Cardiovascular morbidity and metabolic signature in patients with rheumatoid arthritis

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ABSTRACT

Background and objectives: Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with excess risk for cardiovascular morbidity and mortality. The treatment of RA by anti-inflammatory drugs has dramatically been improved over the resent decades. Although, articular inflammation and disability burden has been reduced, cardiovascular disease (CVD) still accounts for more than half of all the death in RA population. This cannot entirely be explained by traditional cardiovascular risk factors and inflammatory activity. The general aims of this thesis were to study the role of essential metabolic pathways and their interplay with inflammation and cardiovascular morbidity in RA.

Methods: A cohort of female RA patients was clinically studied and prospectively followed up under 5 years. Cardiovascular risk (CVR) in relation to metabolism regulating molecules in fat tissue and in the peripheral blood was investigated. Blood samples and white fat aspirations were used to study different cytokines and gene expressions. Quantitative PCR was used for transcriptional analysis of proteins. As receptor for insulin-like growth factor 1 (IGF1R) has a key role to connect inflammation and metabolism, the consequences of its inhibition on regulation of T cell balance in experimental RA were explored.

Results: Low serum levels of IGF1 were associated with higher CVR, hypertension and metabolic signature such as adiposity and hyperlipidemia. The levels of IGF1 were constantly low independent to age in IGF1 low group and under 5 years follow-up, the frequency of hypertension and CV events were significantly higher in this group particularly, in younger patients compared to control group. RA patients with hypertension showed unfavorable metabolic profile including higher levels of plasma glucose and insulin followed consequently by higher inflammation and disease activity.

Accumulation of signal transducer and activator of transcription 3 (STAT3) in white adipose tissue (WAT) promotes metabolic activity on leucocytes. Interleukin 6 (IL6) and leptin induce metabolic disorders and increased CVR by activating STAT3 in WAT, in RA. Low serum levels of soluble receptor for advanced glycation end products (sRAGE) were associated with both previous and new cardiometabolic events (CME). Younger patients with low sRAGE levels showed higher CVR and adverse metabolic factors. Inhibition of IGF1R on the levels of insulin receptor substrates (IRS1/2) in experimental model resolved the inflammation and arthritis followed by reduced production of IL6 and STAT3 by spleen T cells with consequent up-regulation of the regulatory T cells.

Conclusion: A range of essential metabolic factors interplays with inflammatory pathways and CVR in RA. This underlines the need to apply biomarkers in clinical practice to improve CVD risk factors management in RA female patients. The patients would also benefit of monitoring for hyperglycemia, hypertension, and obesity.

Keywords: Rheumatoid arthritis, cardiovascular risk, cardiometabolic event, IGF1

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SAMMANFATTNING PÅ SVENSKA

Bakgrund: Ledgångsreumatism (RA) är en inflammatorisk systemsjukdom. Dödlighet i kardiovaskulär sjukdom (CVD), jämfört med befolkningen i allmänhet, är fortfarande hög vid RA, trotts utveckling av effektiva mediciner som minskar inflammation och destruktioner i lederna. De traditionella riskfaktorerna eller inflammatoriska parametrar kan inte helt förklara den ökade kardiovaskulära dödligheten. Detta kan bero på samspel mellan olika metaboliska och inflammatorisk molekyler och receptorer som fungerar på olika sätt i RA. Det övergripande syftet med denna avhandling var att studera samspel mellan kardiovaskulära riskfaktorer och metaboliska och inflammatoriska aktiviteter in RA.

Metoder: Studierna som ingår i denna avhandling baseras på en kohort av 184 patienter med RA (alla kvinnor) som rekryterades från reumatologenheten i Västra Götaland (Göteborg, Uddevalla). Patienterna undersöktes av en reumatolog beträffande sjukdomsaktivitet och kardiovaskulära risker. Blodprover och fett aspiration togs vid besöket och efter 5 år följdes patienterna upp för kardiovaskulära händelser eller ändring i kardiovaskulära risker. IGF1 receptor är en viktig länk mellan inflammation och metabolism. Av den anledning, undersökte vi påverkan av hämning av denna receptor på balansen mellan T celler (Th17/Treg) i ett experiment.

Resultat:
Den första studien visade att lägre uttryck av insulinliknande tillväxtfaktor 1 (IGF1) var associerad med högre risk för hypertoni, hjärt-kärlsjukdomar och metaboliska störningar såsom fetma och höga värden av blodfetter. Patienter i IGF1-låggrupp hade en konstant låg nivå av IGF1 oberoende av ålder. Under 5 års uppföljning var frekvensen av hypertoni och hjärt-kärl händelser signifikant högre i denna grupp, särskilt hos yngre patienter jämfört med kontrollgruppen. RA patienter som samtidigt led av hypertoni visade ogynnsam metabolisk profil sådan som höga nivåer av blodsocker och insulin och följaktligen högre inflammation och sjukdomsaktivitet.
Den andra studien visade att ökad intracellulär transkriptionssignalering såsom signal transducer och aktivering för transkription 3 (STAT3) i vit fettvävnad stimulerar metabolisk aktivitet i vita blodkroppar i blodet hos RA patienter. Interleukin 6 (IL6) och leptin aktiverar STAT3 i vit fettvävnad som följaktligen orsakar metabolisk obalans och ökad risk för hjärt-kärl sjukdomar i RA.
Den tredje studien presenterade att låga serumnivåer av löslig receptor för avancerad glykerade slutprodukter (sRAGE) var associerade med både föregående och nya kardiometabola händelser. Yngre patienter

med låga nivåer av sRAGE visade högre kardiovaskulär risk och ogynnsamma metaboliska faktorer.
I den fjärde studien rapporterade vi att hämning av IGF1R insulinreceptorsubstrat (IRS1/2) nivå i experimentell modell kunde minska inflammationen och artriter tack vare minskad produktion av IL6 och STAT3 av T-celler i mjälten tack vare uppreglering av de regulatoriska T celler.

Konklusion:
Ett flertal olika metaboliska faktorer har stor roll i reglering av olika inflammatoriska aktiviteter som är delaktiga i kardiovaskulära sjukdomar i RA. Detta understryker behovet av att använda biomarkörer i klinisk praxis för att förbättra hanteringen av riskfaktorer för hjärt-kärl sjukdomar hos kvinnliga RA-patienter. Patienterna skulle också ha förmåner av att kontrolleras avseende blodfetter, blodtryck och fetma.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Erlandsson M.C, Lyngfelt L, Åberg N.D, Wasén C, Espino R.A, Töyrä Silfverswärd S, **Nadali M**, Jood K, Andersson K.M.E, Pullerits R, Bokarewa M.I. Low serum IGF1 is associated with hypertension and predicts early cardiovascular events in women with rheumatoid arthritis. *BMC-Biomed Central Medicine (2019) 17:141*

II. **Nadali M**, Pullerits R, Andersson K.M.E, Töyrä Silfverswärd S, Erlandsson M.C, Bokarewa M.I. High expression of STAT3 in subcutaneous adipose tissue associates with cardiovascular risk in women with rheumatoid arthritis. *International Journal of Molecular Sciences, 2017 Nov 13; 18(11):2410.*

III. **Nadali M**, Lyngfelt L, Töyrä Silfverswärd S, Erlandsson M.C, Andersson K.M.E, Bokarewa M.I, Pullerits R. Low soluble receptor for advanced glycation end products precedes and predicts cardiometabolic events in women with rheumatoid arthritis. *Frontiers in Medicine (Lausanne). 2020; 7: 594622*

IV. Erlandsson M.C, Töyrä Silfverswärd S, **Nadali M**, Turkkila M, Svensson M.N.D, Jonsson I-M, Andersson K.M.E, Bokarewa M.I. IGF1R signaling contributes to IL6 production and T cell dependent inflammation in rheumatoid arthritis. *Biochimica et Biophysica Acta - Molecular Basis of Disease 1863 (2017) 2158–2170*

CONTENT

ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American college of rheumatology
AGE	Advanced glycation end products
AKT1	Serine-threonine kinase 1
APC	Antigen presenting cells
ASCVD	Atherosclerotic Cardiovascular Disease
BMI	Body mass index
CDAI	Clinical disease activity index
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CME	Cardiometabolic events
CRP	C-reaktivt protein
CVR	Cardiovascular risk
DAMPs	Damage associated molecular patterns
DAS28	28 joint count Disease Activity Score
DCs	Dendritic cells
DD	Disease duration
DMARD	Disease modifying anti-rheumatic drug
eCVR	Estimated cardiovascular risk
esRAGE	Endogenous secretory RAGE
EULAR	European league against rheumatism
ERK	Extracellular-regulated kinase
ESR	Erythrocyte sedimentation rate
FRS	Framingham risk score
HAQ	Health assessment questionnaire
HDL	High density lipoprotein
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IFNγ	Interferon gamma
IGF1	Insulin like growth factor 1
IGF1R	Insulin like growth factor 1 receptor
IL	Interleukin
IRS	Insulin receptor substrate
IQR	Interquartile range
JAK	Janus kinase
LDL	Low density lipoprotein
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase

mBSA	Methylated bovine serum albumin
MHC	Major histocompatibility complex
mSCORE	Modified Systemic Coronary Risk Evaluation
MTX	Methotrexate
NFkB	Nuclear factor kappa B
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
qPCR	Quantitative PCR
QRISK	Q Research Cardiovascular Risk Calculator
RA	Rheumatoid arthritis
RELA	Transcription factor p65
RQ	Relative quantity
SBP	Systolic blood pressure
sRAGE	Soluble receptor for advanced glycation end products
STAT	Signal transducers and activators of transcription
TC	Total cholesterol
TCR	T cell receptor
Th	T helper
TNF	Tumor necrosis factor
Treg	Regulatory T cells
T2D	Typ 2 diabetes
WAT	White adipose tissue
WBC	White blood cells

1 RHEUMATOID ARTHRITIS

1.1 Introduction

Rheumatoid arthritis is a systemic chronic inflammatory disease characterized by swelling, pain and stiffness in the joints. The inflammation causes cartilage and bone damage that leads to dysfunction and disability. In RA, symptoms develop slowly, usually over several months. Serological indicators of inflammation such as white blood cell count (WBC), platelet count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are usually elevated. Small joints in hands and feet are involved symmetrically at the initial phase of the disease and then other joints can be involved under progression of the disease. The immunological hallmarks of RA are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). In the recent decades, better insight in inflammatory pathways has improved the assessment and treatment of the disease. Using treat-to-target strategy appeared to be successful to improve prognosis in early-diagnosed RA.

1.2 Epidemiology

Prevalence of RA is reported to be about 0.5-1% in general population worldwide. The studies showed no decline in RA prevalence under the recent decades (Minichiello et al., 2016; Myasoedova et al., 2020). The mean age at the disease onset is 40-50 years and the incidence is 2-3 times higher in women compared to men. Older age is in general a risk factor for development of RA, but the disease can also affect younger individuals. Despite several broad studies in RA, the etiology of the disease remains not fully understood. Common risk factors identified so far are smoking, obesity, female gender and genetic factors.

1.2.1 Genetic

Strong association with some types of human leucocyte antigen (HLA) class II molecules, namely HLA-DRB1*01 and HLA-DRB1*04 has been seen with RA. HLA-II molecules are expressed on the surface of macrophages, B cells and dendritic cells. A register-based case control Swedish study reported three times higher prevalence of HLA-DRB1 positive genotype in first-degree relatives of RA patients and two times in second-degree relatives (Frisell et al., 2013).

1.2.2 Smoking

Smoking separately or in combination with ACPA raises the risk for developing RA and is related to increased severity of the disease (Källberg et al., 2011). Both, duration and amount of cigarette smoking seem to increase mutually development and severity of RA (Hutchinson et al., 2001).

1.2.3 Hormones

Hormones have also important impact on development of RA. RA is more common in females. Hormonal changes such as menopause increase the incidence of RA in women. Susceptibility to develop RA and the severity of the disease are reduced during pregnancy often followed by flare of the disease postpartum (Brennan et al., 1994). Hormone replacement may modify severity of disease in RA (Forsblad d'Elia et al., 2003).

1.2.4 Obesity

Obesity is a predisposing factor for RA and CVDs. You are going to find more information in section “4.1. Adipose tissue and cardiovascular morbidity”.

1.3 Clinical features of RA

The main symptom in RA is polyarthritis, which usually is symmetrical and predominant in small joints. Commonly, the disease onset is insidious with presence of pain, swelling, tenderness and stiffness in metacarpophalangeal, proximal interphalangeal and metatarsophalangeal joints. Engagement of larger joints such as shoulders, knees and wrists is frequent. Extra-articular symptoms such as cutaneous nodules, vasculitis as well as hematological, neurological, pulmonary, cardiac, renal, and ocular manifestations are common (Cojocaru et al., 2010).
There is no single laboratory test specific for RA. It may be particularly challenging to diagnose the RA disease at the early stage. To identify a homogenous group of RA patients suitable for clinical studies, a set of classification criteria is usually applied (Arnett et al., 1988; Aletaha et al., 2010).

2 CARDIOVASCULAR RISK IN RA

Despite better understanding of RA pathogenesis and improved pharmacological treatment, cardiovascular (CV) morbidity and mortality remains a major challenge in RA. Epidemiological studies have reported a 50% higher mortality of cardiovascular disease (CVD) in RA patients compared with general population (Avina-Zobieta et al., 2008), while CVD mortality in general population declines. Elevation of pro-inflammatory factors, consistently increased activation of B and T cells in RA patients in combination with traditional CV risk (CVR) factors drive CVD and promote atherosclerotic lesions. EULAR recommends annual CVR assessment using national guidelines for all patients with RA (Agca et al., 2017). However, the optimal approach to estimate CVR remains unclear. The existing guidelines focus on disease activity control to reduce the excess of CVR, rather than suggesting an accurate algorithm for choice of therapy. The guidelines recommend aggressive treat-to-target approach in order to reduce the systemic effect of chronic inflammation. However, we still miss a big population of RA patients with increased CVR despite advanced control of inflammation. The mechanisms linking together inflammation, metabolic imbalance and RA therapies with respect to CV outcomes are still untouched (DeMizio et al., 2020). Analysis of modern observational studied demonstrated that high risk of CVD in RA might result from both traditional risk factors and RA specific CVD risk factors (Wang et al., 2019). The overview about different risk factors is illustrated in Figure1.

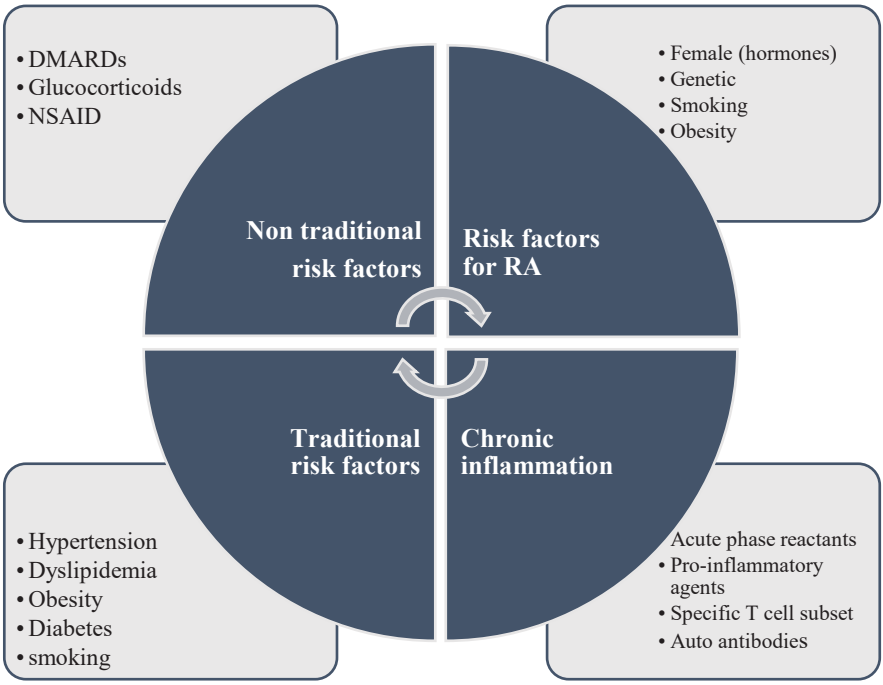

Figure 1. *Risk factors for RA and CVD.*

2.1 Prevalence of CV mortality in RA

Population-based studies show about 1.5-fold higher CV mortality in RA patients compared with the general population (Van den Hoek et al., 2017). RA disease is suggested to be an independent CVR factor comparable to diabetes mellitus (Peters et al., 2009; Lindhardsen et al., 2011). Other studies have provided evidence for an increased incidence of atypical infarction and silent ischemic heart disease in RA patients. Higher mortality after myocardial infarction has also been observed in RA population (Maradit-Kremers et al., 2005; Douglas KM et al., 2006). Similar trends are reported for cerebrovascular events and venous thromboembolism (Bacani et al., 2012; Avin˜a-Zubieta et al., 2008).

