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PREDICTIVE BIOMARKERS IN RHEUMATOID ARTHRITIS

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Predictive biomarkers in rheumatoid arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D)

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The public defence will take place on the 24th of August, 2018, at $9^{\underline{00}}$ am, at the Centre for Molecular Medicine (CMM) Lecture hall, L8:00, Karolinska University Hospital, Solna.

Those who have knowledge, don't prediet. Those who prediet, don't have knowledge

Lao Tzu 6th century BC, Chinese poet

ABSTRACT

Being a very heterogeneous disease, rheumatoid arthritis (RA) is challenging for treatment. On a group level, some therapy options might be superior compared with others, however, this does not mutually exclude the less effective option to be more suitable for some patients, if the superior therapy option does not help. In different patients the concentration of biomarkers may vary dramatically, however, translation of meaning of these variations for each patient is not feasible yet. Observations and comparisons of these biomarkers before start of therapy between patient groups with different outcome after therapy can help to understand their role and make individualised approach for the therapy choice.

Low or moderate levels of a multi-biomarker disease activity (MBDA) score measured at baseline or follow-up visits could identify RA patients at very low risk of radiographic progression (RP). Moreover, in patients with high MBDA score at the start of treatment escalation, those on infliximab+methotrexate (IFX+MTX) therapy had significantly less RP than patients on non-biological triple therapy (TT) (papers I and II).

In treatment-naive, early RA, patients who failed respond to MTX were randomized to IFX+MTX or non-biological TT. The categories of the MBDA score at the time of randomisation were differentially associated with treatment outcome after 1 year for these two therapies. Patients with low MBDA score benefited more from TT, while those with high MBDA score responded better to IFX (paper III).

Furthermore, when the 12 component biomarkers of the MBDA score were analysed at baseline, four of those (paper IV) as well as two of 177 proteins retrieved from an affinity proteomic study (paper V) were associated with treatment outcome: low disease activity (LDA) and EULAR good response after 3 months of MTX therapy. Combination of these biomarkers within each study also showed improved prediction of treatment outcome.

In patients failing on MTX monotherapy who were randomised to addition of biological TNF inhibitor IFX, very low serum IFX (sIFX) level and anti-drug antibody (ADA)-positivity were associated with poorer outcome. Among baseline parameters, female gender predicted very low sIFX level and ADA-positivity at follow-ups, with similar trend for RF-positivity (paper VI).

In summary, using combination of serum biomarkers helps to predict and identify preferential therapy option for subsets of patients. Further studies of these biomarkers, if validated, will facilitate personalised therapy approach for subsets of patients.

LIST OF SCIENTIFIC PAPERS

I. **Hambardzumyan K**, Bolce R, Saevarsdottir S, Cruickshank SE, Sasso EH, Chernoff D, Forslind K, Petersson IF, Geborek P, van Vollenhoven RF. Pretreatment multi-biomarker disease activity score and radiographic progression in early RA: results from the SWEFOT trial. Annals of the rheumatic diseases. 2015 Jun;74(6):1102-9.

II. **Hambardzumyan K**, Bolce RJ, Saevarsdottir S, Forslind K, Wallman JK, Cruickshank SE, Sasso EH, Chernoff D, van Vollenhoven RF. Association of a multibiomarker disease activity score at multiple time-points with radiographic progression in rheumatoid arthritis: results from the SWEFOT trial. RMD open. 2016;2(1):e000197.

III. **Hambardzumyan K**, Saevarsdottir S, Forslind K, Petersson IF, Wallman JK, Ernestam S, Bolce RJ, van Vollenhoven RF. A Multi-Biomarker Disease Activity Score and the Choice of Second-Line Therapy in Early Rheumatoid Arthritis After Methotrexate Failure. Arthritis & rheumatology (Hoboken, NJ). 2017 May;69(5):953-63.

IV. **Hambardzumyan K**, Bolce RJ, Wallman JK, van Vollenhoven RF, Saevarsdottir S.Serum biomarkers for prediction of response to methotrexate monotherapy in early rheumatoid arthritis: results from the SWEFOT trial. Manuscript

V. **Hambardzumyan K**, Hamsten C, Idborg H, Lourido L, Saevarsdottir S, Nilsson P, van Vollenhoven RF, Jakobsson P-J. Association of serum protein levels at baseline with response to methotrexate at 3 months in patients with early rheumatoid arthritis. Manuscript

VI. **Hambardzumyan K**, Hermanrud C, Marits P, Vivar N, Ernestam S, Wallman JK, van Vollenhoven RF, Fogdell-Hahn A, Saevarsdottir S. Association of female gender and positive RF with low serum infliximab and anti-drug antibodies, which relate to treatment failure in early RA. Manuscript

LIST OF ARTICLES NOT INCLUDED IN THE THESIS

I. van Vollenhoven RF, Bolce RJ, **Hambardzumyan K**, Saevarsdottir S, Forslind K, Petersson IF, Sasso EH, Hwang CC, Segurado OG, Geborek P. Brief Report: Enhancement of Patient Recruitment in Rheumatoid Arthritis Clinical Trials Using a Multi-Biomarker Disease Activity Score as an Inclusion Criterion. Arthritis & rheumatology (Hoboken, NJ). 2015 Nov;67(11):2855-60

II. Levitsky A, Brismar K, Hafstrom I, **Hambardzumyan K**, Lourdudoss C, van Vollenhoven RF, Saevarsdottir S. Obesity is a strong predictor of worse clinical outcomes and treatment responses in early rheumatoid arthritis: results from the SWEFOT trial. RMD open. 2017;3(2):e000458.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACPA – anti citrullinated protein antibodies

ACR – American College of Rheumatology

ADA – anti-drug andtibodies

BMI – body-mass index

CCP – cyclic citrullinated peptide

CD – cluster of differentiation

CDAI – clinical disease activity index

CIA – collagen-induced arthritis

CNS – central nervous system

COMP – cartilage oligomeric matrix protein

CRP – C-reactive protein

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

CVD – cardiovascular diseases

DAS28 – disease activity score based on 28 joints

DMARD – disease modifying anti-rheumatic drug

EGF – epidermal growth factor

ELISA – enzyme-linked immunosorbent assay

ESR – erythrocyte sedimentation rate

ETN - etanercept

EULAR – European League Against Rheumatism

FGA – alpha-chain of fibrinogen

FOI – fluorecent optical imaging

GM-CSF – granulocyte-macrophage colony-stimulating factor

HAQ – health assessment questioneare

HCQ - hydroxychloroquine

IFX – infliximab

sIFX – serum infliximab

Ig – immunoglobulin

IL – interleukin

JAK – Janus kinase

JSN – joint space narrowing

JSW – joint space width

LDA – low disease activity

LOCF – last observation carried forward

MBDA – multi-biomarker disease activity

M-CSF – macrophage colony-stimulating factor

MFI – median fluorecence intencity

MMP – matrix metalloproteinase

MRI – magnetic resonance imaging

MTX - methotrexate

PatG – patient global assessment of disease activity

PhysG – physician global assessment of disease activity

RA – rheumatoid arthritis

RANKL – receptor activator of nuclear factor kappa-B ligand

RF – rheumatoid factor

ROC – receiver operating characteristic

ROS – reactive oxygen species

RP – radiographic progression

SAA – serum amyloid A

SDAI – simplified disease activity index

SHS – modified van der Heijde score

SJC – swollen joint count

SSZ – sulfasalazine

STAT – signal transducer and activator of transcription proteins

TCZ – tocilizumab

TJC – tender joint count

TNF – tumour necrosis factor

TNF-R – tumour necrosis factor receptor

TT – triple therapy

VAS – visual analogue scale

VCAM-1 – vascular cell adhesion molecule

VEGF – vascular endothelial growth factor

YKL-40 – cartilage glykoprotein-39

1 INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune, chronic inflammatory disease characterised by painful joints and synovitis that can lead to a damage of cartilage and bone. It affects approximately 0.5-1% of total population (1) and occurs more often in women (60-86%) than men (2-4).

Aetiology and pathogenesis of RA are not completely understood. However, recent advances in research have shown important role of interaction of some genetic susceptibility factors (the strongest being carriage of *HLA-DRB1* and *PTPN22*), with environmental triggering factors such as smoking, infections and others (5-7). Better understanding the pathogenesis of RA would help to improve targeted treatment. However, the greatest challenge for this is the heterogeneity of the disease. Therefore, investigation of predictive and prognostic biomarkers is in high need, and identification of such factors could help discriminate subsets of patients with certain pathogenesis and predisposition of response to a specific therapy.

1.1 PATHOGENESIS OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis is usually divided into two main subgroups in regard to auto-antibody status: seropositive and seronegative. Earlier, rheumatoid factor (RF) was the main autoantibody used for diagnosis and classification into sero-(RF) positive and seronegative disease. However, the commercial anti-cyclic citrullinated peptide (anti-CCP) test, used to measure anti-citrullinated protein antibodies (ACPA) that were discovered later than RF, show higher specificity for RA. The pathogenesis of seronegative RA is very unclear. The disease course of these patients is considered to be more heterogeneous compared with seropositive patients. In contrast, seropositive (anti-CCP positive) RA (2/3 of patients) has been studied in more detail and more clear picture has developed for the pathogenesis of anti-CCP positive RA. First, the discovery that anti-CCP antibodies can develop in non-symptomatic individuals several years before the onset of the disease attracted attention of many researchers to investigate the role of these autoantibodies in the pathogenesis. According to current theory (8, 9), development of ACPA starts from tissue located outside of the joints (for example, lungs or mucous membrane) leading to auto-antibody production. Shift of the process from extra-articular compartments to the joints is yet unclear. It is

believed that expression of citrullinated epitopes on precursor osteoclasts attracts ACPA and triggers activation of these cells leading eventually to bone erosion. They also start producing IL-8 which binds to nociceptors in the joints triggering pain. IL-8 also may attract neutrophils (which is commonly seen in early arthritis) giving a start of the inflammation process (activation and migration of other inflammatory cells, vasodilation and production of pro-inflammatory cytokines). Synovial membrane becomes infiltrated with macrophages, mast cells, T and B cells and plasma cells. These cells together with synovial fibroblasts produce pro-inflammatory cytokines (tumour necrosis factor (TNF), interleukin-6 (IL-6), IL-17, macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL)) and matrix enzymes that maintain and progress the inflammation in the joints, facilitate angiogenesis, cartilage destruction and osteoclastogenesis accelerating bone erosion (6). Histologically, RA synovium may have two phenotypes: diffuse, where lymphocytes are randomly infiltrated into synovial tissue without any structural formation and follicular, where T and B lymphocytes form clusters, so called germinal-like centres (10-12). It has been shown in RA patients that joints with follicular type of synovitis are at higher risk of destruction compared with diffuse synovitis (13).

1.2 CLINICAL CHARACTERISATION AND OUTCOME OF RHEUMATOID ARTHRITIS

1.2.1 Course and symptoms

The diagnosis of RA is complex and is based on physical examination as well as laboratory immunological analysis of the presence of auto-antibodies towards IgG (RF-positive) and/or anti-CCP (anti-CCP-positive). Numbers of swollen and tender joints detected by rheumatologist together with molecular components of inflammation such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are other components of the diagnostic work-up.

The course of RA may vary among individuals from mild to more aggressive and destructing features. RA may affect almost any join, however, joints of hands and wrists as well as forefeet are most usually affected ones (14, 15). At the onset, morning stiffness on the joints is often present. Most common symptoms are presented in Table 1. RA may in addition have extra-articular manifestations (Table 1).

