL, scores lower than 50 indicate lower HRQoL, as compared to the referenced population. Data from 154 patients with 2 years of follow-up were analyzed. At baseline 39.0% of patients were in remission. Patients in remission had higher SF-36 scores in all subdomains compared to patients not in remission. PCS was positively associated with remission and employment and negatively associated with SLICC damage index, erythrocyte sedimentation rate, medication, patient global assessment and body mass index. MCS was positively associated with Caucasian ethnicity and negatively associated with patient global assessment. In generalized estimating equation (GEE) models, a gradual and significant increase of PCS was observed from patients not in remission (mean PCS 36.0) to remission on therapy (41.8) to remission off therapy (44.8). No significant difference in MCS was found between remission states. We hypothesized that the lack of association of remission states with MCS could be explained by a high mean MCS in our cohort, which was in fact comparable to the general Dutch population. Secondly, we have a low prevalence of neuropsychiatric SLE in our cohort, which is a manifestation strongly associated with lower MCS. Because several randomized clinical trials in patients with SLE have failed their primary endpoint, possibly by suboptimal selection of outcome parameters, we suggested careful consideration of selecting MCS as an outcome parameter. Nonetheless, the study in chapter 6 shows a strong and persistent association between remission states and PCS according to newly formulated and internationally accepted remission criteria.

Antimalarials

Antimalarials, of which HCQ is most commonly used, have become the cornerstone of SLE treatment. The most important beneficial effects attributed to HCQ are reduction in disease activity, prevention of flares, reduction in damage accrual, and improvement of overall survival. HCQ is generally well tolerated and side-effects are usually mild. (Hydroxy)chloroquine-related retinopathy (further termed HCQ-related retinopathy, as HCQ is usually meant) however is a severe complication with a reported prevalence of 0.5–1% after 5 years of use. Previous studies have demonstrated, however, that still a substantial group of patients do not use antimalarials. In **chapter 7** we studied the reasons for non-use in our cohort and we also assessed the incidence of chloroquine-related retinopathy. Out of 190 SLE patients studied, 73.2% were using antimalarials (nearly all HCQ) during their last study visit. Previous HCQ use was as high as 92.1%. Intolerance was the most frequent reason for not using antimalarials. Two patients had a contraindication for antimalarials. Other common reasons were: discontinued without a documented reason, quiescent disease, or never initiated. Non-use of antimalarials was associated with a longer disease duration, higher damage accrual and a history of lupus nephritis.

Only one patient discontinued HCQ due to HCQ-related retinopathy. As HCQ-related retinopathy was previously associated with use of a HCQ dosage above 6.5mg/kg lean body weight per day, we

assessed the daily dosage in our cohort. We found that 84.6% of patients used 400 mg of HCQ daily. According to lean body weight, 87.5% of patients took a daily dosage above the then recommended dose. The patient with chloroquine-related retinopathy had used 11 mg/kg lean body weight of HCQ (400 mg) daily for 11 years. We concluded that antimalarial use has improved, but is still sub-optimal, especially in patients with longstanding disease or a history of lupus nephritis. Furthermore, we have shown that antimalarials are frequently prescribed in dosages above the then recommended 6.5 mg/kg lean body weight per day, but that these dosages seem to be well tolerated.