The traditional CVR factors including age, gender, high blood pressure, obesity, hyperlipidemia and smoking have not been able to independently explain the excess of CVD in RA. Some of these CVR such as obesity and smoking are identified as a part of RA pathogenesis (Symmons et al., 1997). Several studies indicate that other underlying factors than traditional risk factors are responsible for CVD in RA, for example inflammation. RA is a systemic inflammatory disease with high levels of CRP, TNFα, IL6 and IL1. On the other hand, recent studies highlighted the novel role of inflammation in impaired endothelial function resulting in formation of atherosclerotic plaque and increased intima-media thickness (Gonzalez-Gay et al., 2005). Endothelial dysfunction has been observed both in younger patients with no risk for CVD and in patients with established RA (Vaudo et al., 2004). A meta-analysis reported high risk for stroke independent of other traditional CVR factors or RA treatment in younger patients (< 50 years) with rheumatic disease (Wiseman et al., 2016).

Solomon and colleagues conducted a prospective study among a big population of women (n= 114 342), who were free from CVD and RA at the baseline and found that the women prospectively diagnosed with RA had more than 2-fold higher risk of developing ischemic cardiac infarction, even after adjusting for traditional CVR factors (Solomon et al., 2003).

Contribution of traditional CVR factors in estimating for newcoming CV events has only a limited value in RA population. Appropriate and modified diagnostic methods are needed for better detection of CVR in the patients with RA (Del Rincon et al., 2001). In other words, we need to have a better understanding for the interaction between the inflammatory mechanisms and the traditional CVR factors that initiate atherosclerosis in RA.

2.2 CVR factors

Hypertension is one of the predominant predictors of CV events, both in general population and in patients with chronic inflammatory diseases. High blood pressure can occur as consequence of inflammation or as side effect of disease modifying anti-rheumatic drug (DMARD) and disease-specific treatments like glucocorticoids.

The incidence of undiagnosed hypertension, dyslipidemia, and type II diabetes is high in patients with RA and treatment target goals are achieved in roughly half of RA patients with CVD. Because of tight interaction between different traditional risk factors, CVR estimation calculators have been composed to help clinicians to assess the effects of risk factor combinations. However, there is a lack of consensus concerning CVD risk estimation in RA. Some of risk calculators underestimate, while some others overestimate the CVR (Wagan et al., 2016).

2.3 Cardiovascular risk estimation

Various CVR estimation methods have formed based on traditional CVR factors. These methods predict the percentage of CV event in the next 10 years. Gender, blood pressure, total cholesterol, smoking habits and diabetes are included in most of the algorithms. However, family history of premature CVD, physical inactivity, high calorie diet, obesity, lipid profile are often not included in the risk estimation.

One of the pioneers in development of the estimation algorithm is the Framingham risk score (FRS) that includes the body mass index. This is one of the most frequently used algorithms to predict the CV morbidity and mortality. Variables such as age, gender, and traditional CV risk factors such as presence of diabetes, current smoking, as well as treated hypertension, values of systolic blood pressure and BMI are included in FRS BMI. It can be used for calculation of CVR for individuals aged 30 to 74 years (D'Agostino et al., 2008).

Reynold's risk score has been developed by using data from the Framingham Heart Study (Bitton et al., 2010). Most of the epidemiological findings in CVD are based on this study. This study is based on three generations of research on heart disease. It has been started at 1948 in Framingham town in Massachusetts with over 5000 men and women between the ages of 30 and 62. In 1971 second generation, and in 2002 the third generation of original participants were included (https://framinghamheartstudy.org/fhs-about/history/). Over the years, careful monitoring of study subjects has led to the identification of different major CVD risk factors. Correction factors for patients with a family history of early CVD, high triglycerides or low HDL-cholesterol are used.

Other estimating scores have been developed. The Systematic Coronary Risk Evaluation (SCORE) was developed in collaboration of 12 European counties (Figure 2). The SCORE index is a flow chart constructed both for the low and high-risk regions of Europe. The parameters used to calculate the SCORE value include age, gender, total serum cholesterol level, smoking history and the systolic arterial blood pressure (Arts et al., 2016; Conroy et al., 2003). However, SCORE is recommended for use in the general population and therefore underestimates the CVR in patients with RA. A multiplication of the estimated CVR by 1.5 for patients with RA is recommended to correct for this underestimation (Peters et al., 2010). A limitation with SCORE is that risk assessment is valid between the ages 40-70. Younger people can show low values for a 10-year risk, therefore preventive measures should still be employed. No correction factors are used but existing cardiovascular disease, diabetes, family history, very high levels of total cholesterol, and blood pressure are used as exclusion criteria.

The European League Against Rheumatism (EULAR) has recently provided recommendations for cardiovascular risk assessment and prevention in patients with RA (Agca et al., 2017). This includes risk assessment using index or other risk assessment instruments. Expanded Risk Score for cardiovascular disease in RA (ERS-RA) has been developed using the North American patient cohort CORRONA (Solomon et al. 2015). The 10 year risk of developing CVD is calculated based on traditional CVR included disease related parameters such as disease duration, cortisone treatment, disease activity index (CDAI), functional ability as evaluated by the health assessment questionnaire (HAQ). One of the latest risk score estimator, Q Research Cardiovascular Risk Calculator (QRISK), is based on observational study from UK (Hippiley-COX et al., 2010) and includes socioeconomic status, ethnicity and presence of RA as modifier to estimate CVR.

In 2003, American College of Cardiology and American Heart Association (ACC/AHA) developed a 10-year atherosclerotic CVD risk algorithm for the patient group with high CVR (Conroy et al., 2003). In the cross-sectional study, Ozen and his colleague compared Atherosclerotic Cardiovascular Disease (ASCVD) estimator, mSCORE and QRISK with gold standard intima-media thickness or carotid plaque by ultrasound (Ozen et al., 2013). The study found the estimation of ASCVD much closer to the ultrasound result compared to QRISK and mSCORE. The comparison of different CVR estimation algorithms is highlighted below in Table 1.

Table 1. *Comparison of different CVR estimation algorithms.*

Algorithm / Indicator	FRS	FRS - BMI	ASCVD	mSCORE	ERS-RA	QRISKII	Raynold
Age	+	+	+	+	+	+	+
Sex	+	+	+	+	+	+	+
Race	−	−	+	−	−	+	−
Systolic BP	+	+	+	+	−	+	+
Smoking	+	+	+	+	+	+	+
BMI	−	+	−	−	−	−	−
Lipid profile	TC, HDL	TC, HDL	TC, HDL, LDL	TC, HDL	TC, HDL	TC, HDL	TC, HDL
Diabetes	+	+	+	−	+	+	−
Family history of premature CVD	−	−	−	−	−	+	+
Incident CV events	+	−	−	−	−	+	−
DD>10	−	−	−	+	+	−	−
Extra-articular engagement	−	−	−	+	−	−	−
Seropositivity	−	−	−	+	−	−	−
Statin medication	−	−	+	−	+	−	−
BP treatment	+	+	+	−	−	+	−
Asprin medication	−	−	+	−	−	−	−
Corticosteroid treatment	−	−	−	−	+	−	−
CDAI	−	−	−	−	+	−	−
HAQ	−	−	−	−	+	−	−
CRP	−	−	−	−	+	−	+

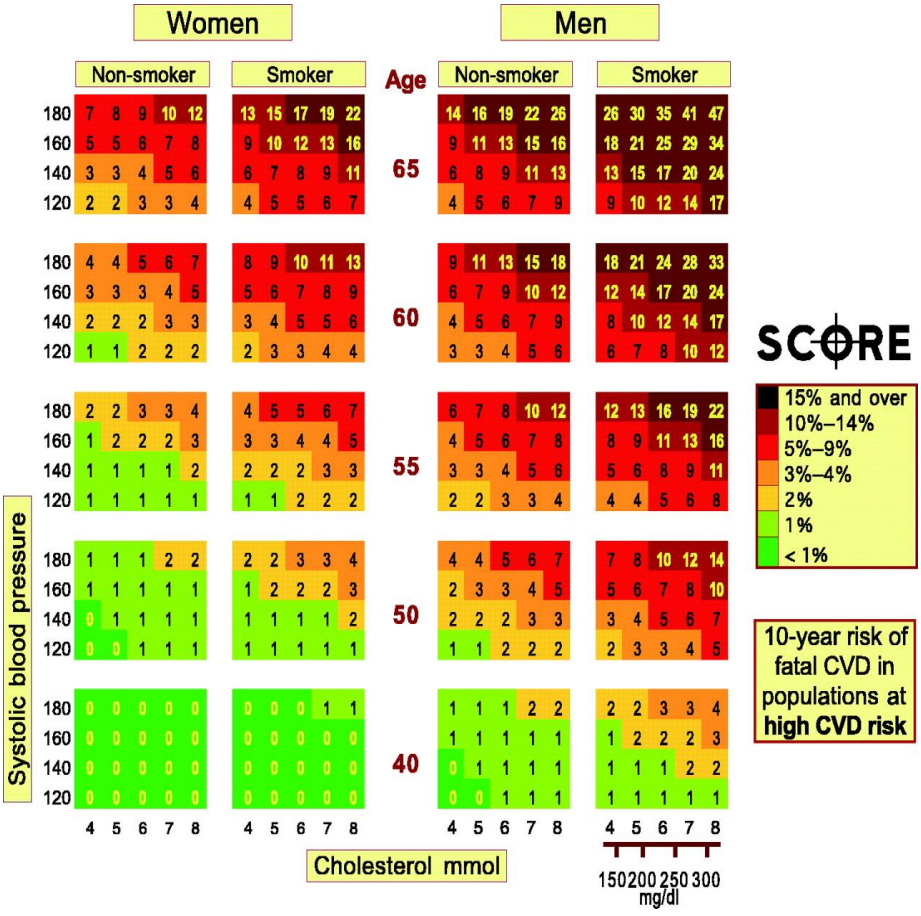

Figure 2. *Estimation algorithm for 10-year risk of fatal CV (in populations at high cardiovascular disease risk).*
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3 INFLAMMATION IN RA PATHOGENESIS

RA is a chronic inflammatory disease. Decades of research have failed to demonstrate a single trigger for RA. A range of soluble inflammatory molecules, autoantibodies, cytokines and signal transduction pathways contribute in different ways to the disease. Despite numerous studies within this field, it is still not clear exactly which pathway has the key role in induction and progression of inflammation. From one side, the innate immune system recognizes pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) in response to infections. On the other side, increasing number of endogenous host-derived molecules, named damage associated molecular patterns (DAMPs), will be identified by various innate immune receptors in response to cellular stress, or tissue damage and promote inflammation and immune responses in the absence of infection. It is proposed that continuous exposure to DAMPs may cause loss of tolerance to self-antigens in autoimmune diseases such as RA (Álvarez et al., 2017). In context of non-sterile inflammation, a cascade of inflammatory pathways is identified under the past decades. They stimulate immune system by activating PRRs.

Toll like receptors (TLRs) are the most studied of PRRs with established role in chronic autoimmune diseases. Diverse TLRs are present on the surface of different antigen presenting cells (APCs) and initiate different signaling pathways. Sterile inflammation is essential for regeneration and tissue repair in the organs, but it may lead to development of a range of autoimmune, metabolic and dysplastic disorders (Gong et al., 2020). Other receptors such as receptor for advanced glycation end products (RAGE) can induce DAMP-initiated inflammatory response independently of PRRs.

In the context of RA, joints are the natural targets for inflammation. The synovium in normal joints is a thin layer, which is poor on cells. It serves the cartilage with nutrients and produces joint lubricants. It comprises intima, which is the continuous surface layer of cells, and subintima, the underlying tissue, which contains blood and lymphatic vessels. The major population of synovial cells are fibroblast-like synoviocytes, which contribute to production of lubricating agents and joint homeostasis. The other cell subset inhabiting joints is macrophages with phagocyting and antigen presenting function, i.e. interacting with T cells. Under the conditions of inflammation, the layer of synovial fibroblasts expands and inflammatory T cells, B cells and macrophages invade subintimal layer. Formation of new blood vessels supports the hyperplastic synovial tissue. The extensive proliferation of the synovial tissue and vessels results in formation of granulation tissue known as “pannus”. Pannus is overloaded with cytokines and inflammatory mediators capable of invading adjacent tissues as cartilage and bones and causing erosions. The structure of healthy and RA joint is depicted in Figure 3.

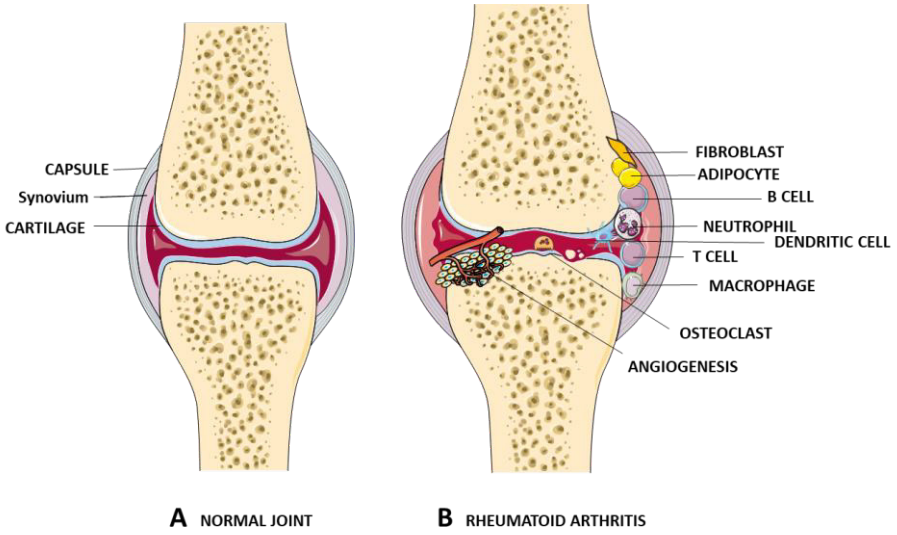

Figure 3. *Schematic view of (A) a normal joint and (B) a joint affected by RA*

3.1 Autoimmune inflammation in RA

CD4+ T helper cells are central organizer of the immune response in RA. They amplify or suppress the intensity of inflammation, recruit and stimulate other immune cells, and make B cells to generate high affinity antibodies. CD4+ T cells comprise functionally distinct subsets of T helpers recognized as Th1, Th2, Th17, and T follicular helper (Tfh) cells. Cytokines direct the differentiation of these subsets of helper T cells, and selective cytokine secretion is the major mode of helper T cell effector function. Figure 4 illustrates different T cell subsets and their derived cytokines in RA.