Table 1. Clinical features of rheumatoid arthritis*

Symptoms	Joint pain Joint stiffness Weakness Deformity Fatigue Malaise Fever Weight loss Depression
More common distribution	Symmetrical Distal PIP MCP MTP Wrist Ankle
Extra-articular manifestations	Skin (rheumatoid nodules) Ocular (keratoconjuctivitis sicca, iritis) Oral (salivaty inflammation) Respiratory (pulmonary fibrosis, pleural effusion, cricoarytenoid inflammation) Cardiac (pericarditis, valvular nodule formation, myocarditis) Neurological (mononeuritis, nerve entrapment, cervical instability) Hepatic (increased aminotransferase concentration) Haematological (anaemia, thrombocytosis, leukocytosis, lymphadenopathy, slenomegaly, thrombocytopenia Vascular (vasculitis)

MCP – metacarpophalangeal join, MTP – metatarsophalangeal joint, PIP – proximal interphalangeal join

* The table is adapted from Lee & Weinblatt, *The Lancet* 2001 and modified

1.2.2 Disease activity measures

Disease activity of RA is estimated using different clinical measures. Disease activity score based on examination of 28 joints and acute phase reactant (DAS28) is one of the routinely used outcome to monitor the disease (16). DAS28 is based on number of tender and swollen joints (TJC and SJC, respectively) of 28 examined joints (hands, wrists, elbows, shoulders and knees), patient's global health on visual analogue scale (VAS), and CRP or ESR (Table 2).

Table 2. Disease activity measures in rheumatoid arthritis

	DAS28-CRP	DAS28-ESR	SDAI	CDAI
SJC	+	+	+	+
TJC	+	+	+	+
CRP	+	-	+	-
ESR	-	+	-	-
PatGH VAS	+	+	-	-
PatGDA VAS	-	-	+	+
PhysGDA VAS	-	-	+	+

CDAI - clinical disease activity index, CRP - C-reactive protein, DAS - disease activity score, ESR - erythrocyte sedimentation rate, PatGH - patint global health, PatGDA - patient global disease activity, PhysGDA - physitian global disease activity, SDAI - simplified disease activity index, SJC - swollen joint count, TJC - tender joint count, VAS - visual analogue scale.

Formulas for calculation of DAS28 based on CRP or ESR are expressed, respectively, as follows:

$$DAS28\text{-}CRP = 0.56\sqrt{(TJC28)} + 0.28\sqrt{(SJC28)} + 0.36ln(CRP+1) + 0.014(GH) + 0.96$$

$$DAS28\text{-}ESR = 0.56\sqrt{(TJC28)} + 0.28\sqrt{(SJC28)} + 0.70ln(ESR) + 0.014(GH)$$

The scale of DAS28 helps to determine patient disease activity (Table 3). Cut-off values used for determining disease activity categories by DAS28-CRP or DAS28-ESR are most often the same. However, it has been shown that DAS28-CRP indicates somewhat lower values compared with DAS28-ESR (17-19). Fleischmann et al illustrated that threshold values of DAS28-CRP of 2.4, 2.9 and 4.6 corresponding to remission, low and high disease activity, respectively were equivalent to established cut-offs for DAS28-ESR (2.6, 3.2 and 5.1, respectively) (20, 21).

Table 3. Disease activity categories

Categories	DAS28	SDAI	CDAI
High	>5.1	>26.0	>22.0
Moderate	3.3-5.1	11.1-26.0	10.1-22.0
Low	2.6-3.2	3.4-11.0	2.9-10.0
Remissoin	<2.6	<3.4	<2.9
Minimum value	0.49	0	0
Maximum value	9.07	86	76

CDAI - clinical disease activity index, DAS - disease activity score, SDAI - simplified disease activity index

Clinical or simplified disease activity indices (CDAI or SDAI) are other measures similar to DAS28 (Table 2), but easier to calculate without computer (22). Both include TJC and SJC based on 28 joints, patient global disease activity VAS and physician global disease activity VAS. SDAI includes in addition CRP, too. Both CDAI and SDAI are calculated by simple summing all the components together and have a range between 0-76 and 0.1-86, respectively.

1.2.3 Response criteria and functional assessment

Change and improvement of patients health is measured by the change of disease activity measures. For example, the European League Against Rheumatism (EULAR) has developed response criteria based on the change in DAS28 as well as the value of DAS28 at the follow-up (Table 4). According to the EULAR response criteria, patients are classified in no responders, moderate or good responders (23, 24). Similarly, the American College of Rheumatology (ACR) developed another criterion for evaluation of improvement during therapy. According to ACR criteria, patients achieving at least 20%, 50% or 70% of improvement in TJC and SJC, and in any 3 out of 5 other clinical parameters (pain VAS, patient's global assessment, physician global assessment, ESR or CRP and functional questionnaire), are classified as having respectively ACR20, ACR50 or ACR70 response to the therapy (25). To assess functional activity, health assessment questionnaire (HAQ) is used (26). The HAQ is self-assessing tool which reflects the ability of patients to perform daily activities. For each question patient may receive scores: 0 (without any difficulty to perform), 1 (with some difficulty), 2 (with much difficulty) and 3 (unable to perform). The total HAQ score is a mean of all obtained scores and therefore it has also the range between 0 and 3.

Table 4. EULAR response criteria*

Improvement of DAS28	DAS28 at end time-point		
	>5.1	>3.2 - ≤5.1	≤3.2
≤0.6	No	No	No
>0.6 - ≤1.2	No	Moderate	Moderate
>1.2	Moderate	Moderate	Good

DAS - disease activity score, EULAR - European League Against Rheumatism
* The table is adapted from van Gestel et al., 1996. and modified

1.2.4 Imaging

Apart from symptomatic and laboratory data, patients are evaluated by imaging techniques, such as X-rays, ultrasound and magnetic resonance imaging (MRI). The most routinely used is X-ray examination of joints. It helps to detect pathological changes, such as joint space width (JSW), and bone erosion, which are not detectable by physical examination. The difference in JSW at earlier and later time-points is called joint space narrowing (JSN). Assessment of JSN and bone erosion allows to monitor the dynamic of these changes during a given time-period, so called radiographic progression of joint damage (RP). These detected pathological changes are due to processes with shift of bone and cartilage turnover towards degradation. There are different scoring systems developed for the evaluation of bone and cartilage damages and disease progression (27). In 1971 Sharp developed a method of assessment of hands and wrists (28), which was later modified in 1985 and is known as standard Sharp method (29). In 1989 van der Heijde modified the standard Sharp scoring method of evaluation of hands and wrists (van der Heijde modified Sharp score), which includes also evaluation of feet and is based on bone erosions and JSN (30-32). Maximum erosion scores of hands and feet (per joint) are 5 and 10, respectively, and maximum JSN score is 4 per joint. The total van der Heijde modified Sharp score has a range between 0 and 448 per patient. Another radiographic evaluation method of joints was introduced by Larsen in 1974 (33). This score also has been modified several times. Initially it contained also soft tissue swelling and periarticular osteoporosis which was removed during later modifications (34). The x-ray technique, however, has some limitations. For example, alteration in erosion size compared with previous picture might be due to slightly different position of joints and it indicates past events in patients, but no conclusion can be done for the current situation.

While radiography gives the possibility to detect pathological changes occurring on surfaces of hard tissue (bone and cartilage) and reflects the past of the patient, other methods such as ultrasound and MRI are able, in addition, to scan soft tissues of the inflamed joints. These tools can measure and illustrate pathological thickening of synovial membrane, tendons sheaths, ligaments and bursae, which indicates the degree of ongoing inflammation (35, 36). With the help of power Doppler and colour Doppler tools, ultrasound may also illustrate increased blood flow in the joints. In addition, MRI may detect pathological alterations in the bone, so called bone marrow oedema. Another useful aspect for both ultrasound and MRI is that they have been shown to identify subclinical inflammation, therefore are considered as important tools for early detection of pathological changes as well as monitoring patients in remission.

A new method for imaging, fluorescent optical imaging (FOI), is being tested for its utility in early diagnosis and monitoring of patients with RA (37, 38). The method is based on a contrast media which is injected intravenously followed by scanning of hands for detection of the fluorescence intensity. The hyperaemic tissues give higher level of fluorescence intensity compared with normal (uninflamed). The method showed high correlation and agreement with other imaging techniques in one study (38), and weaker findings were in another study (39). A good agreement of FOI with ultrasound for detecting clinically silent synovitis was reported by Kisten et al (40). Higher sensitivity but lower specificity of FOI in RA patients compared with MRI or power Doppler ultrasound was observed by Krohn et al (41).

1.3 THERAPY OF RHEUMATOID ARTHRITIS

Since the aetiology and pathogenesis of RA is not fully understood, the treatment is challenging and is directed towards suppression of immune system by using corticosteroids and disease modifying anti-rheumatic drugs (DMARDs). Even introduction of biological DMARDs (bDMARDs) in treatment of RA did not result in 100% response of patients. The inconsistency of clinical outcome during therapies and presence of such a variation in therapy options highlights the heterogeneity of RA and a need of prognostic biomarker research.

Based on results of many studies and current international guidelines, methotrexate (MTX) is the most recommended non-biological conventional DMARD (cDMARD) for first-line therapy (42, 43). MTX is a folate antagonist leading to reduction of pyrimidine and purine synthesis and stop in DNA replication; therefore it is being used in treatment of oncological

diseases. In RA, the beneficial effect of lower doses of MTX is not completely clear (44, 45). However, it is believed that its anti-proliferative action on lymphocytes leads to immune suppression. MTX can also inhibit JAK/STAT pathway (46), which is also a target of recently developed therapies. Taking into account that majority of pro-inflammatory cytokines are synthetized through this pathway, this property of MTX may explain its anti-inflammatory effect on RA. MTX was also found to lead to apoptosis of activated lymphocytes via increasing reactive oxygen species (ROS) (47-49). Another hypothesis of beneficial effect of MTX on RA patients is its ability to release endogenous adenosine, which binds its A2a receptor leading to immunosuppression (50, 51).

In clinical practice of RA therapy approximately 30-40% of patients responds and improves their symptoms and lab outcome. According to EULAR and ACR recommendations, for those patients who do not achieve low disease activity or remission, the MTX therapy is intensified by adding other non-biological cDMARDs, such as sulfasalazine (SSZ) and hydroxychloroquine (HCQ) or biological DMARDs (bDMARDs) (42, 43). It has been proven by many investigations that the use of combinational cDMARD therapy is significantly effective than MTX monotherapy. For example, O'Dell and colleagues demonstrated that addition of SSZ and HCQ to MTX led to significantly better outcome compared with MTX monotherapy (52, 53). Similar result was observed by de Jong et al from tREACH trial (54).

