SUMMARY AND DISCUSSION
SCOPE OF THIS THESIS

The first section of this thesis focuses on thyroid dysfunction in rheumatoid arthritis (RA) patients and the relationship between thyroid abnormalities and cardiovascular risk. This section also describes changes in thyroid function during the immunomodulating treatment of RA. The second section of this thesis aims to investigate whether the lipid profile improves during B cell depleting therapy. The other aim of this section is to describe pharmacogenomics effects on RA patients during rituximab treatment (RTX) in order to clarify, firstly, the biological processes underlying any clinical outcomes during RTX in RA, and, secondly, whether baseline gene expression profiles discriminate between responders and non-responders in RA patients treated with RTX.

MAIN FINDINGS FROM SECTION I. THYROID DYSFUNCTION AND RHEUMATOID ARTHRITIS: MORE THAN COMORBIDITY.

RA is a chronic inflammatory disease affecting the various smaller joints of the hands and feet. The inflammatory burden of RA extends beyond the joints however, as extra-articular manifestations of RA also include well-known comorbidities like osteoporosis and cardiovascular disease (CVD).(1-3) The question arises whether thyroid dysfunction might be considered as new extra-articular involvement.

In chapter 1.1 the prevalence of hypothyroidism is investigated in both RA patients attending a specialized rheumatologic referral centre as well as patients attending a general practitioner in primary care. Chapter 1.1a finds that arthritis patients face a twice as high risk of hypothyroidism compared to the general population. This chapter also observes a female predominance of hypothyroidism. Furthermore, the prevalence of CVD in arthritis patients with and without hypothyroidism was compared in a cohort, studying prevalence and incidence of CVD and cardiovascular risk factors, of more than 350 RA patients attending an outpatient rheumatology clinic. This study observed a more than threefold increased risk of CVD prevalence in female hypothyroid RA patients compared to female euthyroid RA patients. In chapter 1.1b the prevalence of hypothyroidism in inflammatory arthritis patients is reassessed in a sample of more than 175,000 patients in primary care (attending general practitioners). The observed risk for hypothyroidism was more than doubled in inflammatory arthritis patients compared to the control group. Furthermore, this study additionally found a similar twofold increased risk of CVD in hypothyroid arthritis compared to euthyroid arthritis patients and an almost fourfold increased risk compared to the euthyroid non arthritis control group. Traditional CVD risk factor assessments did not explain this elevated CVD risk, suggesting an independent role of hypothyroidism in CVD risk elevation in RA.
In the general population, thyroid dysfunction has been associated with several cardiometabolic abnormalities (i.e. central adiposity, high triglycerides, insulin resistance, jointly known as metabolic syndrome (MetS)). Since MetS has been associated with higher CVD risk and increased onset of diabetes mellitus (DM), a higher prevalence of MetS in hypothyroid RA patients can be an important mediator of the pronounced CVD risk. Therefore, the occurrence of MetS was highlighted in chapter 1.2 in 257 CVD event naïve RA patients. This study observed a more than threefold elevated risk of MetS in hypothyroid RA patients compared to euthyroid RA patients. This study also demonstrated an increased 10-year CVD risk estimation, i.e. higher Framingham score, in hypothyroid RA patients compared to euthyroid RA patients. As a result this chapter underlines the amplified CVD risk in hypothyroid RA patients, and reflects a higher prevalence of MetS and higher Framingham risk scores.

The most common form of autoimmune thyroiditis is Hashimoto thyroiditis, characterized by the elevated presence of antibodies against thyroid peroxidase (TPOabs). As hypothyroidism is a common disease in RA, the prevalence of TPOabs were studied in Chapter 1.3. Additional explorations were made into whether TPOabs were associated with an amplified CVD risk and whether these antibodies were associated with a certain RA phenotype. TPOabs were found in 15% of 322 RA patients, predominantly in females, compared to published reports showing the prevalence to be approximately 8% in the general population.(6) In addition, this study found a significantly higher increase in carotid intima medica thickness (cIMT) in TPOabs positive RA patients compared with TPOabs negative RA patients. Moreover, it should be noted that, TPOabs positive patients had significantly higher disease activity scores.