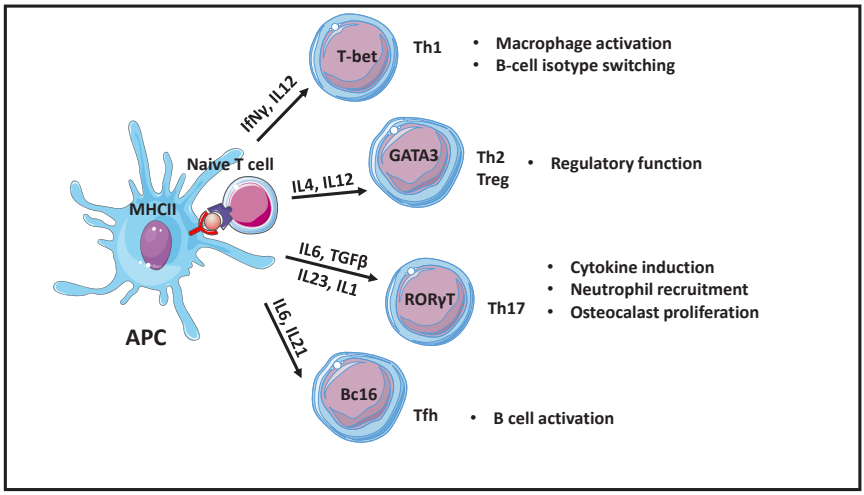

Figure 4. *CD4+ T helper cell subsets in RA*

As synovitis is the main manifestation in RA, numerous studies focused on the role of immune cells in synovia. T cells, B cells and APCs such as macrophages, and dendritic cells are accumulated in the inflamed synovia. All these cells produce proinflammatory cytokines and chemokines, activating and regulating each other´s function. Memory CD4+ T cells are expanded in the inflamed joint.

IFNγ and IL1 are produced by Th1 cells and have an important role in the pathogenesis of RA and cartilage destruction. One of the crucial roles of IL1 is promoting macrophages to produce pro-inflammatory cytokines, such as TNFα which is macrophage- and endothelial cell activator and is a powerful stimulator for other pro-inflammatory cascades, IL6, IL1β and IL8. (Van den Berg et al., 1999). Th2 cells are responsible for extracellular immunity, and Tfh are needed to maintain germinal centers of B cells and help them to differentiate into plasma cells and memory B cells (Crotty et al., 2011). Another subset of T cells is Th17 cells. These cells are involved in host defense at mucosal site. In RA, they are implicated in the pathogenesis by producing proinflammatory cytokines interferon gamma (IFNγ), IL17 and IL6.

A complex interplay between different T cell subsets and pathways is characteristic for RA. This presents a serious obstacle for resolution of RA inflammation by intervention on level of a single pathway (Chemin et al., 2019). Thus, RA has no cure.

In healthy immunity, self-reactive T cells are destroyed in thymus through a negative selection. However, some self-reactive T cells escape this process and keep the capacity to initiate autoimmune inflammation. Thus, regulatory T cells (Tregs) are required to protect against auto-inflammation. Tregs are characterized by low proliferative capacity and by their ability to suppress CD4+ and CD8+ T cell immune responses. Tregs in RA synovial tissues are characterized by expression of FOXP3, CD25, and CD4 receptors. In RA, their function is affected by multiple cytokines as TNFα and IL6. Treg and Th17 cells have opposing functions. In the presence of proinflammatory cytokines like IL6 the activity of Tregs declines. Hence, intervention on IL6R contributes to improvement of RA disease (Samson et al., 2012).

3.2 Activation of T cells by antigen-presenting cells

When T cells are introduced to peptide antigens through major histocompatibility complex (MHC) class II molecules, they become activated. When they get activated, they divide rapidly and produce cytokines that regulate or assist the immune response. Dendritic cells (DCs) are important APC that act as a link between innate and adaptive immune system. In the individuals with genetic and physiological susceptibility for RA, MHC-II molecules are used by DCs to present antigens to the T cell receptor. This induces activation of naive T cells and transforms them into effector CD4+ T cells. Activated effector T cells cross the endothelial cell wall barrier and move into the joint tissue. T cells expand in the synovial tissue. The expanded colony of T cells produces cytokines, which attract other cells into the joint. Inflammatory signals from DCs stimulate the transcriptional activity of nuclear factor kappa B (NF-κB). NF-κB can be induced by TNFα signaling or by other proinflammatory molecules. NF-κB activates signal transducer and activator of transcription 3 (STAT3). STAT3 increases production of IL6 by promoting Th17 cells. This results in cell survival, cellular growth, apoptosis and maintenance of immune homeostasis and inflammatory responses (Neumann et al., 2000; Bettelli et al., 2007)

4 INFLAMMATION CAUSES CV MORBIDITY IN RA/ ADVERSE METABOLIC PROFILE IN RA

The interplay between inflammation, metabolism and CV morbidity in RA is a complex issue. The widespread systemic impact mediated by pro-inflammatory cytokines in RA alters metabolism in RA via the production of cytokines, adipocytokines and metabolic intermediates. The high-energy demand by invading immune cells causes the accumulation of metabolites and stimulates adipocytokines, which carry out signaling functions such as activating transcription factors, which act as metabolic sensors. These events drive inflammatory pathways, which in turn perpetuate chronic inflammation. It is still an unsolved question if metabolic changes are a consequence or the cause of RA pathogenesis. (Pucino et al., 2020).

Inflammation underlies all steps of atherosclerosis from plaque formation to plaque rupture. From inflammatory point of view, synovial changes in RA and unstable endothelial atherosclerotic plaque share common pathways (Libby et al., 2008). For instance, TNFα, IL1 and IL6 are known to be raised in both conditions. Animal and human data suggest that multiple environmental factors influence mucosal immune function through the host genetics by enhanced mucosal permeability and induction of pro-inflammatory agents and the amplification of autoimmune responses. (Bergot et al., 2019).

Obesity is a potent CVR factor in general population. The European Society of Cardiology has recently reported a 2-3 folds increase of prevalence of obesity (body mass index ≥30 kg/m^2) and T2D in Europe over the last 30 years (Timmis et al., 2020). The prevalence of RA is also higher in the countries with simultaneously higher prevalence of obesity (Myaesodova et al., 2007; Alamanos et al., 2005). Several epidemiological and observational studies have identified obesity as a common risk factor for RA with impact on diagnosis, treatment and long-term adverse outcomes for CVD in the patients (Pedersen et al., 2006; Ljung & Rantapää-Dahlqvist et al., 2006), while other studies have reported the opposite (Turesson et al., 2016; Cerhan et al., 2002). Considering that weight is a dynamic parameter and changes over time, especially in chronic inflammatory diseases, it is not sufficient to look at the patients ordinary BMI. Individuals with similar BMI can present different body fat distribution. The phenomena “cachectic obesity “has been introduced to describe the proportion between skeletal muscle and adipose tissue (AT) in chronic inflammatory diseases such as RA. The patients develop excessive fat mass while they present decreased or normal BMI by losing fat-free mass (Summers et al., 2008). Accordingly, the clinicians encouraged to remember that many RA patients might have significant adiposity despite normal BMI. Kita’s group showed that using BMI as an indicator for assessment for

overweight in RA patients should be adjusted to lower cut-off point compared to general population (Stavropoulos-Kalinoglou et al., 2007). In contrary, successful control of inflammation may result in weight gain (Jurgens et al., 2013).

Another challenge in assessment of CVR in RA is divergent lipid profile compared to general population. The association between dyslipidemia and CVR in RA is complex. Systemic inflammation appears to be a potential contributor to the deviated lipid profile changes in RA. Untreated RA disease may result in paradoxical lipid changes such as lower levels of total cholesterol, low-density cholesterol (LDL) and high-density cholesterol (HDL) (choy et al., 2009). However, intensified RA treatment and decline in inflammation increases the lipid levels in serum (Steiner et al., 2009). This lipid increasing effect has reported in RA patients using traditional and/or biologic DMARDs (Daien et al., 2014).

The mechanisms by which the inflammation causes lipid changes are not fully understood. Systemic inflammatory state in RA may cause both quantitative and qualitative changes in HDL and thereby induce alteration of its anti-inflammatory and atheroprotective properties.

Production of CRP and other inflammatory mediators may increase the oxidation and uptake of LDL by macrophages and hepatocytes (Singh et al., 2008). Giles et al reported 3-fold higher CVR in RA patients with extremely low LDL levels (Giles et al., 2019). All the patients underwent cardiac computed tomography to quantify the coronary arterial calcification score (CAC) to assist estimation of CVR (Giles et al., 2020).

The athero-protective function of HDL to transport cholesterol from circulation to the liver and to stop oxidation of LDL is impaired in RA, which accelerates CVR (Charles-Schoeman et al., 2012). Alternative hypothesis is that an increase in total cholesterol, LDL, HDL and triglycerides in RA patients during DMARD treatment may reflect anti-inflammatory effect of the treatment and normalization of lipid levels to those seen in the general population. Paradoxically, the observed increase in lipid levels was not associated with a rise in CVD (Choy et al., 2009). Another population-based longitudinal study in RA patients (n=651) with tight follow up of inflammatory parameters and lipid profile presented also a reversed relationship between lipid profile and CVD with high ESR as a probable confounder for this association (Myasoedova et al., 2010). This complex interplay between lipids and inflammation in RA includes an impact of inflammation on lipid composition as well as the phenomenon of the lipid paradox contributes to difficulties with interpretation of lipid profile and limits their practical usefulness for estimation of CVR. One of the aspects contributing to this underestimation may be the lipid paradox and the other is utilizing disease duration over10 years as increasing criteria for CVR, while the studies report

increased CVD already earlier in the disease course (Nikiphorou et al., 2020). Thus, more accurate estimation tools for identifying CVR in RA patients are needed.

4.1 Adipose tissue and metabolism in CV morbidity

RA is associated with significant changes in body composition, lipid profiles, adipokines and insulin sensitivity. Metabolic changes, such as alteration of lipid profile occur even in preclinical RA. On one hand, paradox impact on lipids, decreased muscle mass and alteration in glucose metabolism have been recognized in active RA. On the other hand, RA is not just limited to inflammation in the joints, but it affects different organs including the AT. Changes in adipocytokines have important role in stimulation of inflammatory mediators underlying increased CVR in RA.

4.1.1 Adipose tissue as an endocrine organ

AT is categorized into white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the most abundant type of AT found throughout the body in different subcutaneous and visceral depots. WAT participates in energy regulation by homeostasis of fatty acids in adipocytes and regulation of the immune system. BAT is found in the neck, above the clavicle and around the spine in adult humans. It generates heat and regulates body temperature. Visceral fat is located inside the peritoneal cavity, whereas subcutaneous fat beneath the skin is called subcutaneous AT. Both consist of WAT.

Not too long ago, AT was regarded a passive organ for energy reservoir and for protection of body organs. The interest in AT increased when first signal molecule leptin was discovered 1994 by Zhang et al. Subsequent discoveries revealed a broad spectrum of other proteins and molecules collectively termed adipokines that are pivotal for hemostasis and survival of the whole organism. Adipokine production provides a biologic link for association between adiposity and inflammation. AT is a key player in immune response. For instance, it can activate monocytes and has impact on both adaptive and innate immune system and increases the levels of TNFα. IL6, IL12 and other inflammatory mediators (Gomez et al., 2011).

AT consists mainly of adipocytes, but other cells such as macrophages, neutrophils, CD4 and CD8 T cells, fibroblasts, endothelial cells are also present. All those cells signal inside the adipocytes to trigger adipokine production.

WAT has a high nerve and vasculature density and has tight interaction with liver, muscles, brain, bone, pancreas, vasculature and reproductive system (Lee et al., 2009). Adipocytes express mediators common for macrophages and

osteoblasts and can be transdifferentiated into these cell types. In obese individuals, low grade chronic inflammation is observed in WAT where anti- and pro-inflammatory balance is critical.
Different receptors and transcriptional genes in AT are tightly associated with adipokine production. Toll like receptor 4 (TLR4) is expressed on adipocytes. Its activation increases the levels of proinflammatory factors such as TNF, IL6, IL1 and IL8. This can trigger angiogenesis and promote differentiation of effector T helper cells in RA (Rogero et al., 2018; Elshabrawy et al., 2017).

4.1.2 Adipokine members

Leptin is the most widely studied adipokine involved in both metabolism and immune responses. Leptin is produced mainly from adipocytes and suppresses appetite and energy intake (Farooqi et al., 2009). Leptin receptor is expressed in many essential organs and cells for instance bone marrow, vascular endothelium and T cells. Hence, it possesses pivotal role in immune system, acute phase reactants, sex hormones, insulin and lipoprotein metabolism. Under critical conditions such as infection, leptin increases the levels of inflammatory mediators, downregulates T cell apoptosis and derives T cells differentiation towards Th1 response and production of cytokines through different immune cells (Derdemezis et al., 2011).

Adiponectin is also synthetized by adipocytes and because of its anti-diabetic and anti-atherogenic properties has been an attractive molecule to study. Adiponectin levels are lower under disturbed metabolism homeostasis such as insulin resistance, T2D and obesity.

Visfatin is produced mainly in visceral fat. It was known primarily as a growth factor for early B cells. Visfatin has insulin-mimetic effects and is elevated in metabolic syndrome and diabetes (Filippatos et al., 2008).

Resistin is an adipokine that acts as potent proinflammatory inducer. It is mainly produced in adipocytes but other cells such as macrophages can synthetize it. In human, pro-inflammatory cytokines such as IL6, TNF, IL12 and lipopolysaccharides, (LPS) upregulate expression of resistin on mononuclear cells but lower expression is found in WAT. Bacterial infection and acute phase proteins induce expression of resistin as well. Resistin mediates its pro-inflammatory effect via TLR4 that induces NF-κB activity. Resistin stimulates PI3/AKT signaling in response to growth factor or IGF and has a role in inflammation. Resistin induces cascade of inflammatory mediators, which helps in maintenance of inflammation. Activation of TLR4/NFκB through resistin generates insulin-resistant adipocytes.

4.1.3 Adipokine and obesity in RA

Several studies have linked obesity as predisposing factor for development of RA. Symmon et al. reported association between BMI> 30 and development for RA in a large population-based case-control study from the Norfolk Arthritis Register in England (OR=3.7). A meta-analysis of 11 studies presented that the relative risk for RA was 1.15 among overweight and 1.31 for obese subjects, compared with normal weight control group (Qin et al., 2015). Another meta-analysis reported the increased risk of RA by 13% for every 5 kg/m increase in BMI. Furthermore, the association between BMI and RA risk showed to be stronger among women than men (Feng et al., 2016).
Obesity is usually associated with more severe disease activity and a reduced probability of response to DMARD such as TNFα-inhibitors. Overweight increases the risk for developing RA in women in younger age (Lu et al., 2014). Body composition in RA patients is different from general population. Lean body mass decreases in all body parts whereas the fat distribution makes a clear shift to abdominal part in these patients (Westhovens et al., 1997). Additionally, it has been evidenced that AT in RA is pathologically changed. The dysfunctional AT produces inflammatory mediators and adipokines that are able to not only influence cartilage and synovium, but even increase CVR in RA populations (Lago et al., 2011).

Elevated levels of leptin have been detected in the serum and synovial fluid in RA patients (Bokarewa et al., 2003) (seven et al., 2009). Leptin is able to modulate multiple immune cells and has impact on regulation of different inflammatory and metabolic pathways. In RA, leptin acts as a proinflammatory mediator (Lago et al., 2011).

Resistin is considered as a potential adipokine in low-grade inflammatory conditions such as RA and synovial resistin levels were found potentially elevated compared to osteoarthritis patients (Schaffler et al., 2003). The production of resistin from different cells in RA in turn upregulates TNFα and IL6 production via NF-κB signaling (Klaasen et al., R 2012). Resistin induces in human's inflammatory response through central receptors i.e. TLR4 and IGF1R and constitutes a potential link between obesity and diabetes (Steppan et al., 2001).