Biological therapy of RA became possible after Köhler and Milstein developed a method of monoclonal antibody production from hybridoma fused cells 4 decades ago (55). Today, there are 9 biological DMARDs used in RA care, of which 5 are TNF inhibitors (infliximab, etanercept, adalimumab, certolizumab and golimumab), one is against B lymphocytes (anti-CD20; rituximab), one against T lymphocytes (abatacept), one against interleukin-6 receptor (IL-6 inhibitor; tocilizumab) and one IL-1 receptor inhibitor (anakinra) (56). The first study on biological therapy (anti-TNF) of RA was published in 1994 illustrating superiority of both higher and lower doses compared with placebo (57). The results from another study confirmed efficacy of addition of infliximab (IFX) to MTX monotherapy (58). In several randomized controlled trials significant effectiveness of etanercept was also proven (compared with placebo groups) on cDMARD-non-responders (59-61). The application of adalimumamb and its efficacy compared with MTX+placebo have been shown in numerous of trials (62-65). On 36 patients from a British double-blinded study, single intravenous infusion of certolizumab showed improvement by ACR20 and ACR50 in a dose-dependent manner (66). Other study applied sub-cutaneous administration of certolizumab, showing that

400 mg dosing resulted in the best improvement compared with placebo or lower doses (67). Keystone and colleagues have demonstrated that certolizumab in combination with MTX was significantly more effective therapy option than MTX monotherapy (68). In an American study, RA patients with inadequate response to MTX received in addition double-blinded subcutaneously golimumab at two different doses or placebo (69). After 16 weeks of the initiation of the therapy, significantly higher proportion of patients achieved ACR20 response from golimumab group, compared with placebo. Similar results were reported from GO-MORE clinical trial, where majority of patients who did not respond to cDMARD therapy, receiving subcutaneously golimumab achieved EULAR good/moderate response (70). Rituximab was developed for treatment of lymphocytic leukemia. In 2001 first results were published on the application of rituximab in few patients with RA (71). All of them achieved response according to ACR70 criteria during therapy. Later the effectiveness of rituximab was shown in other RA patietns (72-77). Anakinra have also been shown to be significantly superior to MTX+placebo in improving clinical and radiological outcomes (78-81). First background evidence that CTLA-4 pathway may be a reasonable target for therapy in RA was demonstrated in 1995, when symptoms of collagen-induced arthritis in rats were prevented by CTLA-4-Ig (82). In 2002 Moreland and colleagues published first evidence of CTLA-4-Ig safety and effectiveness on patients who failed DMARD therapy (83). Later came more studies confirming effectiveness of anti-T cell therapy (abatacept) in cDMARD and bDMARD non-responders (84-89). The high levels of IL-6 in autoimmune diseases led to more investigations on it ending up with development of antibodies targeting IL-6 receptor (90). First evidence of tocilizumab on RA patients was illustrated on a Japanese study by Nishimoto et al, where they demonstrated that patients with persistent symptoms achieved ACR20 response in dose-dependent manner (91). This was followed by a European multi-center study where MTX inadequate responders were randomized to different doses of tocilizumab with or without MTX and MTX+placebo (92). Patients on tocilizumab monotherapy achieved better outcome than placebo group, but those on combination were the best.

The relative effectiveness of combined cDMARD versus bDMARD therapy has been studied in several trials. However, it is not so clear which of these alternatives is better option. In the double-blinded randomized trials TEAR and RACAT, the edition of etanercept was generally not more effective than addition of sulfasalazine and hydroxychloroquine (triple therapy), establishing formal non-inferiority in the latter trial but identifying some clinical outcomes

that differed between the treatments (93, 94). Addition of infliximab for 6 months to triple therapy showed a clinically beneficial trend at 2 years compared with triple therapy+placebo group of patients from the NEO-RACo clinical trial (95). Following further yearly examination, however, the slope between the two arms merged closer – resulting in disappearance of the trend at 5 years. In the open-label randomized control trial SWEFOT, the addition of infliximab to MTX was significantly more effective after one year than escalation to triple therapy, but the effectiveness was no longer significant after two years (96).

1.4 PREDICTIVE BIOMARKERS

All these results were obtained on group level. However, it was shown in RACAT trial that some patients who failed to respond to combined cDMARDs did well after switching to bDMARD therapy, and some bDMARD-non-responders did better after they switched to combined cDMARD therapy (94). This suggests that cases are different and an individual approach is needed for more optimal therapy choice for each individual case. For this reason, predictive biomarkers, including demographic and molecular parameters have been studied extensively.

1.4.1 Clinical and demographic factors as predictive tools for disease outcome

Studies on different patient populations show that presence of several factors, including some of the risk factors for aetiology of RA, is indicator of more severe disease and poorer prognosis in RA. For example, smoking and female sex have been shown by many researchers to be poor prognostic factors for disease outcome in patients with RA on different therapies (97-99). Baseline DAS28, on which the treatment outcome is based, is considered one of the strongest and consistently illustrated predictors of response. Results from other baseline clinical and demographic factors are less consistent.

1.4.2 Molecular biomarkers

1.4.2.1 Auto-antibodies

Autoantibodies are one of the earliest components of the disease. They arise several years before disease onset. Most commonly known and used are RF and anti-CCP antibodies, which

have diagnostic role. RF is found in many other autoimmune diseases, but anti-CCP is more specific for RA (100). Being positive for these autoantibodies (but more often for anti-CCP) have been shown in most of studies, relatively consistently, to predict higher risk of radiographic progression (100-106).

Data regarding the association of RF and anti-CCP with subsequent clinical response or remission is more controversial. For example, in predominantly male US veterans with established RA, both RF- and anti-CCP-positivity (but more RF-positivity) were predictive for not achieving remission (107). In an Irish study, anti-CCP-positivity was associated with more lymphocytic infiltration in the synovium, lower proportion of EULAR response and higher proportion of radiographic progression (108). In a Thai study, being double-positive was an indicator of lower likelihood for achievement of remission at 1 year follow-up, compared with double-positive patients (109). Being positive for either of the auto-antibodies and having low likelihood of achievement of remission was shown by other researchers too (110-112). In contrast, several researchers found no association with auto-antibody status at baseline and subsequent clinical outcome (113-116). In addition, higher titers of anti-CCP antibodies have been shown to be predictors of better clinical outcome for some biological therapies (117, 118). The last observation makes anti-CCP antibody interesting possible tool for personalised therapy.

1.4.2.2 Acute phase reactants

C-reactive protein (CRP) is an inflammatory marker produced mainly by hepatocytes during inflammation. Secondary sources might be also monocytes, lymphocytes, adipocytes and some other cells (119, 120). Not being specific for RA, nevertheless, it has a diagnostic role for RA, but also is used to monitor patient inflammation intensity during therapy. Apart from being an inflammatory marker, CRP contributes in maintaining the inflammatory process, by affecting endothelial cells (upregulates VCAM-1 and E-selectin expression) and facilitating migration of leukocytes from blood stream to tissues (121).

CRP is correlated with many symptoms of RA patients and is associated with subsequent radiographic progression (122-125). CRP might have a direct involvement in the joint damage process via increasing RANKL leading to osteoclatogenesis (126).

Similarly to CRP, *erythrocyte sedimentation rate (ESR)* is an indicator of inflammation, correlates with CRP and symptoms of patients with RA, and its high level is associated with

radiographic progression (127-129). It measures rate of sedimentation of erythrocytes in mm per hour which depends on concentration of CRP and other inflammatory molecules in the blood. Apart from CRP, ESR is sex-specific and the threshold values for high category for male versus female is different (>20 versus >30 mm/hr, respectively). In a study, lower baseline levels of CRP (<3 mg/L), but not low ESR (<20 mm/hr) was independently associated with higher likelihood of remission and LDA during anti-TNF treatment (130). In contrast, another study demonstrated higher levels of both CRP and ESR (>10 mg/L and >30 mm/hr, respectively) to be predictive for remission to TCZ (131). Similarly controversial results were observed by Navarro-Compan et al in their systematic literature review (132).

Serum amyloid A (SAA) is another acute phase protein synthesised mainly by hepatocytes and is correlated with ESR, CRP and disease activity in patients with RA (133, 134). Extrahepatic sources, such as synoviocytes and chondrocytes of RA patients, have also been demonstrated (135, 136). In 1983, Chambers et al showed in 185 RA patients that all those with high SAA also had high CRP, however, 40% of patients with low CRP still had high SAA and they were similar clinically with those with high CRP (137). Similar result regarding SAA having higher sensitivity than CRP and ESR have been observed by others (138, 139). This may be caused by the method used (probably, SAA was measured with more sensitive method compared with CRP method). But if the discrepancy is true, both may be useful discriminative biomarkers since in the liver, CRP synthesis is driven by IL-6 and synthesis of SAA by IL-1 (140). SAA has been shown to correlate with 28SJC, and induce expression of MMPs and cell adhesion molecules by synovial fibroblasts and endothelial cells, and facilitate leukocyte migration and angiogenesis (141-145). SAA but not CRP or ESR was also shown to correlate with cartilage turnover markers and predict 1 year radiographic progression (145). However, the pro-inflammatory property of SAA shown *in-vitro* previously was detracted by Björkman et al showing that natural SAA behaves differently from recombinant one (146). The reducing effect of anti-TNF therapy on SAA has been shown by Charles et al, however, not at that magnitude as on IL-6 and CRP (147). Tofacitinib was also shown to decrease both IL-6 and SAA (148). Decrease of SAA by golimumab+MTX during first 4 weeks might be predictive for response at 16 weeks in RA (149). SAA was higher in anti-CCP-positive RA patients compared with negative ones. Its better correlation with DAS28 compared with anti-CCP autoantibodies might make it better biomarker for monitoring RA patients (150). Higher levels of SAA were shown to be associated with a risk of CVDs and renal involvement in

patients with RA (151). SAA was more decreased by ETN+MTX than by conventional DMARDs (152).

1.4.2.3 Inflammatory cytokines, metalloproteinases, growth factors and cell adhesion molecules

In RA patients, there is a communication between immune cells and joint synoviocytes. One of the way of these communications occurs via cytokines. Cytokines are molecules that are released by some cells and affect the same or other cells via their specific receptors. The cytokines may have pro-inflammatory or anti-inflammatory role. In RA the equilibrium is shifted towards predominantly pro-inflammatory cytokines.

Interleukin-1 (IL-1) is an inflammatory cytokine that is commonly produced by different cell types, including synovial fibroblasts in RA patients, and induces production of other inflammatory mediators including TNF-α. In RA, being produced by synovial macrophages and fibroblasts, IL-1 facilitates migration of the inflammatory cells to the joints. By this and inducing proteoglycan degradation, IL-1 plays a fundamental role in joint damage (153). However, Eklund et al has demonstrated that the association of baseline high IL-1β with the presence of erosions does not predict radiographic progression after initiation of DMARD therapy (154). Natural regulator of IL-1 is IL-1 receptor antagonist (IL-1Ra). In RA, the balance between production of IL-1 and IL-1Ra is altered (155-157). Treatment of RA patients with recombinant IL-1Ra (anakinra) have been shown to drop clinical disease activity and slow down radiographic progression (78, 79, 158). IL-1 was also shown to be associated with anaemia in RA patients (159). In 48 RA patients treated with MTX, having high levels of baseline IL-1β AND lower levels of IL-8 was associated with better clinical outcome (based on Ritchie-index, duration of morning stiffness and CRP) (160). Low level of IL-1β expression was observed in synovial tissue of patient with diffuse lymphoid infiltrates, which was associated with seronegative mild form of the disease course, while high expression of IL-1β was seen in patient with granulomatous synovial tissue – associated with extra-articular manifestation with nodules formation (10). Seitz et al showed that in patients with good ACR response (over ACR50) to 6 months of MTX, the ratio of IL-1Ra/IL-1β produced by PBMCs was significantly lower compared with non-responders (<ACR20) (161). Polish researchers showed that MTX+prednisone decreased serum levels of IL-1β and IL-6 but there was no association between the change and clinical response to the treatment (162). In 15 patients with RA and temporomandibular joint pain, high pre-treatment level of plasma IL-1β was a

poor prognostic factor for reduction of the pain after treatment with IFX (163). In another study on 51 RA patients, pre-treatment synovial expression of IL-1β did not differ between ACR20 responders and non-responders to IFX (164). Kayakabe et al, on the other hand demonstrated in white blood cells collected at baseline that their LPS-stimulation caused significantly higher production of IL-1β in 6-month post-anti-TNF treated responders (EULAR good/moderate) than non-responders (165). Baseline low or early (after 4 weeks) achievement of low serum IL-1β levels could predict response to tocilizumab after 1 year of therapy in advanced RA patients (166).