Chapter 1.4 evaluated whether (chronic) inflammation plays a pathogenic role in the development of thyroid disturbances. Changes in thyroid hormones were assessed in 138 RA patients during treatment with the immunomodulating agent adalimumab (a tumour necrosis factor (TNF) blocking agent). In hypothyroid patients the thyroid stimulating hormone (TSH) levels improved during adalimumab treatment. These beneficial effects were most apparent in L-thyroxine naïve and TPOabs positive hypothyroid RA patients. In addition, this chapter reports on a case of biochemically hyperthyroidism development in a RA patient 4 months after RTX treatment (a B lymphocyte depletion therapy) with both clinically longstanding manifested autoimmune hypothyroidism and type I DM. Although both studies are a proof of principle, these studies also underline the close relationship between inflammatory cytokines, immune cells and the thyroid gland. Therefore clinicians should be more alert to any disturbances and advances in thyroid function during different immunomodulating therapies.
This thesis observes that hypothyroidism is present in approximately 7% of RA patients, which is more than twice the proportion in the general population. This observation confirms the hypothesis that there is a positive correlation between the prevalences of hypothyroidism and RA. Clinicians should therefore be actively encouraged to screen and treat RA patients for hypothyroidism. The question arises whether hypothyroidism should actually be considered as an extra-articular manifestation of RA.

This thesis reinforces the general idea that autoimmune diseases have a tendency to cluster, which may point to a common genetic origin of different autoimmune disorders. Intriguingly, patients with the same genetic background of autoimmune candidate genes may ultimately present with a totally different disease manifestation, a concept known as the “mosaic of autoimmunity”.(7;8) Although autoimmune disorders have a wide spectrum of manifestations, the common ground between the different autoimmune entities seems to be the underlying inflammatory state, identifiable by the presence of pro-inflammatory cytokines. Whether inflammation itself has a pathogenic role in autoimmune diseases like autoimmune hypothyroidism, is something that needs further research. This thesis supports the theory that inflammation evokes hypothyroidism, as it is observed that TNF blocking agents are able to improve thyroid function in L-thyroxine naïve hypothyroid patients. This suggests that effective anti-inflammatory therapy may resolve thyroidal dysfunction in at least a subset of patients. Further support for this hypothesis was provided by previous research (in mice) which found that chronic exposure to cytokines without lymphocytic infiltration of the thyroid glands, resulted in a hypothyroid state with extended histological similarities to Hashimoto thyroiditis.(9) Moreover, this thesis observed the intriguing effects of B cell depletion therapy in a patient with longstanding clinically manifest autoimmune hypothyroidism. These findings strongly suggest the direct pathogenic role of inflammation in the evolution of hypothyroidism. These observations need further elaboration to allow more insight into which inflammatory mechanisms are involved in the development of autoimmune thyroiditis and to find out whether and what immunomodulating therapies may be beneficial for hypothyroidism.

This thesis demonstrates that hypothyroidism amplifies CVD risk in RA patients. Clinicians should therefore pay closer attention to thyroid disorders in RA patients who already have a high background CVD risk. In this context, it is also interesting to mention that last year an American study observed a doubled risk of incident CVD in hypothyroid RA patients, independent of traditional CVD risk factors.(10) Hypothyroidism should therefore be considered as an important mediator in CVD
development in RA patients, and clinicians should be urged to actively screen and treat hypothyroidism in RA patients. As this thesis shows an amplified prevalence of cardiovascular risk factors in RA patients with comorbid hypothyroidism, the active screening and treatment of these risk factors should be advocated.

Several factors may be responsible for this elevated CVD risk. Firstly, this thesis shows an elevated prevalence of traditional CVD risk factors in hypothyroid RA patients, reflected by the higher prevalence of MetS. Secondly, this study observes higher cIMT progression in TPOabs positive patients. Thirdly, the role of chronic inflammation in hypothyroid patients should receive more attention, because this thesis shows higher disease activity and higher inflammatory parameters in RA patients with TPOabs and we know that inflammation deteriorates cardiovascular risk factors. Fourthly, the question is raised as to whether patients with hypothyroidism are adequately treated for their thyroid dysfunction. Finally, both RA and hypothyroidism are associated with endothelial dysfunction and microvascular dysfunction. All these aspects seem to contribute to the elevated cardiovascular risk in female hypothyroid RA patients. In conclusion, hypothyroidism amplifies cardiovascular risk in RA patients and therefore intensive cardiovascular risk management is needed in these patients.

FUTURE RESEARCH SECTION II. HYPOTHYROIDISM AND RHEUMATOID ARTHRITIS: MORE THAN COMORBIDITY.

The findings of this thesis suggest further investigations are needed to explain what pathogenic mechanisms are involved in autoimmune thyroiditis and CVD development.