4.2 IL6 and TNF in adipose tissue metabolism

IL6 is a member of the gp130-related cytokine family. Different cell types, such as T and B cells, monocytes, fibroblasts, osteoblasts, keratinocytes,

endothelial cells, mesangial cells and some tumor cells, produce IL6. IL6 activates cells by first binding to the α-chain of IL6R, which dimerizes with gp130 and activates receptor-associated kinases (JAK1, JAK2, and Tyk2) within the cell. IL6R functions in a membrane-bound and soluble forms, which are detected in the circulation and at sites of inflammation. IL6 is traditionally considered as an acute phase proinflammatory cytokine. It is also engaged in glucose metabolism, inflammatory homeostasis and hypothalamic-pituitary-adrenal axis (Jones et al., 2011). The increase in IL6 and sIL6R in synovial fluid elevates the risk for joint destruction in RA (Kotake et al., 1996). IL6 stimulates B cells to differentiate into antibody producing plasma cells (Jogo et al., 2001) and in combination with IL1β and IL23 derives differentiation and proliferation of T cells to IL17 producing Th17 cells (Acosta-Rodriguez et al., 2007). This would suggest that IL6 has a key role in the development of the adaptive immune response. Additionally, IL6 shifts acute inflammation to chronic by activating neutrophils that express membrane-bound IL6R to release sIL6R as they reach the site of inflammation. This recruits leucocytes through activation of trans- signaling in adjacent endothelial cells and results in a shift from neutrophil to monocyte infiltration (Marin et al. 2001). IL6 has a key role in systemic as well as in articular symptoms in RA and is implicated in damage to the articular cartilage. IL6 is also involved in lipid metabolism and development of coronary artery disease.
The successful treatment of autoimmune conditions with tocilizumab, the humanized IL6R antibody, emphasizes the importance of cytokines that signal through the gp130 which in turn activates JAK/STAT pathway. This pathway has been target for research as cytokines that signal via this pathway such as IFNγ, GMCSF, IL6, IL10, IL15, IL23 have become linked with the pathogenesis of chronic inflammatory diseases and cancer (McInnes et al., 2007). Biologics are able to target these cytokines (e.g., IL6R blockade by tocilizumab). Selective small molecule JAK inhibitors have also favorable therapeutic effect on RA.

STAT3 expression is correlated to cell proliferation and is associated with tumor growth, survival, and angiogenesis. The regulation of STAT3 pathway by IL6 has received substantial attention in the studies of cancer and autoimmune disorders. Alteration of STAT3 activity pathways have prognostic value of metastatic processes in certain cancers (Grivennikov et al., 2010). Furthermore, pharmacogenomic studies have identified genetic links between STAT3 and chronic disease. For instance, meta-analysis of a genome-wide association of European patient cohort found seven new rheumatoid arthritis risk loci. These included gene products associated with STAT3 signaling/activity and an additional suggestive risk allele in the IL6R gene (Stahl et al., 2010).

There is a cluster of active signaling molecules that modulate production of adipokines, of which classical cytokines such as TNF-α and IL6 are intensively studied with regard to inflammation and metabolic disorders. Although lipid metabolism and immune response are highly integrated, immunological intermediators and excess of harmful lipid products can interfere with immune-metabolic regulation. Hotamisligil et al. reported that in the obese individuals, overexpression of TNFα in fat tissue regulates insulin signaling negatively which indicates obesity as a low-grade chronic inflammatory state. Both IL6 and TNFα crosstalk with the cells that contribute to the attraction of macrophages and CD4+ T cells to adipocytes and control their differentiation. TNFα and IL6 increase vascular permeability and endothelial expansion and maintain hypertension and blood volume (Lauper et al., 2017)

5 IGF1R AND INSULIN SIGNALING

5.1 Effect of inflammation on IGF1R/insulin signaling

The role of IGF1R-AKT1 pathway in CVR and metabolic disorders has been attracting attention in decades. Insulin-like growth factor 1 (IGF1) is a potent growth factor highlighted in RA (Zhao et al., 2019). IGF1 has similar peptide sequence to insulin and has endocrine, paracrine, and autocrine effects. It is predominantly produced in the liver, but it is produced locally by a large number of tissues. In the recent decades, IGF1 has been linked to many physiological effects such as tissue growth and development, lipid metabolism, anti-aging, anti-inflammatory, anabolic, and antioxidant with hepato-protective properties. It is also implicated in bone and brain development, glucose homeostasis and insulin sensitivity. IGF1 enhances lymphocyte maturation. Deregulation of IGF1 is studied in cancer, T2D and CVD (Denley et al., 2005). IGF1 regulation in response to increased glucose production is slow in contrast to insulin and activates a couple of hours after food intake. Only 1% of total IGF1 is free and IGF1 function is regulated by IGF1 binding proteins (Frystyk et al., 2004). The main part of circulating IGF1 molecules is compounded with IGF binding proteins. Bound IGF1 is considered to be inactivate. This prolongs IGF1 half-life from minutes to hours. IGF-binding proteins modulate IGF1 binding to IGF1R or insulin receptor.

IGF1R belongs to the family of tyrosine kinase receptors. The biological functions downstream of IGF1R and insulin receptors are not always distinct as they share several intracellular signaling steps. The IGF1R generally mediates mitogenic while the IR metabolic functions. They have a high affinity for own ligands and somewhat lower affinity for other ligands. IGF1/insulin hybrid receptor combines the structure of both receptors with high binding affinity particularly for IGF1. The affinity of the hybrid receptor for its ligand modifies tissue homeostasis. The expression of IGF1R/insulin is identified in AT, skeletal, endothelial and smooth muscle tissues (Chisalita et al., 2005, 2006, 2009).

Activation of the IR/IGF-1R through IR substrate is followed by activation of phosphatidylinositol 3-kinase (PI3K) and consequent stimulation of AKT (protein kinase B). Activating of several transcriptional factors supports altered glucose regulation in liver and impair adipocyte differentiation. Other pathway downstream of IGF1R/IR is via extracellular-regulated kinase/ mitogen-activated protein kinase (ERK/MAPK) signaling. This has mitogen function and is involved in cell growth, survival and differentiation (Taniguchi et al., 2006). Intracellular effects of IGF1R are mediated by Janus kinases (JAK)-

STAT3 activation. In human and experimental RA, intervention of the IGF1 pathway caused the reduction of IGF1 levels and increased expression of IGF-binding proteins, which decreases bioavailability of IGF1 despite elevated IGF1R expression. RA is characterized by low levels of IGF1 in serum, suggesting deep impairment of IGF1 system. Further studies focused on competitive ability of cytokines and adipokines to promote inflammation through IGF1R, while IGF1 levels are low (Boström et al., 2011). IGF1R expression is detected in synovial fibroblast and leucocytes in inflamed joint (Verschure et al., 1995). However, the role of IGF1R in immune response is not entirely clear. Expression of IGF1R has shown a considerable increase on peripheral T cells following their activation via T cell receptor and co-stimulatory CD28 molecule and it is pivotal for survival of activated T cells. Increased generation of activated B cells in spleen has reported by stimulation of CD4+ T cells followed by IGF1 administration (Walsh et al., 2000). Activated IGF1R modulates inflammatory process by promoting IL10 and IL4 producing Th2 cells and inhibiting interferon δ that in turn induce regulatory T cells (Bilbao et al., 2014).

IGF1 counteracts inflammation. Suppression of inflammation in diabetes type I, in the early stadium of autoimmune encephalopathy and in psoriasis has been reported by IGF1 administration (Gluckman et al., 1992; Ristow et al., 1993). Smoking and adipokines led to alteration of IGF1 levels and triggered inflammation in RA (Erlandsson et al., 2016). In contrast, higher IGF1 has a favorable effect on inflammation, thereby several intervention studies have been conducted, both *in vitro* and *in vivo*, to modulate the inflammation. Forsblad d'Elia and colleagues observed increased levels of IGF1, and inversely lower IL6 levels in RA patients who received hormone replacement therapy (D'Elia et al., 2003). In the present thesis (paper IV), we highlight the role of IGF1R signaling on inflammation and RA disease activity by manipulating IRS1/IRS2 in experimental setting.

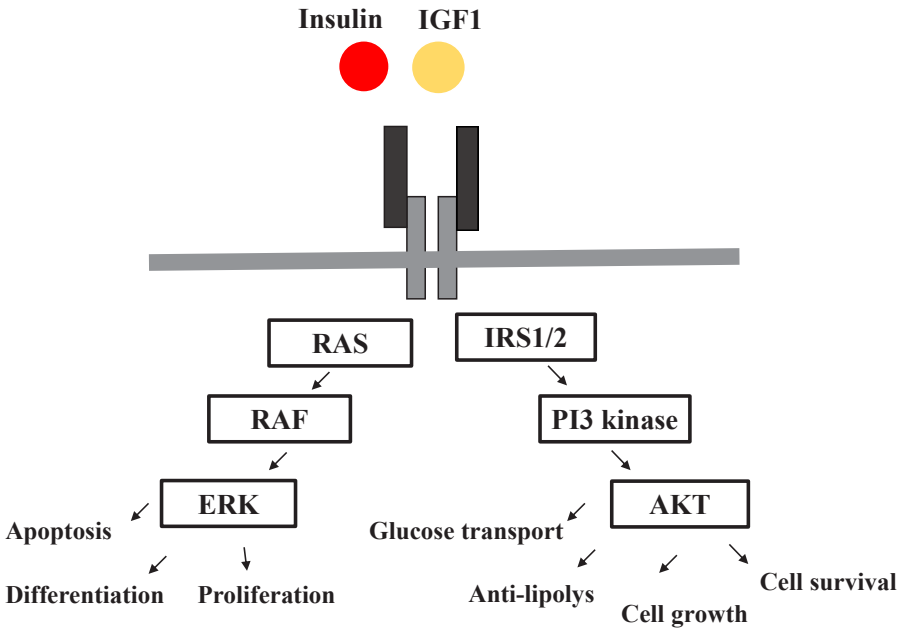

Figure 5. *Schematic illustration of intracellular signaling through IGF1 receptor.*

5.2 IGF1R and insulin signaling in CV morbidity

IGF1 is connected to regeneration and reparation of endothelial cells by inhibiting oxidative stress. It causes also vasodilatation and improvement of hypertension in experimental studies (Higashi et al., 2012). In clinical studies, disorder in GH/IGF1 production has direct association to hypertension (Bondanelli et al., 2001). Other studies on general population have reported that low levels of circulating IGF1 have inverse impact on blood pressure and cardiovascular mortality and morbidity (Conti et al., 2004). Lower levels of IGF1 are tightly associated with aging, obesity and diabetes. Recent studies have indicated that IGF1 plays a profound role in improvement of cardiovascular diseases by anti-inflammatory function, stimulation of angiogenesis and improvement of cardiac contractility during exercise (Higashi et al., 2019).

The role of IGF1R/IR on cardiovascular system is complex. Studies in animal models have presented that absence of IR in the heart has impact on cardiac metabolism while deletion of IGF1R cause no basal cardiac impairment but disturbs exercise induced cardiac function. Absence of both receptors cause fatal cardiomyopathy early after birth (Laustsen et al., 2007).
Endothelial cells express both IR, IGF1R as well as hybrid form of IR/IGF1R. Current studies suggest that the concentration of IR/IGF1R is determined by monomeric ratio of IGF1R and IR. Accordingly, higher IGF1R expression results in elevated formation of IR/IGF1R and lower concentration of IR. This phenomenon results in metabolic dysregulation in different cells such as endothelial cells, smooth muscle cells and adipocytes. These cells have critical role in pathophysiology of CVDs (Belfiore et al., 2009).
In paper I, we have comprised two independent cohorts to study IGF1 related molecules that increase cardiovascular risk.

6 INFLAMMATION AND AGE/RAGE SIGNALING

Under the condition of hyperglycemia, non-enzymatic glycation is carried out by the attachment of sugar to biological macromolecules such as proteins and lipids in order to reduce sugar. This non-enzymatic reaction is irreversible and attributes to higher production of advanced glycation end products (AGEs). Unfavorable environmental factors such as smoking, air pollutants and highly processed foods accelerate production of AGEs. In endogenous manner, metabolic disorders such as hyperglycemia, hyperlipidemia and obesity are the substantial factors inducing AGEs.

Receptor for AGEs (RAGE) was introduced by Schmidt et al. 1992. RAGE is a member of the immunoglobulin superfamily of cell surface molecules, which is present in a range of cells involved in innate immune response (Bucciarelli et al., 2002). AGE-RAGE combination is a major modulator of inflammation. Soluble RAGE blocks this combination by acting as a decoy and binds AGEs without activating inflammatory pathways. The consequent is that the level of sRAGE is reduced due to utilization in the metabolic states. Monocytes, macrophages, endothelial cells, adipocytes and vascular smooth muscle cells express RAGE. Full-length RAGE comprises immunoglobulin-like regions as V domain, which has key role in ligand binding and C domain for intracellular signaling. C domain connects to intracellular sphere via transmembrane domain and cytosolic tail (Yonekura et al., 2003). Soluble RAGE (sRAGE) has multi-ligand structure and has the ability to bind to different molecules. Endogenous sRAGE is the spliced form generated by RAGE mRNA transcription, additional variants of sRAGE are formed by proteolytic shedding of the extracellular domain of membrane-bound RAGE. sRAGE acts as a decoy and can inhibit the connection between RAGE ligands and membrane-bound RAGE by itself binding the RAGE ligands. It thereby modulates the pro-inflammatory effect of cell-bound RAGE signaling (Schmidt et al., 2001). Low levels of sRAGE have observed in a variety of disorders such as early stage of endothelial dysfunction, coronary artery disease, hypertension, atherosclerosis, and diabetes with well-preserved renal function. Low levels sRAGE are reported in RA patients compared with patients with non-inflammatory joint disease and healthy controls (Pullerits et al., 2005). Brix et al (2012) reported significantly lower levels of serum sRAGE in morbid obese compared to healthy lean individuals. After two years of follow-up, the obese group presented elevated levels of sRAGE after bariatric surgery and weight loss, which was related to the improved homeostatic assessment of insulin resistance (HOMA-IR) and glucose tolerance.

A considerable number of studies investigated association between serum sRAGE and metabolic disorders. Those studies reported negative correlation between sRAGE with BMI in obesity, with recently diagnosed T2D, IR, and HOMA-IR (Miranda et al., 2017, Hagen et al., 2015).
There is a growing interest to understand a connection between AGEs and exacerbating metabolic dysregulation. It is well established that AGE accumulation leads to the development of diabetic complications. Accumulation of AGEs is associated with development of cardiomyopathy. In an experimental study, mice on western fast-food diet developed heart injury and inflammation. Prevention of RAGE activation showed favorable effect on cardiac disease and inflammation. This indicates the importance of monitoring of AGE/RAGE axis through dietary and pharmacological strategies for prevention or management of cardiac disease (Tikellis et al., 2008).