IL-6, together with TNF-α, are considered as disease driving main molecules. IL-6 is a pro-inflammatory cytokine that is produced by variety of cells including lymphocytes, fibroblasts, osteoblasts and endothelial cells. Its action occurs via both membrane bound IL-6 receptor (IL-6R) and soluble receptor (sIL-6R) affecting different cell types. In RA, IL-6 is increased in synovium and blood, and its level correlates with clinical disease activity and joint damage. Its proliferative effect on endothelial cells via increase of VEGF (167) suggests that IL-6 may contribute to angiogenesis in inflamed synovium of patients with RA. T- and B-cell responses are also require IL-6 stimulation. For example, IL-6 helps in differentiation of B cells into plasma cells and antibody production (168, 169), an event that is considered as a start point in the pathogenesis of RA. Moreover, a correlation was observed between IL-6 and RF titres both in synovial fluid and serum of seropositive patients (170, 171). IL-6 together with IL-1β also differentiate naïve T-cells into Th17 subset (172), which has been shown to have a fundamental role in autoimmune diseases. Expression of IL-6R on neutrophils suggests that IL-6 may play a role in neutrophil infiltration of inflamed synovium seen in RA. Indeed, increased IL-6, caused by co-culturing of RA fibroblasts with endothelial cells, and enhanced adherence of neutrophils to endothelium have been reported (173). Moreover, biopsies of synovial tissue from knee joints of 51 RA patients showed positive correlation between synovial IL-6 levels and infiltration of inflammatory cells in synovium (174). IL-6 trans-signalling (interaction of IL-6 with sIL-6R) facilitates osteoclastogenesis via RANKL pathway, leading to bone resorption and joint destruction in RA while the osteoclastogenesis was inhibited by anti-IL-6R antibodies (175, 176). In collaboration with IL-1, IL-6 induces production of MMPs by synovial cells, leading to cartilage degradation (177). Via direct stimulation of hepatocytes, IL-6 may enhance acute phase response (178, 179) and production of hepcidin (180) – a peptide disturbing iron metabolism and causing anaemia, which is

commonly seen in RA. Fatigue, which is a common accompanying symptom in RA was also shown to be achieved in healthy volunteers after IL-6 injection (181).

Methotrexate and other conventional DMARD treatment of patients with RA decreased levels of IL-6 (182, 183). Reduction of IL-6 level during first year of DMARD therapy was shown to correlate with clinical and radiographic outcome at 3 years (184). However, a study on 33 RA patients treated with etanercept and 46 RA patients treated with rituximab did not show statistical difference in baseline serum IL-6 levels between responders and non-responders at 3 months (185, 186). Similarly, a post-hoc analysis of 2 randomised control trials (CIMESRTA, N=150 and OPERA, N=180) failed to illustrate predictive capacity of baseline serum IL-6 level for clinical remission of radiographic progression during 2 and 5 years of follow-up (187). Surprisingly, another study of 73 established RA patients showed better clinical outcome from ETN therapy among those, who had higher baseline IL-6 level (188). Contrary, a study on incomplete responders to conventional combination DMARD or anti-TNF therapy also illustrated association of low IL-6 (<15 pg/ml) at start of RTX with higher likelihood of good EULAR response at 6 months of follow-up (189). Japanese researchers demonstrated in biologic-naïve RA patients that lower pre-treatment serum IL-6 levels were associated with response to TCZ (190). Moreover, they reported that patients with low, but not high pre-treatment IL-6 had more benefit from TCZ than from IFX therapy. Wang and colleagues, on the other hand demonstrated that baseline serum IL-6 levels was not predictive for remission to TCZ therapy based on data from five clinical trials (191). Nevertheless, another Japanese study in DMARD-incomplete responders demonstrated that patients with low serum IL-6 levels (<30 pg/ml) at 3 and 6 months after TCZ start had significantly higher proportion achieving CDAI- and DAS28-based remission at 1 year (192). The association was independent of age and sex. Results from a Japanese study on 33 RA patients with incomplete response to MTX and starting IFX therapy demonstrated poorer clinical outcome in those patients who had higher pre-IFX treatment serum IL-6 level (>5.45 pg/ml) (193). Another Japanese study of 25 RA patients with incomplete response to IFX (3 mg/kg treatment dose) that underwent dose escalation, demonstrated that patients with pre-escalation serum IL-6 level of <5.16 pg/ml experienced more increase in sIFX level with more response compared with patients with ≥5.16 pg/ml level of serum IL-6 (194). This led to better 1-year drug survival after escalation in patients with low compared with high serum IL-6 level. A study on 253 eRA patients showed that serum IL-6 levels were associated with radiographic progression during 4 years of follow-up, independently from BMI (195). However, the

association became a non-significant trend when anti-CCP was included in the model. Gottenberg and colleagues, on the other hand, observed an independent from anti-CCP status association of baseline IL-6 with 1 year radiographic progression in 578 DMARD-naïve eRA patients (196). In another study of 173 RA patients urinary IL-6 showed independent prediction of RP at 1 and 3 years follow-ups (197). Patients with high levels of IL-6 had approximately 3 times higher risk of RP. This association remained even when looking in patients with low ESR only. In 88 newly diagnosed untreated RA patients pre-treatment higher IL-6 levels (>7.6 pg/ml, based on ROC curve) were associated with MRI-detected bone erosion during 1 year of follow-up (198). The association was independent from, and even stronger than seropositivity. Baseline low IL-6 levels, on the other hand, predicted MRI-detected erosion repair.

As mentioned earlier, *TNF* is another key, and mostly studied cytokine in pathogenesis of RA. Similar to IL-6 it affects many different cell types (pleiotropic) and is produced mainly by monocytes, macrophages, some T cells and other type of cells such as synoviocytes. Being a classical pro-inflammatory cytokine it can also express anti-inflammatory effects. Its action is expressed via binding one of the two receptors: TNF-RI (p55) and TNF-RII (p75). TNF-RI is expressed on many cell types, while TNF-RII mainly on immune and endothelial cells, as well as synoviocytes. TNF-RI has death domain and can activate caspase system leading to cell apoptosis, while TNF-RII lacks this domain. Both receptors may also induce cell proliferation and inflammation (199). A natural way of regulation of TNF-signalling is proteolytic cleavage of the membrane bound receptors, which still can bind TNF but do not induce intracellular signalling (decoy receptors) (200). Disability of cleavage of TNF-RI caused by mutation, has been shown to induce auto-inflammatory conditions (201). Signalling from TNF leads to production and secretion of many other inflammatory mediators such as IL-1, IL-6, GM-CSF and IL-8 (202-205). TNF facilitates also osteoclastogenesis and bone erosion through RANKL/RANK and other pathways, as well as cartilage degradation, angiogenesis and attraction of neutrophils (204). Blocking TNF in RA patients and mice with arthritis lead to decrease in nociceptive activity in the CNS detected by MRI already 24 hours after first injection of the anti-TNF drug (206). Notably, clinical and serological inflammatory signs were still unaffected. This suggests that TNF also contributes to pain feeling via directly affecting the CNS in patients with RA.

TNF has been shown to be associated with histologically different disease phenotypes. For example, a study on 25 RA patients showed that subjects with follicular synovitis had

significantly higher levels of serum TNF compared with patients with diffuse infiltrates (207). A similar study on 47 RA patients by the same investigators showed similar results: higher serum levels of TNF, TNF-RI and TNF-RII in patients with follicular synovitis compared with diffuse histological type (208). In 50 RA patients, higher baseline production of TNF-RI from PBMC and higher serum levels of TNF-RII were observed in better responders to 6 months of MTX therapy, when compared among 4 response groups (>ACR70 and ACR70-50, ACR20-50 and <ACR20) (161). However, no significant association was observed for TNF. In an American study, pre-treatment sTNF levels were significantly higher in RA patients who did not respond to IFX therapy (assessed by Richie score index) compared with those who responded (209). In non-responders, increasing frequency of administration improved better clinical outcome than increasing the dose of IFX. A study in 60 RA patients demonstrated in multivariate analysis that synovial tissue mRNA level of TNF (among other cytokines) was predictive for joint damage progression (210). Contrary, a study of 99 serum biomarkers (including TNF) in 152 RA patients from GO-BEFORE trial did not reveal significant correlation between baseline levels of TNF or in the change of TNF at week 4 and radiographic damage progression at week 28 or 52 (211). Another study of 44 RA patients did not reveal any association of peripheral blood mRNA level at baseline with subsequent response to IFX at 22 weeks (212). Immunohistochemical examination of pre-treatment synovial biopsies of 143 RA patients indicated association of higher expression level of TNF with subsequent response (delta-DAS28>1.2) to IFX at week 16 (213). Responders also had higher number of infiltrated macrophages and T cells (potential sources of the TNF). In 33 RA patients treated with ETN, serum level of TNF at baseline was not significantly different among responders (improvement in 3 of 4 ACR criteria) and non-responders at 3 months (185). In contrast, a study on 93 early RA patients from Japan, Sweden and USA demonstrated predictive capacity of pre-treatment serum TNF for response to ETN (higher in responders) (214). In 42 RA patients treated with non-biological DMARDs, baseline serum TNF was significantly lower in ACR20 responders than non-responders at 6 months (215). A Japanese study done on 327 RA patients from a double-blinded randomized control trial (RISING), (patients started IFX after MTX failure), illustrated a clear association of baseline plasma TNF levels with response (EULAR good or DAS28 remission or ACR-based) at 54 weeks (216). In patients receiving the highest dose of IFX (10 mg/kg) significantly higher proportion of patients responded if they had lower baseline TNF levels (dose-response relationship among patients with low, intermediate and high levels of plasma TNF levels). It

was also observed that patients with high baseline levels of TNF had lowest level of serum IFX at 54 weeks, which was also associated with poorer outcome.

IL-17, another cytokine, relatively recently have been shown to be involved in pathogenesis of autoimmune diseases. It is mainly produced by Th17 subset of CD4+ T cells and can be considered as opposing balance to Treg cells (217). IL-17 acts via its dimerised receptor leading to production of other inflammatory cytokines and chemokines, which could partially explain migration of neutrophils and monocytes (218, 219). Increased level of IL-17 has been reported in synovial fluid and serum of patients with RA (220, 221). Surprisingly, a nested case-control study has demonstrated that levels of IL-17 in pre-RA individuals (subjects without any symptoms and complains, who developed later RA) was significantly higher compared with the levels at the diagnosis (222). It can also induce osteoclastogenesis via RANKL pathway. The inflammatory and bone resorptive effect of IL-17 is synergised by the presence of TNF (219). In RA patients, serum IL-17 levels are highly correlated with disease activity, serum levels of TNF and CRP, and synovitis and bone erosions detected by MRI (221, 223). Nevertheless, IL-17 seems to be more important for pathogenesis of other inflammatory diseases such as psoriasis, since blockade of IL-17 or its receptor has been shown to have the best effect and were approved for psoriasis (219).