The first step may be to identify a common genetic origin in hypothyroidism and RA, reflecting the presence of identical genes and genetic polymorphisms (e.g. PTPN22, CTLA4, STAT4 and interferon regulated genes), as these polymorphisms and genes have been described in both hypothyroidism and RA. In addition, the question arises whether other autoimmune disorders like celiac disease, autoimmune diabetes, addison’s disease, and vitiligo also tend to coexist in RA patients and whether these patients share the same genetic predisposition. The second step may then be to look at the pathogenic role of inflammation allowing more insight into the development of autoimmune thyroiditis, as this may indicate new therapeutic targets in patients with hypothyroidism. Additionally, cardiovascular risk management strategies are needed in hypothyroid RA patients to lower their amplified cardiovascular risk. Initially, hypothyroid RA patients should be treated in accordance with the EULAR evidence-based recommendations for annual cardiovascular risk management. This...
assessment needs to be targeted at patients suffering from inflammatory arthritis and must be implemented according to national guidelines.

The cardiovascular burden in hypothyroid RA patients needs to be reassessed after the implementation of these guidelines. Future studies should also focus on subclinical hypothyroid patients, and an interesting question is whether L-thyroxine supplementation in a randomized placebo controlled trial (designed for RA patients with comorbid subclinical hypothyroidism) will result in a decreased cardiovascular event rate. Finally, in the coming decade we need to find out which parameters should be used to predict the evolution of hypothyroidism. Identification of these clinical biomarkers and, subsequently identification of ways to deal with them, may eventually enable the prevention of the cardiovascular consequences of hypothyroidism in RA patients.

**MAIN FINDINGS SECTION II. RITUXIMAB AND RHEUMATOID ARTHRITIS: MORE THAN B CELL DEPLETION.**

The recognition of B lymphocytes as contributors to RA pathogenesis - by producing autoantibodies, antigen presenters, cytokine producers and inducing lymphopoeisis, (11) seems to suggest that B cell targeted therapy is a pivotal treatment option for RA patients. RTX is a chimeric monoclonal antibody directed against the cell membrane protein CD20 of B lymphocytes. RTX effectively depletes B cells and seems to be an efficacious and safe treatment modality for RA patients refractory to TNF blocking agents.(12-14) Effective anti-inflammatory therapy is important to arrest radiological progression, but also appears important in the improvement of the increased cardiovascular risk profile, as chronic inflammation perpetuates dyslipidaemia. In fact, effective anti-TNF treatment has been suggested to have a beneficial effect on lipid profiles, although the effects seem transient.(15) It has not been thoroughly studied whether B cell targeted therapy influences lipid metabolism. In chapter 2.1 changes in lipids and HDL protein composition during RTX treatment were studied in 49 RA patients with longstanding RA duration. In this study we observed that the atherogenic index (i.e. the total cholesterol to HDL cholesterol ratio) decreased ~9% (from 4.32 to 3.90) 6 months after RTX treatment in the total group of RA patients. In RA patients who responded well to RTX, this study found a significant improvement of the apolipoprotein (Apo) B to Apo A1 ratio of approximately 9% (from 0.57 to 0.52). Moreover, improvements of HDL cholesterol and atherogenic indexes are reported in those who responded to RTX, by respectively ~5% and ~7%.

Protein compositions of HDL cholesterol were studied at the same time as the quantitatively lipid levels, since certain functional properties of the HDL particle are correlated to inflammatory states
and render HDL proteins that are more pro-atherogenic in high disease activity states. Chapter 2.1 demonstrates that initiating RTX treatment in patients with active disease has a pro-inflammatory and pro-atherogenic HDL compound, illustrated by a large amount of serum amyloid A (SAA) binding of the HDL particle. Furthermore, this study demonstrates a significant reduction of the SAA content of the HDL particle in RA patients who respond well to RTX. These findings ultimately show the beneficial effects on the lipid profiles of RA patients during RTX treatment, particularly in RA patients who respond well to this treatment.

Despite the fact that RTX depletes B cells in all RA patients, only ~65% of RA patients show a clinical response to RTX treatment. These differences in treatment responses between individuals reflect the heterogenic nature of RA and raises the question of whether RTX induces other mechanisms of action, apart from B cell depletion, which can explain the diversity in the response to treatment. Therefore, in chapter 2.2a a pharmacodynamic study was undertaken to clarify whether RTX-induced biological processes could be correlated with the clinical outcome of RTX. This microarray study, with a genome wide approach in 13 RA patients treated with RTX, revealed differences in the expression of gene profiles between individuals, which could be categorised in clusters, representing 6 different biological pathways: 1) type I interferon (IFN) response genes (IRG), 2) translation, NK cell mediated immunity and T cell receptor signalling, 3) B cell mediated immunity, 4) extra-cellular matrix modelling and iron transport, 5) chemotaxis and adhesion, and 6) cytoskeleton and coagulation. When arranging patients by response status, 6 months after RTX initiation, this genome wide microarray study showed a selective increase in the gene profile representing type 1 IRG expression at 3 months, in RA patients who responded well. Less responsive patients showed no induction of this IFN gene expression profile.