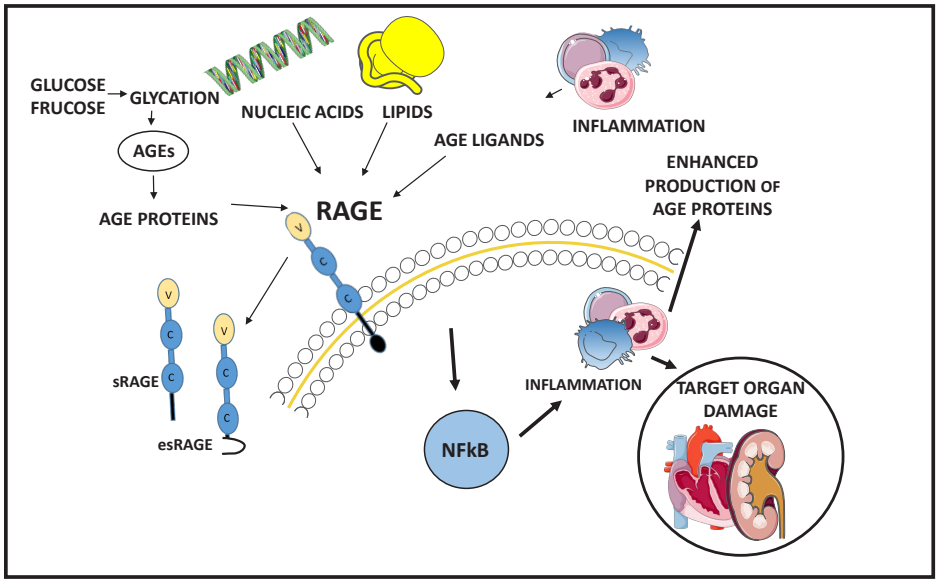

Figure 6. *The receptor for Advanced Glycation End Products (RAGE) is a key pathway for inflammatory complications.*

7 AIMS OF THE THESIS

- PAPER I :
  to investigate the role of IGF1 and IGF1/IGF1R signaling in CVD in RA

- PAPER II:
  to compare IGF1R signaling in fat tissue and blood leucocytes of patients with RA

- PAPER III:
  to assess the association between soluble RAGE levels and CVD in RA

- PAPER IV:
  to investigate the impact of IGF1R signaling in experimental arthritis

8 MATERIALS AND METHODS

An overview of the main methods used in this thesis is summarized below. Further detailed information is found in section "Materials and methods" in paper I-IV.
The studies were approved by the Swedish Ethical Review Authority and were conducted in accordance with the ethical principles of the Helsinki Declaration and Good Clinical Practice. All patients provided written informed consent before enrolment into the study.

8.1 Study subjects

8.1.1 Patients and controls

The cohort involved 184 female RA patients, consecutively recruited at the Rheumatology Clinic of Sahlgrenska University Hospital, Gothenburg, and the Northern Älvsborg County Hospital, Uddevalla. All the participants fulfilled the ACR 1987 classification criteria for RA (paper I-III).
Additionally, in paper I, we studied clinical and serological data of 132 consecutive female patients recruited at the Sahlgrenska University Hospital in the frame of the Sahlgrenska Academy Study on Ischemic Stroke at 3 months after index IS. The clinical and laboratory characteristics of patients are presented in Table 2.

Table 2. *The clinical and laboratory characteristics of RA patients (PAPER I-III)*

n=184 RA Female	Median [1:th-3:th Quartile]	n, Percentage
Disease duration,y	3.5 [4-14]	
Age,y	42 [45-62]	
SBP, mmHg	115 [120-140]	
DBP, mmHg	70 [70-81]	
BMI, kg/m^2	25.6 [22.4-28.1]	
Body fat content, %	35 [32.5-41.3]	
Cholesterol, mmol/L	4.4 [4.6-5.9]	
TG, mmol/L	1.2 [0.69-1.2]	
LDL, mmol/L	2.4 [2.68-3.83]	
HDL, mmol/L	1.7 [1.5-2.1]	
DAS28, arbU	3,xx [2-4.1]	
SR, mm/hour	9 [5-13]	
IGF1, ng/mL	138 [109-181]	
Leptin, ng/mL	23.9[11.2-37.5]	
Adiponectin, μg/mL	5.2 [3.2-8.3]	
Resistin, ng/mL	21.3 [12.6-36.5]	
Visfatin, ng/mL	2.6 [0.9-4.3]	
IL6, pg/mL	2.3 [0.1-7.9]	
MTX, mg/week	17.5 [12.5-20]	
bDMARD		(64) 34.8%
Current smokers		(28) 15%
Former smokers		(94) 52%
RF+ or ACPA+		(8) 21.4%

PAPER IV

Blood samples were collected from 84 female patients with RA. All the patients were treated with methotrexate. Clinical and demographic characteristics of the patients are shown in Table 3.

Table 3. *Clinical characteristics of the patients with RA grouped after IGF1R expression in WBC. The expression of IGF1R above the lowest tertile comprised the high IGF1R group. Data are shown as median [IQR].*

RA patients	Low IGF1R, $n = 28$	High IGF1R, $n = 56$	
Age, years	48.5 [40–56.2]	62 [51–64]	$p < 0.001$
Disease duration, years	6.5 [4–11]	8.0[5.0–11.5]	ns
BMI, kg/m^2	23.7 [22.4–24.9]	26.1[22.5–28.8]	$p = 0.01$
DAS28, arbitrary U	3.12 [1.81–3.69]	3.26 [2.42–4.26]	ns
Tender joints, n	1.5 [0–8]	3 [0–6]	ns
Swollen joints, n	2 [0.8–6]	2 [0–4.2]	ns
ESR, mm/h	7 [4.8–11.2]	12 [7–17.8]	$p = 0.003$
MTX-treated, monotherapy n (%)	28 (100%)	17 (31%)	ns
MTX-treated, Dose, mg/week	15 [12.5–20]	12.5 [8–20]	$p = 0.05$
Biological treatment, n (%)	9 (32%)	27(49%)	ns
TNF-inhibitors, n (%)	7 (25%)	18 (32%)	ns

8.1.2 Prospective follow-up (PAPER I and III)

A telephone interview was conducted 5 years after enrolment to study for each patient according to a standard questionnaire to collect information about any recent CVD events, T2D and current medications related to hypertension, diabetes and hyperlipidemia. The reported data about CVD events and medications were then controlled against medical records and the Swedish National Patient Registry.

The BP burden was calculated as a sum of systolic and diastolic BPs. Hypertension was considered by systolic BP was >140 mmHg, or diastolic BP was > 90 mmHg or current pharmacological treatment for hypertension. DAS28 of RA was calculated based on 28 tender and swollen joints and ESR.

8.2 Methods

8.2.1 Gene expression analysis (PAPER I-IV)

Quantitative PCR (qPCR) makes it possible to both detect and quantify the expression levels of the genes. Total mRNA was prepared using PAXgene RNA tubes and then High Capacity cDNA Reverse transcription kit was used to make complementary DNA. Quantitative PCR was performed on a ViiA™ 7 Real-Time PCR using SYBR Green qPCR Mastermix. Melting curves between 60 °C and 95 °C for each PCR were performed to ensure specificity of the amplified product. Relative expression levels of target genes were controlled to reference genes to obtain dCt. Relative quantity (RQ) was estimated using the ddCt method.
Gene expression for IGF1R, AKT1, STAT3, IRS1 and IRS2 on human WBC were calculated by qPCR (**PAPER I)**. The expression activity of selected genes, IGF1R, STAT3, AKT1, RELA, RETN and TLR4 were analyzed on human WBC and WAT (**PAPER II)**. The expression of IGF1R, NF-κB, STAT3 on human WBC and RoRγT, FoxP3, Tbet and STAT3 on murine spleen were anlyzed by qPCR (**PAPER IV)**.

8.2.2 Synovial tissue

Human synovial tissue of wrist joint of RA patients was collected in connection to hand surgery in RA patients and examined histologically. The synovial tissue samples were fixed with formalin and decalcified with TRIS-buffered EDTA followed by paraffin embedding, and thereafter sectioned in 4 µm-thin slices. Immunohistochemistry was completed by incubation with titrated rabbit antibodies for CD3, pSTAT3 and pIGF1R-Tyr1131. Staining was visualised using ImmPress anti-rabbit staining (Vector laboratories, Burlingame, CA, USA) and counterstained with Mayer's haematoxylin.

8.2.3 White adipose tissue (WAT) biopsy

The biopsy from WAT was performed on the right side of the pre-umbilical area on the abdominal wall by needle aspiration. A 40 mm long 18-gauge hypodermic needle was adjusted to a 20-ml syringe adapted to a piston. The sample was aspirated by providing vacuum through pulling back the plunger on the syringe several times. The samples were kept in reagent and stored in -80°C.

8.2.4 Serological measurements

In **PAPER I**, serological measurements In RA samples, serum IGF1, TC, triglycerides, HDL and LDL were measured by photometry. In the IS samples, serum IGF1 was determined by a radioimmune assay. Plasma glucose levels were measured by FreeStyle Lite. Sandwich ELISAs were used to measure insulin and IL6 and IL1b.
In **PAPER II,** specific Sandwich ELISAs were used to measure the serum levels of resistin, adiponectin, leptin, visfatin, IGF1 and IL6.
In **PAPER III,** the levels of sRAGE were detected by specific ELISA. ESR, blood lipids and RF/ACPA antibodies were analyzed at the Laboratory of Clinical Chemistry at the Sahlgrenska University Hospital according to clinical routines.

8.3 Animal study (Paper IV)

8.3.1 Induction of experimental arthritis

Arthritis was induced in female Balb/c mice by methylated bovine serum albumin (mBSA) which is widely used for experimentally induced antigen-specific inflammation in targeted organs. At the age of 8 weeks, mice were injected subcutaneously with 200 μg mBSA on the flank and after one week a booster dose of 100 μg mBSA was injected subcutaneously in the tail base. On day 21, the mice were injected by 30 μg mBSA intra-articularly in the knee joints and on day 28, they were killed by cervical dislocation. Then, spleen, liver, blood and knee joints were collected for further analysis.

8.3.2 Intervention in IGF1R signaling

In this study, we chose to disrupt IGF1R signaling in two different levels.

A. Inhibition of insulin receptor substrate 1 and 2 (IRS1/2) by injection of synthetic molecule NT157subcutaneously to mice:
NT157 induces inhibitory phosphorylation and degradation of IRS1/2. Mice were immunized by mBSA and from the first day were injected with NT157 five days/week in 28 days. They were injected using two different doses, 1mg/kg (n=8), and 10 mg/kg (n=16) whereas control group received placebo,

cyclodextrine vehicle (n=18). The experiments contained in total 50 mice and were performed twice.

B. Direct inhibition of IGF1R:

Lentiviral particles were applied to inhibit IGF1R directly by targeting small hairpin IGF1R RNA (shIGF1R). Control group received non-targeting RNA (shNT). The day before the first immunization with mBSA, each mouse received $3x10^6$ particles intra-peritoneally. Then on day 9 and 20 before intra-articular injection. The experiments contained in total 38 mice and were performed twice.
At the end of both experiments, blood, spleen, liver and knee joints were collected and the weight of spleen, liver and total body weight were recorded.

8.3.3 Cell culture preparation and stimulation

Single cell suspensions from spleens were prepared. Cells were counted by Sysmex device and then were re-suspended to a concentration of $1x10^6$/ml. The cells were seeded in 96-well plate and were incubated by 1 µg/ml anti-CD3 antibodies to stimulate TCR mediated effect. Then 25 µg/ml mBSA was added in the culture to stimulate antigen-specific T cells. After 48 hours of cultivation, the supernatants were collected and kept frozen.

8.3.4 Flow cytometry analysis

Flow cytometry by Becton Dickinson (BD) FACS Canto II was performed on whole blood and spleen supernatants from mice. Single cell suspensions in FACS buffer were pre-incubated with Fc-block (BD). Additional cell markers to CD3, CD4, CD19, CD, CD11b, CD11c, Ly6G and IGF1R were used to determine the role and activation status of the cells.

8.3.5 Histological evaluation for arthritis

The evaluation of arthritis in collected murine knee joints was performed regarding to cell scattering pattern and engagement of synovial tissue. The synovitis was semi-quantitatively graded as follows: grade 0 – no signs of inflammation; grade 1 – mild proliferation of synovial tissue where more than two intimal lining layers of synovium are invaded by inflammatory cells; grade 2 – multiple inflammatory foci in the sub-lining synovial layer; and grade 3 – scattered invasion of inflammatory cells throughout the synovial tissue (Dehlin

et al., 2011). Signs of cartilage and bone erosivity were evaluated separately (**PAPER IV**).

8.3.6 Cytokine measurements

Cytokines levels of IL17, IFNγ and IL6 of supernatants from murine spleen cell cultures were measured by ELIZA using 384-well plate. Murine IL2, IL10, IL4 and TNF were measured with a cytometer bead assay (BD) in **PAPER IV**.

8.4 Statistical Analysis

Data are presented as mean ± SD, median [IQR], in percentage or number. We used nonparametric methods for statistical analysis throughout this thesis since the distribution of our patient cohorts was skewed. Statistical differences between groups were performed using Mann-Whitney U test.
The patient cohorts were dichotomized into $IGF1^{hi}$ and $IGF1^{low}$ groups split by the median level (**PAPER I**) and by mSCORE <1 and mSCORE≥1 (**PAPER II**). The sRAGE concentrations within the lower 75% in CNRG were chosen as low and were used to dichotomize CME free RA patients to $sRAGE^{lo}$ and $sRAGE^{hi}$ (**PAPER III**). The levels of IGF1R within 2/3 upper part of tertiles indicated $IGF1R^{hi}$ (**PAPER IV**).
All correlations between variables in patients were performed with non-parametrical Spearman rank correlation test (**PAPER I-IV**). Correlation coefficients were assessed using Fisher r-to z analysis at http://vassarstats.net/rdiff.html. Two-tailed p-value< 0.05 was considered as a statistically significant difference between the groups.

9 RESULTS

9.1 Paper I

To study whether low levels of IGF1 are associated with CVD in RA, we chose a group of female RA patients and a control group of female none-RA patients 3 months after ischemic stroke. We divided both patient cohorts in $IGF1^{low}$ and $IGF1^{hi}$ groups in respect to median levels of IGF1 (p values in the $IGF1^{hi}$ column present the difference between IGF1 low and high in RA group in table 4). The frequency of hypertension and incidental measures of systolic BP were significantly higher in $IGF1^{low}$ group in RA. Hypercholesterolemia and higher BMI were also characteristic for the $IGF1^{low}$ group RA patients but not smoking or diabetes.

Table 4. *Clinical characteristics of female RA and stroke cohorts*

	RA		Post stroke	
	Low IGF < 140 ng/ml	High IGF1	Low IGF1 < 145 ng/ml	High IGF1
n	96	88	66	66
Age, years	59.0 [51.2–63]	47.5 $p<0.0001$ [34.5–55.3]	58.5 $p=0.73$ [51–66]	52.7 $p=0.004$ [39.8–61.7]
Serum IGF1, ng/ml	**109.5 [93–127]**	**182.5 $p<0.0001$ [158–225]**	111.6 $p=0.74$ [92–127]	183 $p<0.0001$ [167–223]
Height, cm	166 [161–169]	167 $p=0.041$ [163–172]	163 $p=0.005$ [160–167]	167 $p=0.009$ [161–172]
Systolic BP, mmHg	**135 [120–145]**	**121 $p=0.0027$ [120–140]**	135 $p=0.70$ [120–140]	130 $p=0.114$ [120–140]
Diastolic BP, mmHg	80 [75–85]	80 $p=0.023$ [70–80]	80 $p=0.037$ [70–80]	75 $p=0.058$ [70–80]
TC, mmol/L	5.6 [4.9–6.0]	5.0 $p=0.0022$ [4.1–5.9]	5.1 $p=0.0004$ [4.5–5.8]	4.8 $p=0.063$ [4.3–5.5]
LDL, mmol/L	3.4 [2.8–4.0]	3.0 $p=0.17$ [2.3–3.8]	2.8 $p<0.0001$ [2.1–3.50]	2.6 $p=0.89$ [2.30–3.30]
HDL, mmol/L	1.8 [1.5–2.2]	1.7 $p=0.20$ [1.5–2.1]	1.6 $p=0.0002$ [1.30–1.90]	1.5 $p=0.13$ [1.10–1.80]
BMI, kg/m^2	25.9[23.1–29.7]	23.9 $p=0.0014$ [21.9–26.7]	24.0 $p=0.17$ [21.8–28.3]	24.6 $p=0.94$ [22.5–28.0]
T2D, *n* (%)	2 (2.1%)	4 $p=0.39$ (4.5%)	12 $p=0.0004$ (19%)	7 $p=0.23$ (9%)
Current smokers, *n* (%)	15 (16%)	13 $p=0.88$ (16%)	32 $p<0.001$ (48%)	28 $p=0.49$ (45%)
Hypertension, *n* (%)	**25 (26%)**	**7 $p=0.011$ (8%)**	30 $p=0.012$ (45%)	28 $p=0.17$ (42%)

9.1.1 Low IGF1 and CVR risk

In RA cohort

CVR was significantly higher in IGF1^{low} group. Since both IGF1 and CVR are known to be age dependent, we analyzed the patients separately within different ages (Figure 7). Gradual increasing of eCVR was presented by increased age in both low and high IGF1 groups (Figure 7A). The younger patients (<50) in IGF1^{low} group had significantly higher BP burden, and metabolic disorders such as high BMI, TC, and LDL, compared to the age-matched IGF1^{hi} group and consequently had higher eCVR. The levels of IGF1 in IGF1^{low} RA patient group <50 years reached a low threshold with no further decline with age whereas in other three groups (Figure 7B), IGF1 levels reduced by aging. Among traditional CVR factors, hypertension had the most prominent frequency in IGF1^{low} group. Additionally, both pharmacological treatment for hypertension and values from incidental measurement of BP were significantly higher in IGF1^{low} group.