In 60 RA patients from the DAMAGE study cohort, expression level of synovial tissue mRNA of IL-17 in combination with synovial mRNA of TNF or disease duration was shown to predict joint damage progression detected by MRI at 2 years of follow up (210). In 20 active RA patients (10 treated with MTX and 10 – with adalimumab), Th17 cell levels in peripheral blood at baseline were associated with poor clinical outcome at 3 months (224). Levels of IL-17 were decreased in 25 RA patients after 30 weeks of treatment with MTX+IFX, but not in patients on MTX monotherapy (n=20) (225). A study in 48 RA patients treated with anti-TNF (adalimumab or IFX) demonstrated that baseline IL-17 levels were significantly higher in EULAR non-responders compared to moderate/good responders after 6 months (226). In addition, among other Th17-related cytokines only IL-17 increased during the therapy in non-responders, while others decreased. A multivariate logistic regression analysis indicated IL-17 the only predictor of poor response. Similarly, Alzabin et al demonstrated in 2 groups of RA patients (n=24 and n=19) treated with anti-TNF, that in non-responders there was significant increase of serum IL-17, while in responders the level was stable (227). Also there was an inverse relationship between proportion of Th17 cells at baseline and DAS28 at 4 week after the therapy. A double-blinded randomised control trial of

TCZ therapy in patients with inadequate response to DMARD therapy, demonstrated that baseline low serum level of IL-17 was associated with achievement of remission at 12 weeks (228). Of patients with low IL-17 levels, 48% achieved remission compared with only 17% of those with high IL-17 levels.

In the joints of RA patients, a release of *matrix metalloproteinases (MMPs)* occur, that leads to digestion and destruction of bone and cartilage tissue. There are 23 different types of MMPs in the humans (from MMP-1 to MMP-28, where MMP-4, MMP-5, MMP-6 and MMP-22 do not exist in the nomenclature) (229). Physiologically, the MMPs are proteases with Ca^{2+} or Zn^{2+} active sites. They are playing important role in degradation of components of extracellular matrix, such as collagen, during tissue remodelling at different stages of development of the organisms. Typical source of MMPs are cells of connective tissues of organs, fibroblast, for example, but also other cells: neutrophils, monocytes, macrophages and endothelial cells. Expression of MMPs may be increased by inflammatory cytokines, hormones and growth factors.

In patients with RA, main matrix degrading members of MMPs are considered MMP-1 (responsible for degradation of collagen type I, II, III, VII and X), MMP-3 (degrades collagen type III, IV, IX and X), MMP-7 (degrades collagen type IV and X), MMP-9 (degrades collagen type IV, V, VII and X) and MMP-13 (degrades collagen type I, II, III and IV) (230, 231). It has been shown that MMPs, for example, MMP-1 and MMP-3, reflect clinical disease activity and structural damage of the joints in patients with RA (232-236). Their levels can also decrease during therapy, which is accompanied by clinical improvements (237, 238). MMP-7 has been shown to be linked with tissue remodelling in the lungs in patients with interstitial lung disease. In RA patients with interstitial lung disease, circulating levels of MMP-7 are significantly higher compared to patients without the complication (239). In a Japanese study of early RA patients, higher serum MMP-3 levels were associated with increased risk of joint damage (based on Larsen score) in the future (240). A study in RA patients revealed MMP-1 level more that MMP-3 level to be associated with development of erosions and stronger association of MMP-3 compared with MMP-1 with CRP (241). Early changes in MMP-3 caused by treatment using MTX or tenidap were predictive for later clinical response evaluated by DAS (242). Another study in 98 early untreated RA patients demonstrated that baseline serum MMP-1 and MMP-3 levels correlated with clinical improvements and joint damage progression (delta Larsen score) (243). Even patients with low CRP but high MMP-1 and MMP-3 showed higher risk of erosive disease during

subsequent 1 year. In patients without erosion at baseline, high level of MMP-3 was predictive for progression of Larsen score. A study in RA patients from ASPIRE cohort showed that baseline serum MMP-3 level as well as its reduction during first 6 weeks of therapy were associated with response (based on ACR criteria) at 54 weeks (238). Among other baseline markers, in a multivariate logistic regression model, only MMP-3 was predictive for clinical and radiographic outcome at 1 year. Similar predictive results of MMP-3 (especially for radiographic progression) were seen by other researchers (244-248). However, a study of 45 RA patients treated with etanercept showed that reduction of MMP-3 during 6 weeks of therapy did not differ between responders (patients in remission, n=10) and non-responders (n=35), while the decrease of another cartilage turnover biomarker, cartilage oligomeric matrix protein (COMP) was significantly greater in responders than non-responders (249). Another controversial result was reported by Japanese researchers illustrating that baseline MMP-3 levels had a trend of higher level among progressors than non-progressors, while RF- and ACPA-positivity were significantly associated with subsequent radiographic progression (250). In contrast to this, a study in 118 RA patients that were followed up during 8 years, even though in a univariate analysis many baseline markers (including COMP and MMP-3) were associated with RP at 8 years of follow-up, a multivariate analysis revealed only MMP-3, anti-CCP-positivity and baseline radiographic damage of joints as independent predictors of radiographic progression (251). Significantly greater reduction in serum MMP-9 was observed in responders compared to non-responders to golimumab add-on therapy in MTX inadequate responders (149). In 114 patients with RA, an improvement of MMP-3 during first 4 weeks of therapy with adalimumab was independently from DAS28-CRP associated with remission at 1 year (252).

Human cartilage glycoprotein-39 (YKL-40) is a protein produced by chondrocytes, synovial fibroblasts, macrophages and neutrophils. It is considered as a chitinase enzyme with lost enzymatic activity towards chitin. Increased levels of serum YKL-40 were linked with inflammation and tissue remodelling, however, still the physiological role of YKL-40 is unclear (253). In synovium of patients with RA, compared with healthy controls YKL-40 is also elevated. Even though serum levels are significantly lower than that in synovium, there is a clear correlation between them. Moreover, both synovial and serum levels reflect disease severity and degree of joint damage in RA (253, 254).

In a study of 191 early RA patients treated with DMARDs (MTX or SSZ), baseline serum levels of YKL-40 were not associated with radiographic progression during subsequent 3

years of follow-up (255). Similar non-association of baseline YKL-40 with 2 years radiographic progression was observed in another RA group treated with adalimumab, even though it was correlated with CRP, MMP-1 and MMP-3 (236). Similarly, a Finish study confirmed unpredictability of baseline serum YKL-40 levels for subsequent radiographic progression, however, correlation with inflammatory markers was obvious (256). A study in 136 RA patients also showed no predictive value of baseline YKL-40 levels for RP during subsequent 5-10 years (257). And finally, a study in patients from CIMESTRA trial (n=150) confirmed non-predictive ability of serum YKL-40 for RP, but also for achievement of clinical remission (187). In the same study, the authors confirmed these results in a validation cohort from OPERA trial (n=180).

Leptin is a lipid hormone produced by white adipose tissue and is known as a regulator of appetite and energy expenditure via acting on hypothalamus and inhibiting production of neuropeptide Y. Its production is positively correlated with body weight and decreases appetite in order to avoid obesity (258). Leptin has been found to be related also to inflammation and immune response. This relationship is two-sided: pro-inflammatory cytokines increase leptin level, as well as leptin induces production of pro-inflammatory cytokines and activates phagocytosis (259). Leptin's pro-inflammatory role via impact on innate and adaptive (mostly T cells) immune systems has been shown in many studies (260). In RA, synovial leptin level is significantly lower than in serum, and RA patients have significantly higher level of leptin than healthy controls (261). However, the data about leptin in RA is quite controversial among different studies, as discussed in Tian et al review article (260). Some studies showed protective role of leptin for radiographic progression, while others failed to illustrate that. Similarly, a controversial results about effect of anti-TNF therapy on serum leptin is discussed in the same review, indicating no change in the majority of studies, however, some studies observed increase in leptin level. Probably, its high dependence on proportion of white adipose tissue makes it difficult for any conclusion. In a group of consecutive RA patients treated with non-biological DMARDs, baseline leptin was associated with higher DAS28 at 6 months (262). In patients with normal BMI, the association hold also for 1 and 2 years outcome. The complexity of results regarding leptin as reflector or predictor of disease activity could be explained by its dependence on other parameters, such as proportion of white adipose tissue. This in its turn is affected by body weight and gender. Smoking should also be considered when investigating leptin in RA patient, since it suppresses leptin production (263).

Another adipokine, *resistin*, which was initially thought also to be produced mainly by adipocytes, and to cause insulin resistance, later has been shown to be produced by peripheral blood mononuclear cells and more to be involved in subclinical inflammation processes and autoimmune diseases (264). In RA, levels of resistin in both synovial tissue and serum is elevated compared to healthy controls and even compared to patients with osteoarthritis and spondyloarthropathy (265). Resistin is produced by different immune cells on response to inflammatory cytokines and induces homing of endothelial progenitor cells to the joints, and overexpression of VEGF (266). Consequently, resistin might be involved in the process of angiogenesis in RA. Resistin was also shown to correlate with disease activity markers such as serum TNF and CRP, RF anti-CCP and joint radiographic data (267). Anti-TNF treatment decreases serum level of resistin in RA patients which is associated with post-treatment reduction of CRP and TNF (268). However, as usual with the majority of biomarkers, there are some studies not confirming above mentioned results for resistin. For example, Otero et al showed that leptin, adiponectin and visfatin levels but not resistin levels were significantly different in 31 RA patients compared to 17 healthy controls, and that the mentioned three biomarkers but not resistin correlated with CRP (269).

For prediction of radiographic progression over 4 years, baseline resistin levels had no value (195, 270). Regarding clinical disease activity only correlations have been published between resistin and different disease activity measures from the same time-points, but no predictive results have been reported.

Some of characteristic features of RA are hyperplasia of synovial fibroblasts leading to invasion of the synovial tissue, hypoxia and neovascularisation, leading to pannus formation. *Epidermal growth factor (EGF)* is a known marker for tumour tissue since it induces neoplastic alteration. It also is responsible for angiogenesis. Similarly to tumour, in RA, EGF is upregulated. Both synovial and serum concentrations are higher compared to healthy individuals, but even compared with osteoarthritic patients (271-273). Hyperplasia of synovial fibroblast may partially be explained by increased EGF, since upregulation of EGFR expression on these cells is reported in RA patients. A significant correlation with neovascularisation also indicates its role in the latter. A mouse model of collagen induced arthritis demonstrated that inhibition of EGFR reduces symptoms of the arthritis, and proliferation of synovial fibroblasts and osteoclasts, indicating indirectly that EGF and its signalling may play an important role in the pathogenesis of RA (273). These results were replicated by another research group (274).

Early changes in serum EGF levels were found to be positively correlated with later changes in total van der Heijde-Sharp radiographic score in early RA patients on MTX monotherapy, but not in patients on MTX+golimumab (211).