These observations raise the question whether baseline gene expression profiles could identify responders before RTX initiation. In chapter 2.2b we investigate in a microarray study of 14 RA patients whether gene expression profiles discriminate between responders and non-responders to RTX treatment and if so, which ones. Genome wide gene expression profiling identifies a selective group of genes, representing type I IRG, to be up-regulated in non-responders. To verify the predictive value of the identified IFN signature for non-response, a Receiver Operating Characteristics (ROC) curve analysis was performed in an independent cohort of 26 RTX treated RA patients. ROC curve analysis demonstrated the IFN signature to be a good discriminator between non-responders and responders (area under curve (AUC): 0.82). To determine if the IFN signature can predict clinical outcome independently from other potential predictors, stepwise bivariate logistic regression analysis was performed. This study showed an independent association between IRG set and non-
response. This data demonstrate that type I IRG at baseline can accurately and robustly predict the clinical outcome of RTX treatment.

**CLINICAL PERSPECTIVE AND IMPLICATIONS RITUXIMAB AND RHEUMATOID ARTHRITIS: MORE THAN B CELL DEPLETION.**

This thesis demonstrates the favorable effects on lipid levels in RA patients treated with RTX, as it observes a reduction of approximately 7% of the atherogenic index and approximately 9% of the Apo B/Apo A-I ratio after half a year of RTX treatment. Although the observed changes in lipid profile in this thesis seem small, they have clinical relevance, because the changes in the atherogenic index are similar to the ones achieved by lipid lowering medications like statins, which aim to decrease CVD risk by lowering LDL cholesterol and triglycerides. (17-19) In addition, the reported improvement of the atherogenic index is intriguing, as a recent meta-analysis could not demonstrate beneficial effects on this composite during TNF blocking agents. (20) However, previous studies in RA patients treated with TNF have proven to reduce the CV event rate. In light of the improvement in the atherogenic index the CVD incidence rates after longterm RTX treatment need to be assessed. (21) Moreover, this thesis observes a clear association between inflammatory parameters and improvement of lipid profile. These findings underline the deep relationship between inflammation and dyslipidemia, an important mediator in accelerated atherosclerosis. As inflammation seems the major cause effecting detrimental cardiovascular risk, effective anti-inflammatory treatment is not only necessary to decelerate radiologic progression, but seems essential to decrease cardiovascular risk in RA patients with an elevated background cardiovascular burden. Along with these beneficial effects on the lipid levels, this thesis demonstrates a clear pro-inflammatory HDL composition in RA patients with active disease status initiating RTX, represented by a large amount of serum amyloid a (SAA) content nested on the HDL cholesterol particle. These observations underscore the functional capacities of HDL protein. Previous studies reported the role of the HDL cholesterol particle in anti-inflammatory and anti-oxidative capacities by reducing lipid oxidative products, down-regulating cell adhesion molecules in endothelial cells and regulating TNF production by macrophages. (22;23) Previous investigations also showed that functional capacities are lost in inflammatory states rendering the HDL particle more proatherogenic. (24) In this context, an observation of major relevance is that the HDL proteome alters to an anti-inflammatory and less proatherogenic composition (as shown by a significant reduction of SAA on the HDL protein particle) in patients who respond well to RTX. This thesis outlines the beneficial effects on the quantitative and qualitative lipid profile as a result of RTX treatment.
The data presented in this thesis shows a positive clinical outcome in 55–65% of the RA patients treated with RTX, which in itself is an intriguing observation, as these percentages equal the response rates in RA patients treated with RTX in randomized controlled trials. Despite these positive response rates in patients (with longstanding disease duration and extremely therapy refractory disease), there is still a large proportion of RTX treated patients showing no clinical response. These observations emphasize differences between individuals to B cell targeted therapy as well as the heterogenic nature of RA. Moreover, the heterogeneity of the disease is also reflected in the different inter-individual pharmacogenomic effects of RTX. The data presented in this thesis shows that genes involved in different biological processes are expressed differently, amongst others B cell mediated immunity, type I interferon (IFN) signalling, NK and T cell mediated immunity, and chemotaxis and adhesion. Intriguingly, when we reanalysed our microarray data by stratifying patients on response rates, it became apparent that the only distinction between responders and non-responders is the pharmacodynamic changes in the expression of a selective group of genes that are all regulated by type I IFN. Type I IFN activity seems to be a common denominator in different autoimmune diseases (e.g. systemic sclerosis, systemic lupus erythematosus, Sjogren syndrome and type I diabetes mellitus).(25-28) Type I IFN response genes (IRG) can be up-regulated as a result of a genetic background (HLA-DR, STAT4, Tyk2 and IRF5), exogenous sources (smoking, bacterial and viral infections) and endogenous origins (necrotic or apoptotic material).(29-31) Hence, in patients with a genetic predisposition, CD20 positive B cell depletion produces a pool of apoptotic and necrotic material which promotes the production and release of IFN, This might selectively take place in RA patients with low IFN activity. Moreover, introduction of this type I IFN signature may stimulate factors that increase B cell survival (such as BLYS and APRIL), protecting B cells from further depletion.(32) Therefore, the dynamics of IFN response reflects the biological processes underlying RTX response in responders. The high baseline type I IFN expression, in non-responders, and the ability of this IFN signature to discriminate adequately and robustly between non-responders and responders before RTX treatment, were two particularly interesting findings. Type I interferons also induce survival, maturation and activation of dendritic cells. These induce a cascade of immune responses - by producing cytokines like TNF α, IL-6 and IL-10, as well as B cell activating factors, chemokines and co-stimulatory molecules - and therefore exert effects on all other immune cells.(33-37) These biological processes may explain the persistent disease activity in patients with a IFN high profile before the initiation RTX treatment.