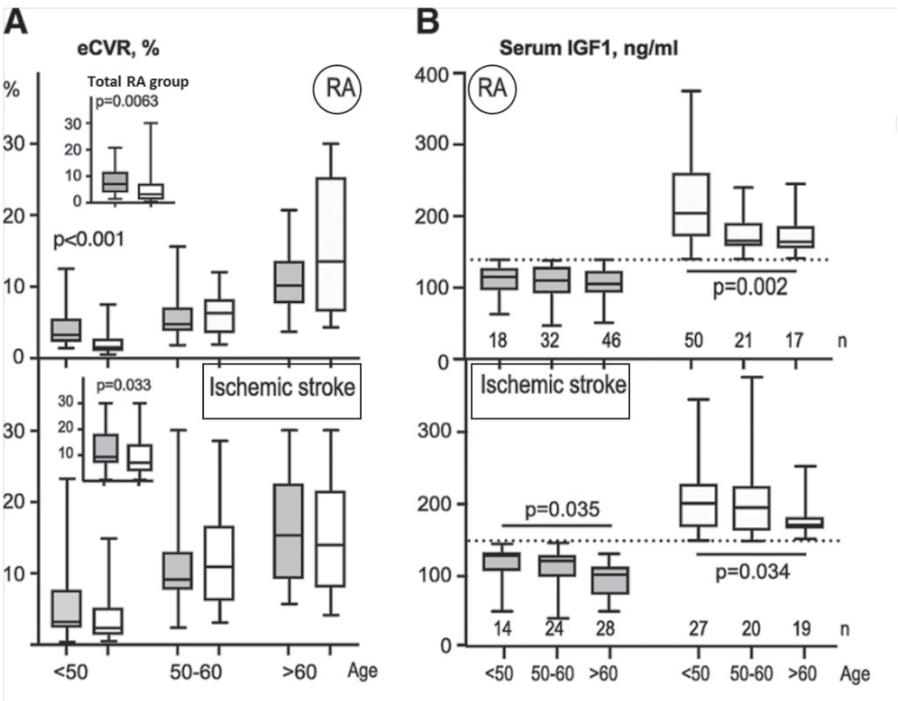

Figure 7. *Serum IGF1 and eCVR in RA patients and post stroke patients as control group. A. Embedded box plots show eCVR in the total cohorts. Box plots show eCVR separately for IGF1low (grey box) and IGF1hi (white box) groups sorted by age. B. Box plots show levels of IGF1 separately for IGF1^{low} (grey box) and IGF1^{hi} (white box) groups stratified by age. Box plots present median, interquartile range*

9.1.2 Low IGF1 implies higher inflammation in RA

In our RA patient cohort, disease-related CVR showed no differences in IGF1^{low} or IGF1^{hi} group. However, ESR and IL6 were higher in IGF1^{low} group, despite the absence of obvious differences in disease activity. We found that the patients in IGF1^{low} group were significantly more often treated with MTX monotherapy (OR 2.26, p = 0.007). The dose of MTX was similar to those used for treatment of patients in IGF1^{hi} group (18.7 vs. 15.0 mg/week, ns) which indicates insufficient inflammation control in IGF1^{low} group and the need to intensify the treatment to achieve remission. The combination of MTX with other DMARDs or TNF-α blocker was not prevalent in the IGF1^{hi} group.

9.1.3 Low IGF1 and prevalence of new CV events

New CVD events were reported in 12 of 184 RA women (6.5%) during the 5-year follow-up time. The baseline characteristics of patients with new CVD events are shown in table 5.

Table 5. *RA patients with CVD events during 5 years follow up.*

IGF1,ng/m	Age, y	Type of event	eCVR, %	DD, y	RA treatment
49	59	IS/TIA	10.0	40	MTX, RTX (14m)
101	65	IS/TIA	8.3	8	MTX
118	65	IS/TIA	7.7	32	MTX, ETC
138	65	IS/TIA	5.7	7	MTX, RTX (8m)
71	66	Atrial fibrillation	10.4	11	MTX, ETC
89	55	Atrial fibrillation	3.3	6	MTX
176	64	Atrial fibrillation	6.2	22	MTX, SLZ
93	48	DVT/LE	12.5	10	None
84	65	DVT/LE => IS	11.1	18	MTX, RTX (12m)
153	64	Aorta aneurysm	6.0	28	MTX, TOCI
119	57	Aorta aneurysm *	15.6	8	AZA
125	63	MI	9.9	17	MTX
109.5	57.5		9.1	14	<= Median

We further asked a question if serum IGF1 has clinical importance for the development of new CVD events. We could present a strong negative relation between CVD events and IGF1 levels by using age and the absolute levels of IGF1 as independent variables in a ROC analysis. (Figure 8A). Interestingly, the age showed no association to CVD events and confirmed that IGF1level independently from age was associated to CVD events. The relative risk of CVD event was 3.47 times higher for the patients in the $IGF1^{low}$ group. Comparison of data for the probability of future CVD events, showed almost 5 times higher probability of CVD event in $IGF1^{low}$ group (OR 4.96, $p = 0.028$) (Figure 8B).

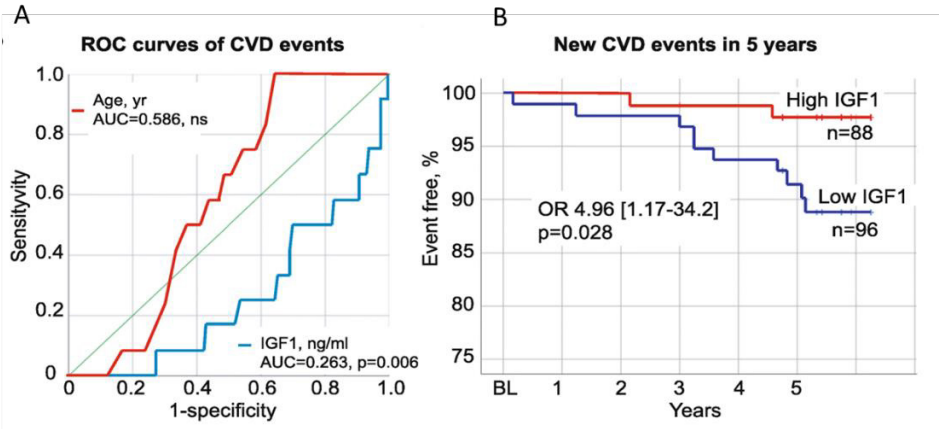

Figure 8. *A. Associations between CV events developed during the 5-year follow-up and age (red line) and absolute serum levels of IGF1 (blue line) at baseline in 184 female RA patients. B. the development of new CV events in IGF1^{hi} or IGF1^{low} groups.*

During the 5-year period, treatment for hypertension showed a tendency to increase significantly, compared to baseline, among RA patients < 50 years and ≥ 60 years in IGF1^{low} group, while treatment for hyperlipidemia or T2D showed no significantly increase in younger patients.

9.1.4 Hypertension and imbalance in the signaling through IGF1 receptor

We studied the transcription of individual proteins within IGF1-IGF1R pathway in IGF1^{low} and IGF1^{hi} groups. We found higher IGF1R and AKT1 and lower IRS1/2 expression in IGF1^{low} group. To identify how these changes are related to hypertension, we looked at the correlation between these components and clinical, metabolic and inflammatory parameters within hypertensive and normotensive patients. In hypertensive condition the correlation pattern with negative correlations between IGF1 and IRS1, IRS2 and plasma glucose was changed and IGF1 was no longer responsible for downstream of IGF1R pathway. Next, unsupervised clustering of 60 RA patients by individual components of the IGF1 signaling pathway presented a group of patient with highest eCVR and similar to the group with new CV events. This group had lower IGF1, IRS1 and IRS2 but higher serum IL6, insulin and p glucose. This

emphasizes the functional role of serum IGF1 in metabolic deviation and development of early CVD in RA.

This cross-sectional, longitudinal study suggests the predictive role of low serum levels of IGF1 for CV events in female RA patients. Low serum IGF1 in association with hypertension increases the risk for metabolic disorders, increasing IL6 and CV events, particularly among the younger female RA patients. The downstream pattern for IGF1-IGF1R pathway is changed in female RA patients with hypertension.

9.2 Paper II

In this study, we investigated the CVR and gene transcription activity in WAT and WBC in 184 female RA patients. CVR was estimated by mSCORE. In our RA cohort, we found 93 patients with increased CVR. This patient group was older and had unfavorable metabolic parameters like hypertension, hypercholesterolemia, the presence of T2D and overweight compared to the group without CVR. There was no difference in disease related parameters or treatment regime between patient groups with and without CVR.
We measured different adipokines and cytokines which are related to the metabolic and inflammatory activity of WAT. The atherogenic properties of leptin and resistin are induced through activation of IGF1R and TLR4 signaling. Under metabolic conditions like obesity, synergic activity of these two pathways activate T cells in the target tissues such as adipocytes (Hursting et al., 2010). Adipokines and mediators related to adipokines were measured. We focused on the gene transcription analysis with respect to the inflammatory axis TLR4, RETN and RELA, and the metabolic axis IGF1R- AKT1 and STAT3. A reference group for gene expression analysis were the young non-smoker female MTX-treated RA patients who had the disease in remission (n=11). All had normal BMI.

9.2.1 Expression of STAT3 is enriched in WAT

We found that the overall expression of STAT3 was significantly higher on WAT than in WBC (Figure 9).

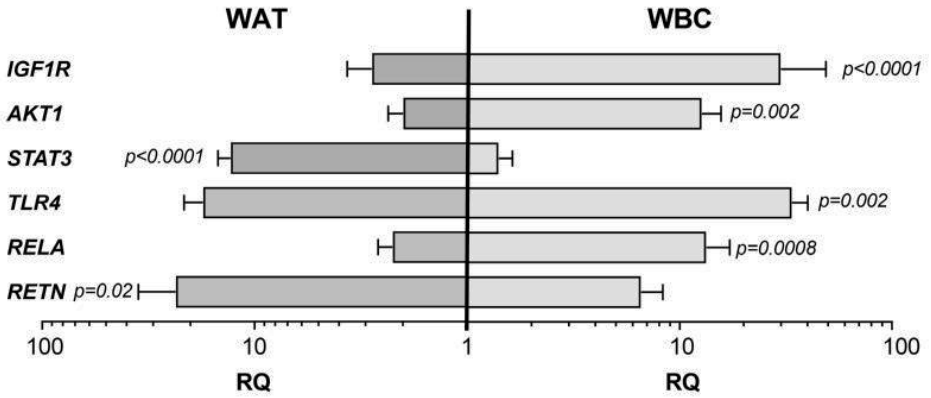

Figure 9. *Gene expression analysis in WAT and WBC of patients with rheumatoid arthritis.*
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STAT3 expression showed to be tissue-related and a significant activation was seen in WAT compared to WBC in RA patients. In CVR group, remarkably higher level of STAT3 was found in WAT compared to patients without CVR (Figure 10)

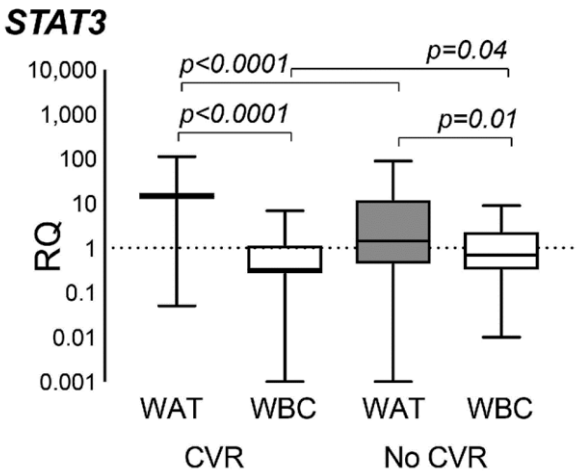

Figure 10. *Gene expression in WAT and WBC of the patients with and without CVR. *Free copy according to the Creative Commons Attribution license*

9.2.2 Metabolic Axis IGF1R–AKT1 in CVR

As signaling through IGF1R activates STAT3 and AKT1 pathways, we studied IGF1R-AKT1 axis in WBC and in WAT. The expression of IGF1R and AKT1 showed similar pattern with correlation in WAT ($r = 0.62$, $p < 0.00001$) and in WBC ($r = 0.51$, $p < 0.00001$). The transcription of both genes was higher in WBC as compared WAT (Figure 11).

Increased WAT-associated products, leptin and resistin and inflammatory markers ESR, IL6 were associated with CVR. In concordance with our study in paper I, serum levels of IGF1 was low in this group. The difference between WBC and WAT for IGF1R was significant in WBC both for the group with CVR and no CVR. For AKT1, the prevalent expression in WBC was higher only in the CVR group. (Figure 11)

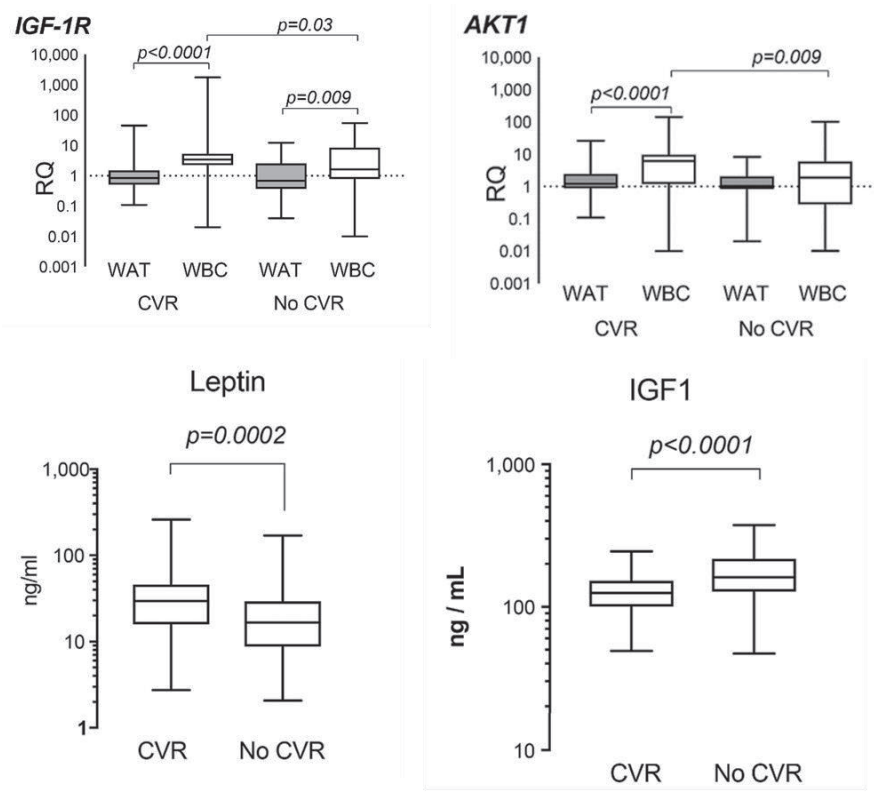

Figure 11. *IGF1R-AKT1 axel gene expression and serum levels of Leptin and IGF1 in the group with and without CVR.*

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9.2.3 Inflammatory Axis TLR4–NF-κB in CVR

The expression of TLR4 was higher in WBC compared to WAT. However, higher TLR4 transcription activity was presented in WAT in CVR group. Similar pattern was also seen for NF-κB (RELA) (Figure 12). Direct correlation was seen between NF-κB and other transcription factors, TLR4, IGF1R, AKT1 and STAT3 in WAT.