Vascular endothelial growth factor (VEGF) is the main angiogenic factor that has been considered in RA patients. It is produced by macrophages, synovial fibroblasts, platelets and endothelial cells (275). Similar to EGF, levels of VEGF have been shown to be higher in synovial tissue, fluid and sera of RA patients compared with healthy people or osteoarthritis patients (276). Fast growth of fibroblast-like synoviocytes causes hypoxic environment in the arthritic joints which activates angiogenesis via upregulation of VEGF (277). Blocking VEGFR on the other hand improved symptoms in collagen induced arthritis in mice (278). In RA, serum levels of VEGF have been shown to correlate with inflammatory markers and clinical disease activity symptoms such as CRP, tender joint count and pain. Pro-inflammatory cytokines such as TNF, IL-1, IL6 and others, induce production of VEGF and therapy of patients with RA (both non-biological and biological DMARDs) decreases the serum level of VEGF and reduce newly built capillaries, but not normal blood vessels (275, 277, 279).

Increased levels of serum VEGF is associated with radiographic progression at 1 year of follow-up and reduction of sVRGF levels – with clinical improvements (280). Another study in two different cohorts of RA patients: CIMESTRA (N=150) and OPERA (N=180), showed correlation of VEGF with DAS28, but could illustrate predictive ability of VEGF for radiographic progression at 2 years of follow-up only for OPERA cohort, and for neither of the cohorts was VEGF predictive for clinical remission (187).

Migration of the leukocytes from blood stream through endothelial layer to the affected tissue is one of necessary and indispensable processes for inflammatory response. The cytokines that are released from macrophages and other cells in the damaged tissue play a crucial role in recruiting the leucocytes. The process of migration of the inflammatory cells occurs due to changes on the surface of both the leukocytes and endothelial cells, which enables the former to become more adherent to the later. In this way the leukocytes start rolling on the blood vessel walls before the migration occurs. Stickiness of the cells is ensured by adhesion molecules that are overexpressed on the membrane, induced by inflammatory cytokines. Here, *vascular cell adhesion molecule-1 (VCAM-1)*, among other is known to have an important role for binding integrins on the surface of the leukocytes. VCAM-1 is mainly

expressed on endothelial cells, but in inflamed environment its expression is detected on other cells such as tissue macrophages, dendritic cells, bone marrow fibroblasts and others (281).

The importance of VCAM-1 in RA has been shown by many researchers, which is discussed in a review article by Kong et al (281). Serum level of soluble VCAM-1 is higher compared with healthy controls. Treatment with DMARD or biological medications decreases its level. Blocking VCAM-1 with monoclonal antibodies dramatically reduces signs of arthritis in the CIA moue models. However, when studying expression of different molecules on synovial membrane in 40 RA patients treated with non-biological DMARDs, an association was found between decrease in expression of other cell adhesion molecules, but not VCAM-1 on the synovial biopsies and improvement of clinical outcome (282). Macias et al demonstrated in RA patients that after MTX monotherapy, responders had significantly lower serum VCAM-1 compared with incomplete responders (283). In 143 RA patients failing two DMARD therapies and starting IFX, expression of VCAM-1 in joint tissue sections, detected by IHC just before IFX start, did not differ between subsequent responders and non-responders at week 16 of IFX therapy (213).

1.4.2.4 Multi-biomarker disease activity score

RA is a very heterogeneous disease. In addition to this, different clinical trials address different questions, using different inclusion and exclusion criteria, which make patients to differ significantly between studies. And finally, the stage of disease could affect variations in pathological processes and treatment responses. These are important facts that one needs to consider when trying to replicate biomarker research on different RA cohorts. The abundance of discrepant results for the same biomarker could be explained by these factors. Therefore using one biomarker would probably always lead to failure in validation of results in a different cohort.

A multi-biomarker disease activity (MBDA) score (Vectra® DA) is a blood-based test that has been developed as a tool for monitoring of disease activity in complement to existing disease activity measures such as DAS28 and radiographic imaging of the joints (284, 285). It has been developed by a multi-step process, starting from identification of 130 candidate molecules from literature searches, and narrowing down during the further prioritisation studies to 24 candidate biomarkers (286). The 24-biomarker-based disease activity score already correlated with DAS28 and radiographic progression. At the next stage of

development, some biomarkers were eliminated because of their high dependence on the conditions of the analyses, resulting in a high variability, and other reasons. After the final optimisation and algorithm training, 12 biomarkers remained in the model. The algorithm of the final MBDA score is calculated from serum concentrations of the following 12 biomarkers: VCAM-1, EGF, VEGF, IL-6, TNF-RI, MMP-1, MMP-3, YKL-40, leptin, resistin, SAA and CRP. The MBDA score is well correlated with DAS28 and other disease activity measures, and has a scale between 1 and 100 indicating from the lowest to the highest disease activity, respectively (284, 287, 288). Its validated cut-offs generate categories of patients in LDA (MBDA score <30), moderate disease activity (MBDA score 30-44) and high disease activity (MBDA score >44). Being correlated with disease activity and inflammatory markers, the MBDA score seems to complement them as shown in the study by Lee et al (289). In their study it was shown that many RA patients with CRP<1.0 mg/dl, had moderate/high MBDA score and there was a linear association between SJC and the MBDA score but not CRP. The dynamic of the MBDA score also reflects the change of disease activity measured by other clinical methods. These associations were independent of a type of anti-TNF treatment (IFX, adalimumab or etanercept) (290). However, assessment of the MBDA score in RA patients treated with tocilizumab should be interpreted cautiously, since tocilizumab causes increase of serum IL-6 leading to increase in the MBDA score, while decreasing other disease activity indices (291).

Predictive studies for the MBDA score have also been conducted by many researchers. For example, the MBDA score predicted progression of joint damage detected by radiographs, ultrasound or MRI in different RA studies (292-296). However, Bakker et al demonstrated that even though the MBDA score was well associated with disease activity, it could not predict subsequent radiographic progression (287). It was also shown that the MBDA score predicting relapse in RA patients that were in sustained remission (297). The prediction was independent from ACPA and their combination increased associations: in patients with low MBDA score and negative ACPA status only 13% relapsed, while among those with higher MBDA score and positive ACPA status 76% relapsed. In the DRESS study, in patients in remission and tapering anti-TNF therapy the MBDA score did not predict flare or successful tapering/discontinuation, however, did predict flares in usual care patients (298). In contrast to these associations mentioned above, another Dutch study (POET) has demonstrated that patients in LDA and tapering anti-TNF treatment, high baseline MBDA score predicted flare at 12 months (299). Another controversial finding was reported in a study of RA patients

treated with abatacept or adalimumab, where the MBDA score was correlated with DAS28-CRP and CDAI only at baseline, but not at follow-ups (300). Moreover, the researchers reported that CDAI was better associated with radiographic progression outcome than the MBDA score. To summarise, it is worth to mention that for interpretation of the MBDA score, consideration of the age, and BMI of the patients have an important role (301).

1.4.2.5 Serum level of therapeutic bDMARD and anti-drug antibodies

Prediction of response to bDMARDs should have been more clear compared to non-biological DMARDs, because of a clearly defined target. However, the prediction is complicated by the immunogenicity of the therapeutic drugs. The structure of the bDMARD molecules triggers the immune system of the patients to respond and generate anti-drug antibodies (ADA). Most of the cases these antibodies are neutralising (binding to the target-binding region of the drug molecule). But even non-neutralising antibodies may create immune complexes leading to accelerated clearance of the bDMARD from the organism of the patient. In any of the cases the immunogenicity will lead to decrease of therapeutic drug concentration in the blood consequently leading to loss of efficiency. Infliximab is considered to be the most immunogenic (302) because of its not fully humanised molecular structure (TNF-binding site is from mouse).

According to different studies the proportion of RA patients developing ADA to IFX fluctuates between 12% and 54% (303-310). Low drug levels in the blood of patients was associated with ADA-positivity (305, 307, 311). In addition, both low drug level and ADA-positivity have been associated with poorer clinical outcome and worse drug survival compared with patients with high drug level or ADA-negative patients (303, 312-315). These observations suggest that monitoring of patient bDMARD level in the blood as well as analysis for ADA may provide essential information to the rheumatologist for optimisation of biological therapy in patients with RA. Garces and colleagues, for example, have suggested an algorithm of treatment of RA patients with bDMARDs based on the availability of information about current disease activity, serum trough level of the therapeutic bDMARD and ADA status (316). Such studies need validation on different RA cohorts using the same methods of analysis for serum drug levels and ADA status. Confirmation of the importance of the blood drug monitoring and ADA status for achievement of good clinical outcome would improve dramatically the healthcare in rheumatology.

In conclusion, RA, as a very heterogeneous disease with unclear aetiology and pathogenesis, is challenging for investigation. The optimal therapy is different for individuals; therefore conducting biomarker studies has an important role in discovery of individuals with different mechanisms of disease. In biomarker discovery a huge role plays the method and patients inclusion criteria since different methods of measurement of biomarkers, serum drug levels and anti-drug antibodies may yield very different results, likewise whether patients were on different therapies during several years or they are newly diagnosed, treatment-naïve early RA patients. Considering these factors in the investigations will provide more reliable data for description of predictors in RA populations.

2 AIM OF THE THESIS

2.1 OVERARCHING AIM

In the presence of challenge of optimal therapy choice and to predict disease course and outcome, the general aim of this PhD thesis was to investigate different blood proteins related to inflammation and immune system as predictors of disease outcome in patients with early RA. The exploration of predictors will contribute in identifying patient phenotypes with certain characteristics, which will enable the best personalised approach for each individual patient.

2.2 SPECIFIC AIM

The study-specific aims of this PhD projects were:

1) For Study I
   a) Using the MBDA score, to identify patients at high versus low risk of subsequent joint radiographic progression.

2) For Study II:
   a) Using the MBDA score at the time of treatment escalation, to identify patients at higher chance of response to IFX or conventional triple therapy (TT), after failure to MTX monotherapy.

3) For Study III:
   a) Using individual biomarkers that comprise the MBDA score, to identify patients at higher versus lower chance of response to MTX monotherapy in early RA.
   b) Using affinity proteomics analyses to explore serum biomarkers that could identify patients at higher versus lower chance of response to MTX monotherapy in early RA.

4) For Study IV:
   a) In early RA patients randomised to IFX+MTX therapy, to study relationship between serum IFX concentration, anti-drug antibodies (ADA) and clinical outcome. We also aimed to investigate baseline predictors for low serum IFX level and development of ADA.

3 MATERIALS AND METHODS

3.1 PATIENT POPULATION

For all study projects materials from Swedish Farmacotherapy (SWEFOT) trial were used (96, 317). The SWEFOT is a randomised-controlled clinical trial of DMARD-naïve, early RA patients (N=493). Newly diagnosed RA patients with active disease (DAS28 >3.2), symptom duration <1 year and age >18 years were included to the trial and started MTX monotherapy for 3 months duration. Patients achieving low disease activity (DAS28 ≤3.2) after 3 months continued the MTX therapy and those patients with DAS28 >3.2 were randomised to conventional triple therapy (MTX-sulfasalazine+hydroxychloroquine) or to biological therapy (MTX+IFX). The patients were followed up 2 years from the inclusion into the trial (Figure 1).