All the findings in these pharmacogenomics studies highlight the heterogeneity of RA. Patients with a high IFN gene expression may represent a different subset of RA patients who display different clinical outcome to treatment modalities. When all these observations were performed in a genome
wide approach, the relationship between IFN activity and treatment outcome was confirmed clearly. (38-41)

FUTURE PERSPECTIVES SECTION II. RITUXIMAB AND RHEUMATOID ARTHRITIS: MORE THAN B CELL DEPLETION

The observations resulting from the studies conducted in this thesis gives rise to the need for further research exploration in the next decade.

In the cardiovascular field a clinically important question is whether a reduced atherogenic index will result in a lower cardiovascular event rate in RA patients after long-term RTX. Moreover, the findings of this thesis underscore the deep relation between inflammation and dyslipidemia. Future studies on dyslipidemia and RA should therefore investigate the cardioprotective functional capacities of the HDL particle in relation to inflammation and atherosclerosis. This will give a better insight into the pathophysiological mechanisms underlying CVD development in RA patients. Finally, the effects on other emerging surrogate markers for CVD during long-term RTX treatment need to be evaluated. Answering these questions will ultimately reveal whether RTX is cardioprotective or cardiogenic.

The identification of IRG as a good predictor of non-response raises the question whether the IFN signature may serve as a biomarker for treatment response before RTX is initiated. The logical next step would be to validate the IFN signature as a predictor of RTX outcome in large scale prospective clinical trials. The outcome of this will determine whether IFN serves as biomarker. In this respect, it should be noted that independent of our cohort, other clinics also observed a clear relationship between IRG and treatment outcome. These findings strongly favor the potential of pharmacogenomics studies to identify new biomarkers like IRG for RTX outcome. Furthermore, future studies should combine other potential biomarkers like ACPA, rheumatoid factor positivity, and antibodies against RTX and RTX levels in order to validate the clinical utility of the IFN signature. Ultimately, these results will bring the personalization of medicine one step closer.
CONCLUDING REMARKS

It its entirety this thesis shows an increased prevalence of hypothyroidism in RA patients. It also demonstrates that hypothyroidism is an important mediator for the development of CVD in RA patients. It proves that the time has come for clinicians to incorporate our understanding of this amplified CVD burden in hypothyroid RA patients into daily practice, to advocate better treatment of hypothyroidism in RA patients and to create greater awareness of the amplified CVD risk. This thesis underscores the close relationship between inflammation and dyslipidemia, emphasizing the need for a tighter control over the disease with pivotal anti-inflammatory therapy to improve the cardiovascular risk profile. The data presented in this thesis highlights that molecular heterogeneity can be related to clinical outcome. Hopefully this will bring the highly desired goal of personalized medication one step closer.
REFERENCES