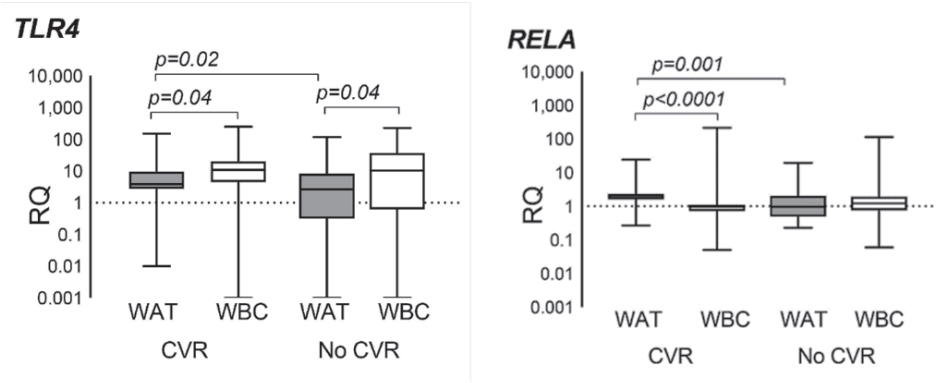

Figure 12. *Inflammatory axel, TLR4-NF-κB in the group with and without CVR. *Free copy according to the Creative Commons Attribution license*

9.2.4 STAT3 in WAT is independently associated to CVR

We performed a binary logistic regression model and identified that CVR was independently associated to STAT3 in WAT, IL6 in serum and body fat content. Next, we used another binary model to identify independent factors associated to high STAT3 in WAT that revealed positive association with inflammatory axis TLR4-NF-κB and disease activity.

The study shows association of CVR with transcriptional activity of STAT3 in WAT and interplay with inflammatory axis on leucocytes. Our finding emphasize on the role of IL6 and leptin in activation of STAT3 on WAT. CVR presented independent association with serum IL6 and STAT 3 in WAT. Further, STAT3 showed to be important for regulation of disease activity and inflammation.

9.3 Paper III

In this study, we assessed whether there is an association between sRAGE levels and cardiometabolic health in RA. The study cohort comprised 184 female RA patients as in Paper II. Our assumption was that lower levels of sRAGE indicate higher risk for cardiometabolic events in RA.

9.3.1 Differences in cardiometabolic and disease-related risk factors in respect to sRAGE

Thirteen patients out of 184 had T2D or previous cardiovascular events. These patients were analyzed separately from main cohort as cardiometabolic reference group (CMRG). The 75th percentile of sRAGE in CMRG defined the arbitrary cut-off level for "low" sRAGE and the RA cohort was dichotomized into two groups: high sRAGE group (n=73) and low sRAGE group (n=98). We found no significant differences in cardiometabolic or RA disease-related risk factors between sRAGE^{hi} and sRAGE^{lo} groups. However, patients in CMRG presented higher eCVR, RA disease activity, dyslipidemia, plasma glucose and adipose tissue inflammatory index, leptin/adiponectin ratio (LAR) compared to the whole group (Figure 13).

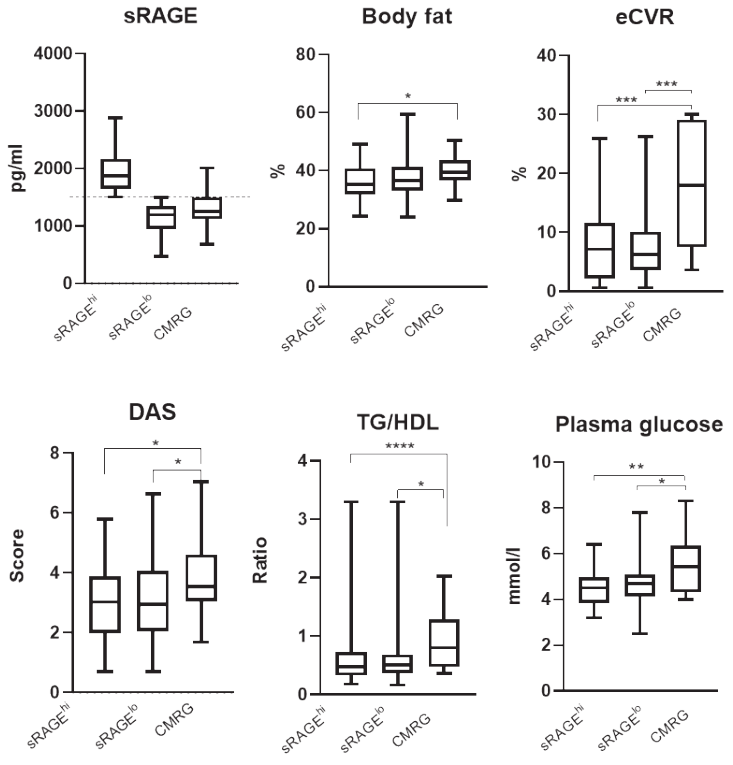

Figure 13. *Metabolic and inflammation-related characteristics of female patients with rheumatoid arthritis.*Free copy according to the Creative Commons Attribution license*

We found a bidirectional correlation between eCVR and sRAGE in un-dichotomized cohort (Figure 14A). Subsequently, we studied differences in correlation between sRAGE and cardiometabolic and inflammatory related parameters in $sRAGE^{hi}$ and $sRAGE^{lo}$ groups. We found using Fisher's r to z that the correlation pattern was significantly different and showed an opposite direction for eCVR, body fat content, age, IL6 and IGF1 between these two groups (Figure 14B). The analysis of traditional CVR factors and disease related risk factors showed no significant differences between the group with low and high sRAGE.

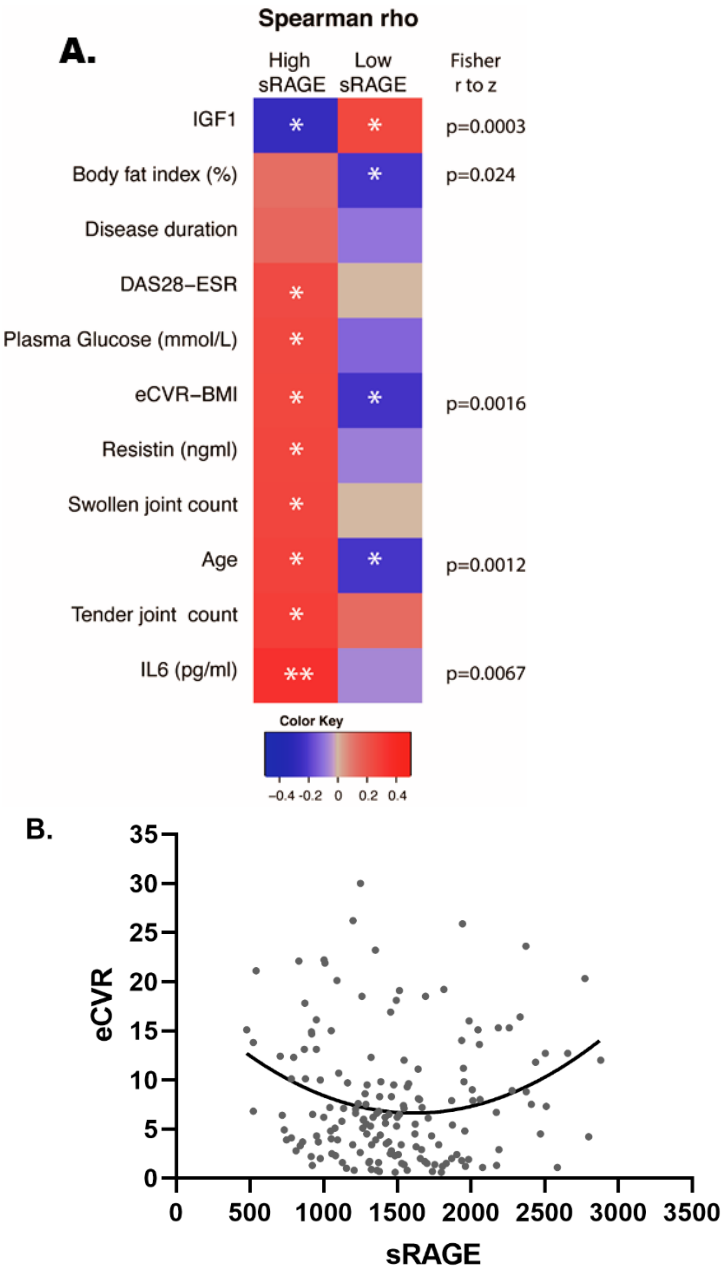

Figure 14. ***A.*** *Fisher's r to z correlation for sRAGE levels and cardio-metabolic and inflammatory parameters within sRAGE high and low groups.* ***B.*** *The univariate correlation analysis in un-dichotomized RA cohort.*

9.3.2 Association between CVR and sRAGE in different age groups

As sRAGE levels have been reported to decrease by increased age and CVR usually increases with age, we looked separately the younger (<50 years) and older (>50 years) RA patients in $sRAGE^{hi}$ and $sRAGE^{lo}$ groups with respect to RA-related and traditional CVR factors. We found significantly higher BMI, IL6 and more tender points in younger patients in $sRAGE^{lo}$ group while older patients in this group presented lower HDL and TC lipid profile compared to $sRAGE^{hi}$ group.

9.3.3 Prospective follow-up

The patients were prospectively followed up for 5 years. During this period, eleven patients presented new cardiometabolic events (CMEs). The prevalence of new events was not different between low or high sRAGE groups. We then studied whether the RA patients who had new events differed at inclusion from those patients who did not have any CME, especially with respect to metabolic and inflammatory factors. Interestingly, the patients in the new CME group had significantly lower sRAGE levels at inclusion than $sRAGE^{hi}$ group. Importantly, patients who presented with new CME had higher CVR and higher frequency of adverse metabolic factors such as increased plasma glucose and higher body fat than patients in the $sRAGE^{hi}$ and $sRAGE^{lo}$ groups (Figure 15). Additionally, the patients with new CME in $sRAGE^{lo}$ group showed significantly higher eCVR and higher frequency of metabolic disorders compared to $sRAGE^{hi}$ group.
Further, we found no differences for CVR factors between CMRG and the group with new CME.

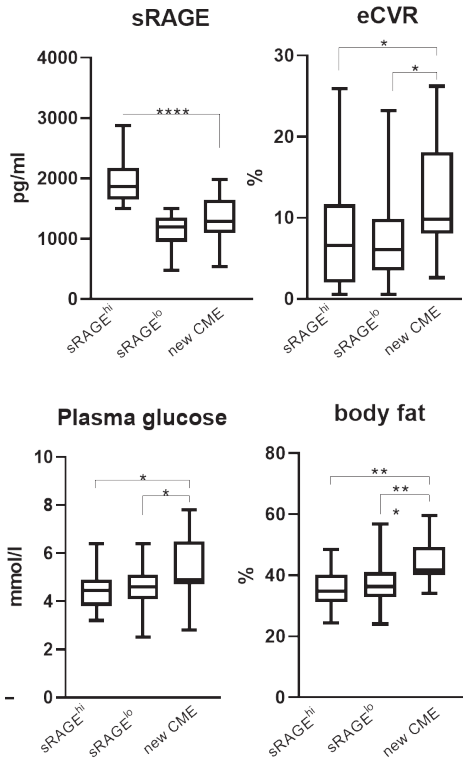

Figure 15. *Comparison of metabolic-related characteristics of RA patients with new cardiometabolic events. During the prospective follow up for 5 years. (CME: patients developed new cardiometabolic events, n=11)*Free copy according to the Creative Commons Attribution license*

This study suggests low sRAGE as a marker of metabolic failure. Decreased sRAGE level was linked with earlier and newly emerged CME in 5 years follow up in our RA cohort. In both cases, low sRAGE was related to increased CVR assessment.in those patients. Low sRAGE was attributed to unfavorable metabolic parameters such as high body fat content and blood sugar. In younger patients, the association of sRAGE and poor metabolic profile was more prominent.

9.4 Paper IV

In this study, we tried to demonstrate the role of IGF1R expression and signaling for development of adaptive immune responses in a cross-sectional RA cohort and in experimental arthritis.

9.4.1 IGF1R signaling in RA patients

We tried to understand the role of IGF1R signaling on leucocytes in RA patients. Immunological staining of the inflamed synovia showed CD3+ T cells as the main cell infiltrate in the joints of RA patients. We found activated pIGF1R and subsequently down streamed activated pSTAT3 at the same location on CD3+ T cells.
We studied clinical relevance of IGF1R expression on leucocytes in the peripheral blood from our RA cohort by dividing the cohort in two groups: low and high expression of IGF1R. The results showed higher pain perception with higher systemic inflammation presented by ESR, serum levels of IL6 and intracellular expression of NF-κB in leucocytes in the patients with higher expression of IGF1R. Interestingly, higher IGF1R expression in leucocytes was concordance with lower levels of IGF1 in serum (Figure 16). In addition, we could find direct correlation between IGF1R expression and intracellular activation through NF-κB, STAT3 on leucocytes. The correlation between IGF1R and STAT3 was only observed in the group with high IGF1R expression. This suggests a direct relation to pain perception, systemic inflammation and expression of IGF1R I leucocytes and points at the role of IGF1R in the intracellular inflammation control.

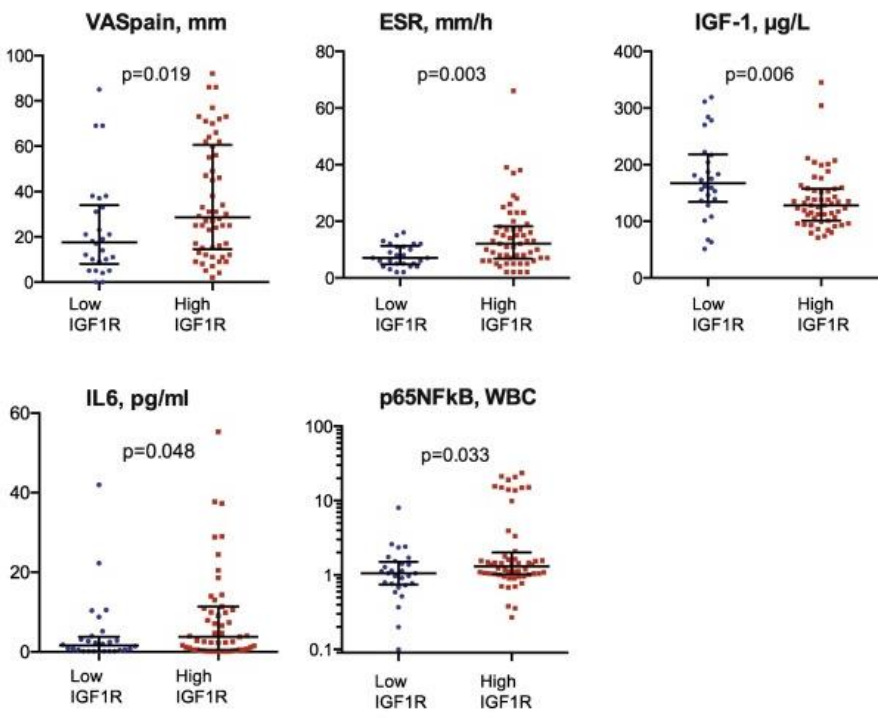

Figure 16. *Comparison between RA patients groups with high IGF1R (*n *= 56) and low IGF1R (*n *= 28).*Free copy according to the Creative Commons Attribution license*

9.4.2 Inhibition of IGF1R signaling

9.4.2.1 Impact of inhibition on splenic cells:

Next, we used a mouse model to study the inhibition of IGF1R signaling. The analysis of the murine splenic T cells by flow cytometry at the end of the mBSA immunization indicated over representation of CD3+CD4+ IGF1R+ memory cells. Further, we found a negative correlation between the expression of IGF1R on CD3+ T cells and the activity of ERK but positive correlation with AKT.