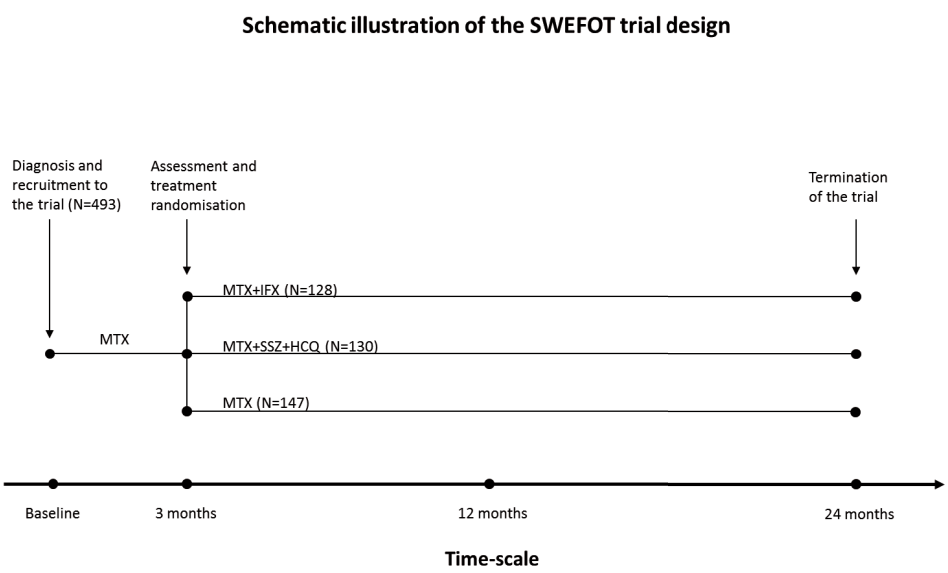

Figure 1. SWEFOT trial design

This thesis is composed of four Study projects (Figure 2) and for each of the project a subset of patients was selected based on availability of data needed for the specific study aim. For the MBDA score projects, totally serum samples of 302 patients were analysed at baseline, of whom 290 were analysed at 3 months and 190 (only randomised patients) at 1 year. However, each of the substudies done on the MBDA score data, different number of these 302 patients were selected (see below for details in each individual project).

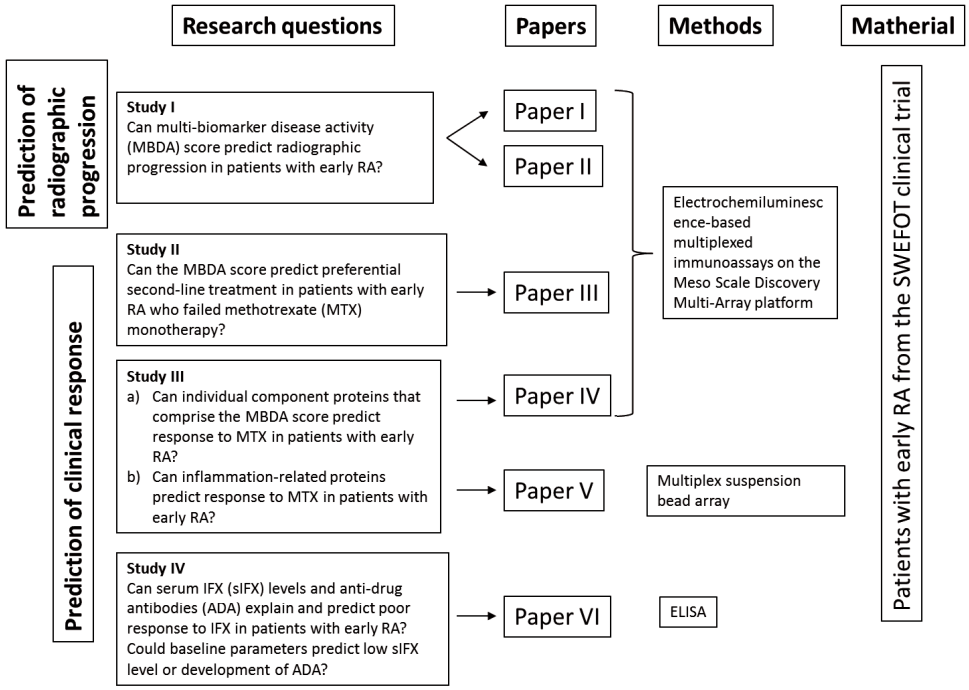

Figure 2. Plan of PhD projects

3.2 STUDY I

In Study I the MBDA score was investigated for prediction of subsequent radiographic progression. In the first paper of the Study I (paper I), the MBDA score of 235 patients at baseline were analysed for prediction of RP at 1 year. For the second paper (paper II) the MBDA scores at baseline, 3 months and 1 year were analysed in 220, 205 and 133 patients respectively for prediction of RP between baseline and 1 or 2 years and between 1 and 2 years. For the RP, van der Heijde modified Sharp scores (SHS) were used. An increase of the SHS >5 was considered as RP. Cut-offs of >3 and >0 were also compared. The Proportion of patients with RP were compared among categories of the MBDA score (low: <30, moderate: 30-44 and high: >44) and other disease activity measures (CRP, ESR and DAS28). In the second paper (paper II) not only MBDA score at a certain time-point, but also the dynamic of it from baseline to 3 or 12 months and from 3 to 12 months was assessed for prediction of RP from baseline to 1 or 2 years and from 1 to 2 years.

3.3 STUDY II

In the Study II, which was published in paper III, the association of the MBDA score at 3 months with second-line treatment outcome at 1 year was assessed. As treatment outcome measures, low disease activity and EULAR good response were used. For patients with missing clinical data at 1 year, last observation carried forward (LOCF) was applied. In this study 157 patients who failed to achieve LDA on MTX monotherapy were included. Of the 157 patients, 75 were randomised to conventional TT and 82, to IFX therapy. Apart from validated categories for the MBDA score (low, moderate and high), new cut-offs were also applied based on receiver operating characteristic (ROC) curve analysis for dichotomisation of patients into high and low MBDA score categories. The proportion of patients achieving LDA or EULAR good response between groups with low versus high MBDA categories were compared for each therapy group separately and then the results were compared between the therapy groups. As reference predictors CRP, ESR and DAS28 at 3 months were compared.

3.4 STUDY III

In the Study III protein biomarkers at baseline were analysed for association with treatment outcome (LDA and EULAR good response) at 3 months in RA patients treated with MTX monotherapy.

3.4.1 Study IIIa

Here we investigated the MBDA score and comprising it 12 individual biomarkers at baseline as predictors of achievement of LDA or EULAR good response at 3 months. Two hundred and ninety-eight patients were included of whom, 104 achieved LDA and 101, EULAR good response. Four biomarkers that were significantly different between patients who achieved and did not achieve LDA. They were analysed by ROC curve analysis for dichotomisation into low and high categories. Then the proportion of patients achieving treatment outcome was assessed. The four biomarkers were also combined into a *combined biomarker score* resulting in a scale between 0 and 4 and indicating respectively, the association with lowest to highest risk for not achieving the treatment outcome. The combined biomarker score was also added to a predictive matrix together with previously established demographic predictors such as sex and age.

3.4.2 Study IIIb

In this study 177 target proteins were assessed at baseline in 135 selected RA patients as predictors of outcome of MTX monotherapy at 3 months. In univariate analyses, eight proteins differed between patients who achieved (n=50) and did not achieve (n=85) LDA at a level of p-value <0.001. In a multivariate analysis, only two were significantly different. These two proteins were analysed by ROC curve and were dichotomised into low and high categories with subsequent comparison of proportion of patients achieving treatment outcomes. The two proteins were combined then generating four categories: low/low, low/high, high/low and high/high, and the proportions of patients achieving treatment outcome were assessed between these categories. In this study, an attempt of reproducing the results for one of the proteins was done in another RA cohort (COMBINE, N=74).

3.5 STUDY IV

In the Study IV, 101 patients who failed MTX therapy and were randomised to MTX+IFX treatment were included for investigation of serum level of IFX (sIFX), anti-drug antibodies (ADA) and their relation with treatment outcome (LDA or remission), as well as to identify baseline predictors of low sIFX level and ADA development. The serum samples were analysed at 3 (missing n=8), 9 (missing n=6) and 21 months after initiation of IFX treatment. All 289 serum samples were analysed for sIFX level, but only samples with sIFX concentration <0.2 μg/ml (n=64) were analysed for ADA-positivity (Figure 3).

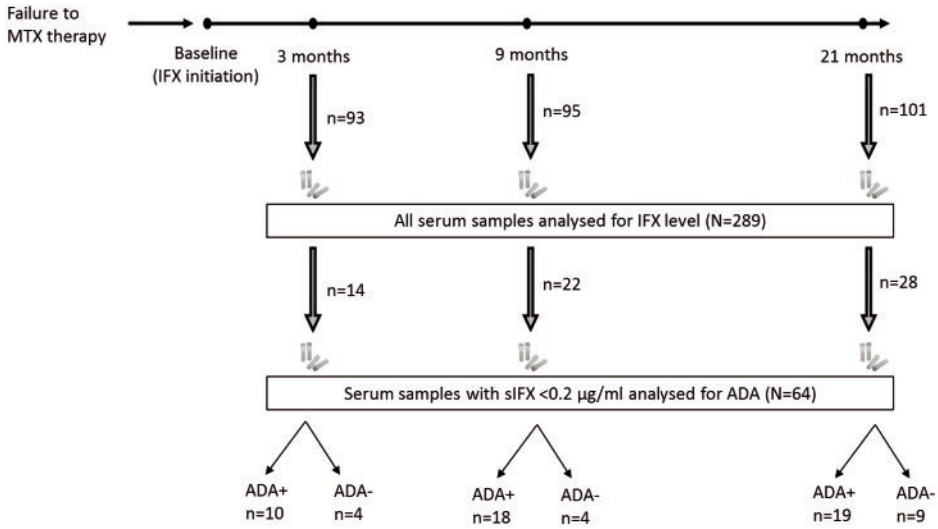

Figure 3. Schematic illustration of serum samples analyses for IFX and ADA in 101 patients from the SWEFOT trial

Patients who were ADA-positive at least ones during the follow-up, were considered as ever ADA-positive and their baseline parameters were compared with parameters of never ADA-positive patients.

3.6 SERUM SAMPLE ANALYSES

Serum samples for all study projects were analysed out of our facility by different laboratory personals.

3.6.1 Analyses of 12 proteins and the MBDA score generation

The serum samples were shipped to Crescendo Bioscience, South San-Francisco, CA, USA for the analysis. The individual 12 biomarkers were analysed by an electrochemiluminescence-based multiplex immunoassay on the Meso Scale Discovery Multi-Array platform. The MBDA score was calculated based on concentration of the 12 biomarkers using Vectra® DA trained algorithm, which is a patent of Crescendo Bioscience and is undisclosed.

3.6.2 Analyses of serum proteins for the affinity proteomics project

Serum samples were sent to Science of Life Laboratory (SciLifeLab) for the analysis of 177 proteins, which was done in Prof. Peter Nilsson's lab. The 177 target proteins were selected based on previous studies in inflammatory and autoimmune diseases. These proteins were analysed using 380 antibodies from Human Protein Atlas. In addition four controls were also used: anti-human IgG and anti-albumin as positive controls, and rabbit IgG and beads without any proteins as negative controls. The levels of the proteins were expressed in median fluorescent intensity (MFI).

3.6.3 Analysis of sIFX levels and ADA

Both sIFX and ADA were measured using in-house validated ELISA methods that are used at Swedish University Hospitals. For sIFX, plates coated with TNF were used. For detection of ADA, only samples with <0.2 µg/ml sIFX levels were analysed, since IFX interferes with the analyts and gives false-positive results. For detection of ADA, competitive ELISA was used: the analyte, which is also IFX, is incubated with TNF-coated plate followed by adding serum, whose unbound ADA competes and displaces with TNF and binds to the analyte.