To investigate the pro-inflammatory role of IGF1R in arthritis, we treated the mBSA-immunized mice with NT157 to inhibit IGF1R signaling on the level of IRS1/2 as the explained in method section previously. IRS1/2 inhibition by

NT157 resulted in reduction of IGF1R+ cells in spleens in treated mice compared to vehicle treated control mice. NT157 treatment had negative impact on the total population of IGF1R^{hi} spleen leukocytes and on CD3+ and CD4+ cells within the naïve and memory T cells. Treatment by NT157 resulted in suppressed downstream of AKT and increased ERK on in spleen which indicates suppression of inflammatory action of IGF1R.

9.4.2.2 Impact of inhibition on experimental arthritis:

Strong correlation was seen between synovial expression of IGF1R and pSTAT3 in the arthritic joints of the control group. This correlation was disturbed by reduction of synovial pSTAT3 with NT157 treatment.

9.4.2.3 Impact of inhibition on development of T cell subsets:

As IGF1R expression is detectable on activated T cells, we stimulated naïve T cells by mBSA or anti-CD3 antibodies. Then we studied the effect of NT157 treatment on T cell function by measuring cytokine production. IRS1/2 inhibition by NT157 significantly reduced the production of IL6, IL17 and IFNγ by antigen-specific T cells (Figure 17).

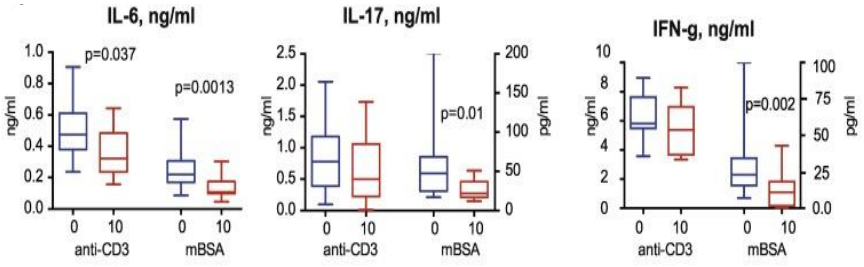

Figure 17. *NT157 treatment inhibits pro-inflammatory cytokine production in spleen T cells of arthritic mice. The objects treated with 10 mg/kg NT157 (red box) or vehicle (blue box) were activated with aCD3 or mBSA.*Free copy according to the Creative Commons Attribution license*

The transcription factors RORγt and FoxP3 correlated to IL6. The ratio between RORγt/FoxP3 was related both to the levels of STAT3 mRNA, phosphorylated STAT3 (pSTAT3) and to the IL17, produced by CD3-activated T cells.

Our observations display that suppression of IL6 production in respect to IRS1/2 inhibition shift the STAT3 dependent balance between RORγt/FoxP3, to Treg differentiation and reduced production of TH17. The present study shows that higher expression of IGF1R on WBC results in higher systemic inflammation such as increased levels of IL6 in serum and higher pain perception, in RA patients.

10 DISCUSSION

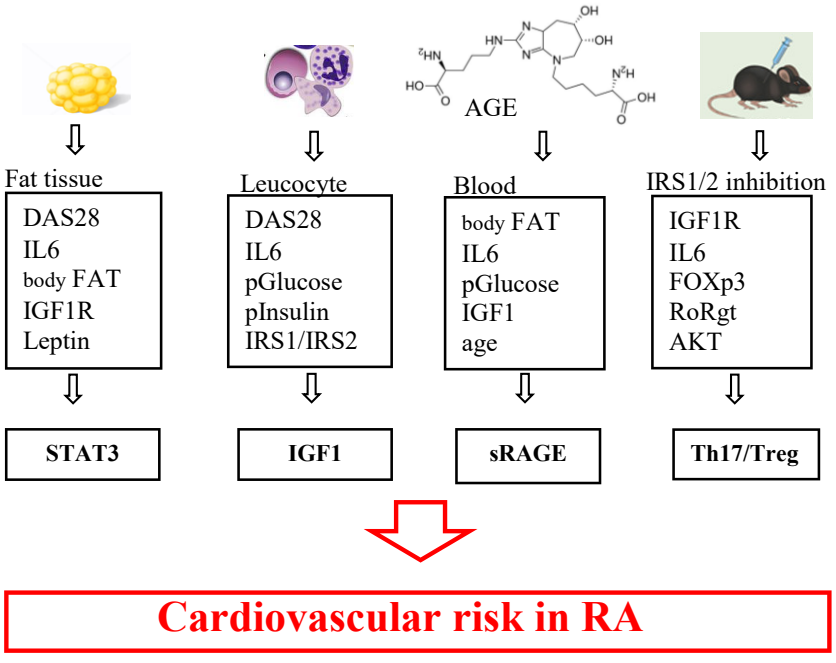

Figure 18. *Receptors and factors associated with increased CVR in RA*

Cardiovascular mortality and morbidity in RA patients remain the major challenge for rheumatologists. Despite advanced pharmacological treatment to control inflammation, CV mortality shows no improvement in RA patients. This attracts attention to metabolic dysfunction in RA as the crucial underlying factor responsible for CVR.

In this thesis, I have studied the role of essential metabolic pathways for the cardiovascular morbidity in RA and demonstrated their interplay with inflammation. We have clinically studied a cohort of female RA patients and investigated CVR in relation to metabolism regulating molecules in fat tissue and in the peripheral blood. As IGF1R has a key role in connecting

inflammation and metabolism, we explored the consequences of its inhibition on regulation of T cell balance (Th17/Treg) in murine experimental RA model.

In **paper I**, I revealed the higher frequency of hypertension and CV events in the RA patients with low serum levels of IGF1. This IGF1-low group had significantly higher metabolic burden like adiposity and hyperlipidemia. Furthermore, IGF1 levels in this group were constantly low and were disconnected from age. Under the 5 years follow-up, the frequency of treatment for hypertension and CV events was significantly higher in this group. Interestingly, the higher frequency was predominantly seen in younger RA patients and not in the control group i.e. post stroke patients. These results suggest that low IGF1 levels indicate metabolic dysfunction in RA. Alternatively, RA disease itself might be the reason for low levels of IGF1 independent of age. The group with low IGF1 had higher inflammation and were frequently treated with MTX monotherapy. This raises the question if measurement of IGF1 during anti-rheumatic treatment could be used to follow the effect of treatment and to improve CVR outcomes in RA patients.
In normotensive condition, serum IGF1 has a negative correlation with the adaptor molecules IRS1/2 down-stream IFG1R and plasma glucose, which supports its protective metabolic function. In RA normotensive group, this correlation remained unchanged. However, the correlation was lost in the hypertensive RA patients with low IGF1. This shows that in the presence of hypertension, IGF1R acts independently to IGF1 to activate the intracellular processes. Accordingly, the unsupervised clustering of the IGF1-related proteins suggested high CVR in combination with low expression of IGF1, IRS1/2 and unfavorable metabolic signature such as increased plasma glucose and serum insulin and higher inflammation and disease activity. This indicates the role of serum IGF1 in the development of early CVD in RA.
Additionally, pro-inflammatory cytokines in RA can also induce the inhibitory phosphorylation of IGF1R and IRS which impedes IGF1 binding to IGF1R (Smith et al., 2010). The promising, therapeutic effect of IGF1 on cardiac remodeling and repairing tissue damage after acute myocardial and cerebral ischemia has been proposed and studied experimentally (Heinen et al., 2019). However, the effect of IGF1 and factors enhancing its production have not been studied in RA. Several small studies reported a lower risk for developing hypertension by using TNF-α inhibitors (Klarenbeek et al., 2010; Desai et al., 2016). Our study suggested that higher IGF1 levels after treatment with TNFα

inhibitors could be a potential mechanism behind this association. Our results showed that poorly controlled disease activity and blood pressure in female RA patients with lower IGF1 predispose to higher CVR. We suggest that careful monitoring of the blood pressure and metabolic parameters during anti-rheumatic treatment could reduce CV events in RA.

In **paper II**, we analyzed an association between the transcriptional activity in WAT and leukocytes with CVR in RA. We found that accumulation of STAT3 in WAT was predictive of higher CVR. STAT3 regulates JAK signaling pathway, which is central for RA pathogenesis. Therapeutic inhibition of JAK-STAT signaling is under special attention of the recent research, partly because of its effect on lipid metabolism (Cohen et al., 2017). Stimulation of STAT3 leads to lipolysis, while inhibition of STAT3 is supposed to increase subcutaneous fat and cause hypertrophy of adipocytes. In concordance with these reports, our study showed a WAT-specific accumulation of STAT3 in non-obese RA patients. Metabolic axis IGF1R-AKT1 presented higher expression on leucocytes in our study compared to WAT. This finding may suggest that STAT3 accumulation in WAT stimulates metabolic factors in leucocytes. STAT3 activation in WAT was independently associated with higher disease activity and CVR.
IL6 and leptin activate STAT3 and regulate lipid metabolism and energy balance. Previous clinical studies reported correlations between leptin levels and CVR factors such as hyperlipidemia and high blood pressure (Bulló et al., 2003). We observed higher serum levels of IL6 and leptin, which could be responsible for the increased transcriptional activity of STAT3 in WAT in patients with increased CVR. Our results indicate a relationship between adipocytokines and inflammation, and suggest that cytokines secreted by adipose tissue contribute to the increased CVR in RA.

Studies I and II demonstrated an association between plasma glucose levels and higher CVR in the presence of IL6. Since glycation of proteins and fatty acids in the presence of hyperglycemia increases the formation of AGEs, we asked if low levels of soluble receptor for AGE (sRAGE) contributed to higher risk for cardiometabolic dysfunction in RA.
In **paper III**, we showed that low sRAGE was associated with both previous and new cardiometabolic events in female RA patients. We observed that low sRAGE was accompanied with metabolic dysfunction such as higher plasma

glucose and overweight that contributed to higher CV morbidity in RA patients. Circulating sRAGE has been suggested as an early marker of cardiometabolic disease. Lower sRAGE were reported in healthy women with obesity and an inverse association between BMI, body fat mass and epicardia visceral fat was described (Doizio et al., 2016). Other studies have shown similar results (Norata et al., 2009). Accordingly, we showed an inverse significant association between sRAGE, eCVR and content of total body fat in younger RA patients (<50 years).Similarly, patients with new CME had low sRAGE and presented significantly higher plasma glucose levels and body fat content than both $sRAGE^{lo}$ and $sRAGE^{hi}$ groups. These findings suggest that low sRAGE levels reflect a metabolic dysfunction preceding a forthcoming clinical CME.

In our study, we measured total circulating sRAGE, which includes both endogenously secreted RAGE and cleaved RAGE. Cleaved RAGE has pro-inflammatory function and endogenous RAGE has metabolic function (Scavello et al., 2019). Under inflammatory states, membrane-bound RAGE is up-regulated responding to ligand exposure and leads to higher production of cleaved sRAGE. It means that increased levels of inflammatory ligands for RAGE result in higher density of the cell-bound receptor via positive feedback loop, which in turn trigger the production of sRAGE. In our $sRAGE^{hi}$ group, we observed positive correlation between sRAGE and inflammatory markers. Under metabolic dysfunction of hyperglycemia, non-enzymatic glycation of proteins and lipids occur in order to reduce sugar. This attributes to higher production of AGEs. AGE-RAGE interaction is a major modulator of inflammation. Soluble RAGE eliminates the access of circulating AGEs without activating inflammatory pathways. This may explain the low levels of sRAGE in RA patients with previous and new CME with high plasma glucose levels. This study shows that low sRAGE is associated with higher CV risk in female RA patients whereas metabolic dysfunction is an eminent factor behind low sRAGE in female RA patients. It suggests sRAGE as a useful biomarker to monitor cardiometabolic health in RA patients.

In paper I-III, we demonstrated the usefulness of different molecules for prediction of CVR in RA. In **paper IV**, we investigated the consequence of inhibition of intracellular IGF1R pathway for the subsets of Th17 and regulatory T cells.

In the material of RA patients, we observed a connection between the upregulation of IGF1R signaling on the peripheral blood leucocytes and systemic inflammation through IL6. We also found intracellular activation of IGF1R and STAT3 in T cells from RA synovia combined with higher inflammation and higher NF-κB associated with higher expression of IGF1R. This indicates a direct relation between IGF1R in leukocytes and inflammation in RA.

In the experimental model of arthritis, we showed that inhibition of IGF1R signaling on the level of IRS resolved the inflammation and arthritis. Our clinical studies, presented in papers I-III, showed that IL6 had essential role in inducing metabolic disorders and was linked to insulin resistance, dyslipidemia and atherosclerosis accompanied by proinflammatory cytokines. The inhibition of IRS proteins in the arthritis mice resulted in reduction of IGF1R signaling followed by the reduced production of IL6 and STAT3 by splenic T cells with consequent up-regulation of the Treg cells. The link between IL6 and insulin/IGF1R demonstrates the important role of this complex in cardiometabolic risk.

Overall, in all our 4 papers, IL6, IGF1, body fat content and plasma glucose demonstrated as essential metabolic parameters modulating various mediators in association with CVR.

11 CONCLUSIONS

The following conclusions can be drawn from the present study:

I – Low levels of IGF1 in association with hypertension result in metabolic dysregulation by activating inflammatory pathways and increase cardiovascular risk in RA.

II – Accumulation of STAT3 in WAT may be a response to activation by adipokines and is associated with higher disease activity and CVR in RA

III – Low levels of sRAGE is associated with higher CVR in RA. Soluble RAGE is down regulated in the metabolic conditions.

IV – Inhibition of the IGF1R by targeting of IRS proteins alleviates experimental arthritis through disruption of IL6 production and alteration of Th17/Treg balance in T cells.

V – Overall, the results underline the metabolic dysregulation and the need to apply biomarkers in clinical practice to improve CVD risk factors management in RA female patients. The patients would also benefit of monitoring for hyperglycemia, hypertension, and obesity.

12 IMPLICATIONS AND FUTURE PERSPECTIVES

This thesis lays the groundwork for future research for improved assessment of CVR by assistance of biomarkers in RA. IGF1 has a key role in integration of metabolic and inflammatory process leading to increased CVR in RA. This suggests the need of prospective investigation about various RA treatment that might improve the levels of IGF1 in the RA patients in respect for CVR factors, particularly in younger and early-diagnosed patients, in whom CVR assessment algorithms underestimate the CVR.
Perhaps, dynamic exercise increases the levels of IGF1 and improves both CVR and disease activity outcome.
Additionally, the results from this thesis point out IL6 as a central link between inflammatory- and metabolic components. During the recent years, increased awareness has pointed towards to the drugs that target IL6 receptors directly or indirectly, through JAK-STAT inhibition. In our study cohort, there were only four patients who received IL6 receptor antagonist, tocilizumab. JAK inhibitors were not approved for treatment of RA in Europe when we started the study. Further studies might enlighten the need to choose favorable RA treatment and follow up different outcomes with respect to different vital markers such as IL6 and IGF1.

IL6 mediates glucose hemostasis. In all our studies, high plasma glucose was a potential, poor prognostic factor for CVR outcome. This suggests the need for regular monitoring of p-glucose in RA.

Our studies were conducted in female RA patient group Therefore, it does not permit any conclusion to implicate the presented markers for CVR in general RA population. Whether the same applies for male RA patients' needs to be addressed in future studies.

The impact of DMARDs on changing lipid profile is still not exactly known and actively discussed. There is still lack of knowledge how various treatment has impacted on metabolic homeostasis and CVR. Further studies with larger population on current biologics are needed to investigate different molecules and pathways we looked at in our studies.

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