3.7 STATISTICAL ANALYSES

For non-parametric variables Mann-Whitney U test was used for all study projects. In all projects also proportion of patients were compared between two different groups, for which Chi-squared or Fisher's Exact test were used. In Study III, the homogeneity of odds ratio was tested by Breslow-Day test. For prediction analyses, uni- and multivariate logistic regression analyses were used (Study I, III and IV). In Study IIIb, of 380 variables (for 177 proteins), those with p-value <0.001 in the univariate analyses were included in multivariate logistic regression model. For cut-of determination, ROC-curve analysis was applied in Studies II and III. The cut-of levels were based on values corresponding to the highest sum of sensitivity and specificity.

4 RESULTS AND DISCUSSION

4.1 STUDY I (PAPERS I AND II)

In Study I, we investigated the MBDA score as a predictor of RP. The selected patients for this study did not differ significantly by their baseline parameters from the entire SWEFOT population. As it was expected, the MBDA score correlated with disease activity markers such as DAS28 and CRP (Figure 4).

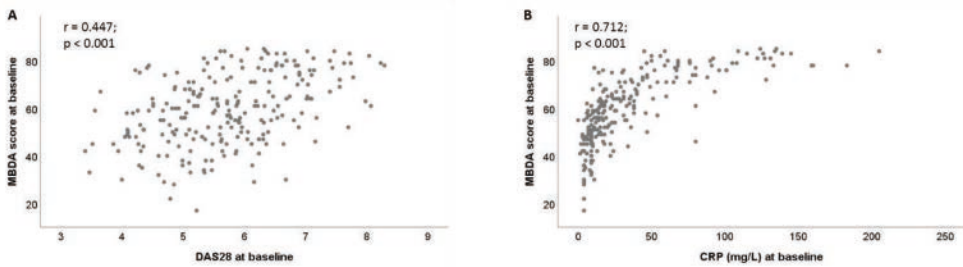

Figure 4. Correlation of the MBDA score with DAS28 (A) and CRP (B) at baseline.

4.1.1 Discordance between the MBDA score and other disease activity markers

The categories of these markers, however, had some discrepancies (Figure 5). For example, of 235 patients 5, 29 and 201 had low, moderate and high MBDA scores, however, of 71 patients with low CRP 42 had high and 24 had moderate MBDA scores and about quarter of patients with low CRP but high MBDA had RP at 1 year (Figure 5C). Patients with low MBDA score (n=5), on the other hand, had low CRP and none of them progressed. No patient had low DAS28 at baseline, since it was an exclusion criteria for the SWEFOT trial. In total, low/moderate versus high categories of the MBDA score at baseline discriminated patients at very low versus high risk of subsequent RP at 1 year (3% and 21%, respectively, p=0.012; Figure 5D).

A

	Baseline DAS28-ESR			
Baseline MBDA	Low (≤3.2)	Moderate (>3.2 - 5.1)	High (>5.1)	Total
Low (<30)	0	0/3	0/2	0/5
Moderate (30 - 44)	0	1/14	0/15	1/29
High (>44)	0	9/51	33/150	42/201
Total	0	10/68	33/167	43/235

B

	Baseline DAS28-CRP			
Baseline MBDA	Low (≤2.7)	Moderate (>2.7 - 4.1)	High (>4.1)	Total
Low (<30)	0	0/1	0/4	0/5
Moderate (30 - 44)	0	1/5	0/24	1/29
High (>44)	0	2/16	40/185	42/201
Total	0	3/22	40/213	43/235

C

	Baseline Clinical CRP			
Baseline MBDA	Low (≤10)	Moderate (>10 - 30)	High (>30)	Total
Low (<30)	0/5	0	0	0/5
Moderate (30 - 44)	0/24	0/4	1/1	1/29
High (>44)	10/42	11/71	21/88	42/201
Total	10/71	11/75	22/89	43/235

D

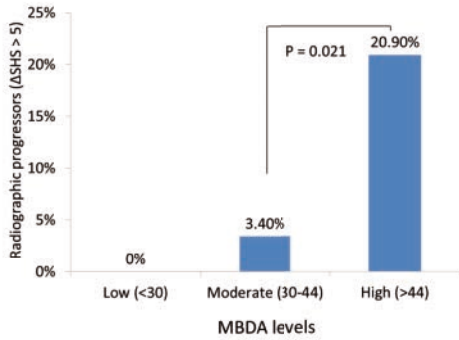

Figure 5. Cross-tabulation of all analysed patients and subset with rapid radiographic progression over one year, by baseline disease activity measures. The denominator in each cell represents the number of patients cross-classified by baseline MBDA and DAS28-ESR (A), baseline MBDA and DAS28-CRP (B) and baseline MBDA and CRP (C) disease activity scores. The numerator in each cell represents the number of patients with radiographic progression at one year. Figure D illustrates radiographic progressors for MBDA low, moderate and high score groups (%). Radiographic progression at one year is defined by increase in SHS > 5 compared to baseline.

4.1.2 The MBDA score in prediction of RP

In bivariate analyses, the MBDA score was independently of other risk factors such as RF, ACPA and sex, predictive for radiographic outcome of the joint damage during 1 year of follow-up (Table 5). In multivariate logistic regression analysis, high MBDA score at baseline compared with low/moderate, was 3.9 times more associated with higher risk of RA at 1 year (adjusted for sex, symptom duration, baseline erosions, current smoking and HAQ score). This was similar with results from the BeSt study, however, for a univariate analysis (for high

MBDA score compared with low/moderate at baseline, RR=3.7) (293). Few more studies, including the BeSt study illustrated predictive ability of the MBDA score for RP (292-295), even though two of these studies (293, 294) used slightly different cut-offs for RP (ΔSHS>0 or >3).

Table 5. Univariate bivariate and multivariate analyses of baseline MBDA score, DAS28 and CRP as predictors of one year radiographic progression.

	Odds Ratio†	95% CI	P-Value‡
Univariate Analyses:			
Baseline MBDA score	1.05	(1.02, 1.08)	<0.001
Baseline DAS28-ESR	1.31	(0.94, 1.81)	0.107
Baseline DAS28-CRP	1.22	(0.88, 1.71)	0.237
Baseline CRP (mg/L)	1.10	(1.02, 1.18)	0.018
Bivariate Models:			
Baseline MBDA adjusted for DAS28-ESR	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for DAS28-CRP	1.05	(1.02, 1.09)	<0.001
Baseline MBDA adjusted for CRP	1.06	(1.02, 1.10)	0.002
Baseline MBDA adjusted for ESR	1.04	(1.01, 1.07)	0.021
Baseline MBDA adjusted for Rheumatoid Factor	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for CCP Status	1.05	(1.03, 1.08)	<0.001
Baseline MBDA adjusted for Total Swollen Joint Count	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for Total Tender Joint Count	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for Global Assessment of Disease Activity	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for SHS	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for Symptom Duration	1.05	(1.02, 1.08)	<0.001
Baseline MBDA adjusted for Sex	1.05	(1.02, 1.08)	<0.001
Multivariate Model*:			
Baseline MBDA adjusted for Sex, Symptom Duration, baseline erosions, current smoking status, HAQ score	1.05	(1.02, 1.08)	<0.001
High (>44) baseline MBDA score adjusted for Sex, Symptom Duration, baseline erosions, current smoking status, HAQ score	3.86	(1.04, 14.26)	0.04

† The odds ratio was estimated from a logistic regression model. The logistic model is estimating the probability of radiographic progression at year 1.
For the univariate model, the odds of radiographic progression increases by 5% for every one-unit increase in the baseline MBDA score. When accounting for other disease activity measures individually (bivariate models), the odds of radiographic progression increase in a cumulative manner, approximately 4-6% for every one-unit increase in the baseline MBDA score.
‡ P-value was calculated using Wald's chi-square test.
*Multivariate model adjusted for significant univariate predictors of one year radiographic progression (n=207), as in Saevarsdottir S et al, Current smoking status is a strong predictor of radiographic progression in early rheumatoid arthritis: results from the SWEFOT trial, ARD in press.

4.1.3 Association of the MBDA score at follow-ups with subsequent RP

In the paper II, we demonstrated that the MBDA score at follow-ups was also informative for RP during subsequent year (Figure 6). None of the patients achieving low MBDA score from moderate (n=11) and only one patient of those who dropped from high category to low (n=17) during 3 months of MTX monotherapy, had RP at 1 year. In contrast, patients who were persistently high at 3 months (n=88), 25% had RP. Similar associations were observed when looking at conversion of the MBDA category from baseline to 1 year and RP during the second year of the follow-up (Figure 6B). CRP and ESR in contrast, could not perform the identification or risk for RP similarly well.

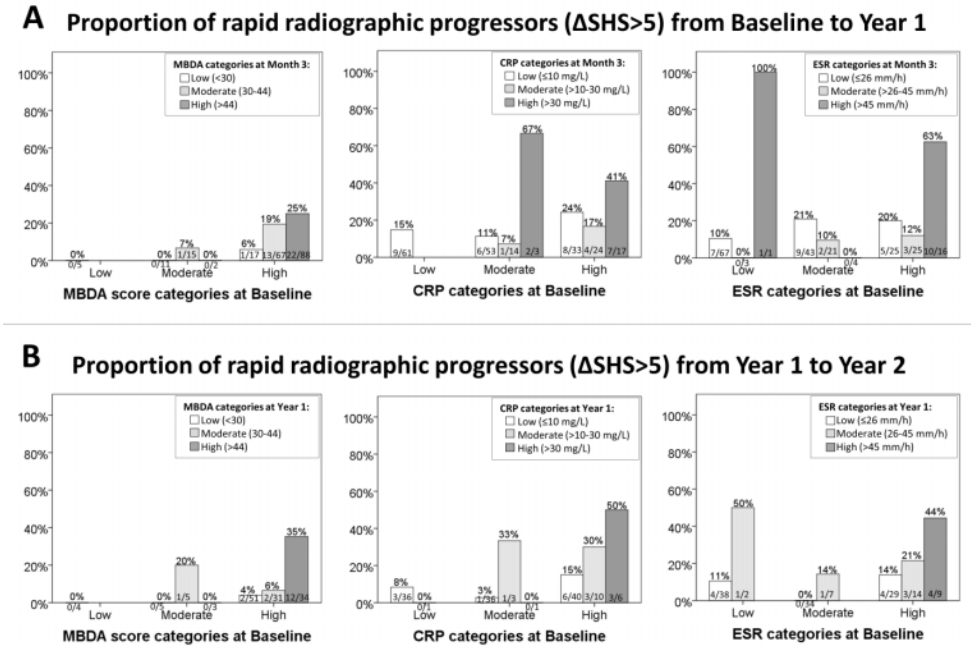

Figure 6. Association of radiographic progression with change in disease activity categories according to the MBDA score, CRP and ESR. Change in categories according to MBDA score, CRP or ESR from BL to Month 3 and radiographic progression from BL to Year 1 (A); and change in categories according to MBDA score, CRP or ESR from BL to Year 1 and radiographic progression from Year 1 to Year 2 (B).

In the second paper, we also observed differential association of the MBDA score at baseline or 3 months with RP at 2 years, for each of the randomised treatment arm. Among patients with high MBDA score, those on TT had significantly higher proportion of RP compared with patients on biological therapy (Figure 7A: 45% vs 25% and 57% vs 32%, respectively; $p<0.05$ for both comparisons).

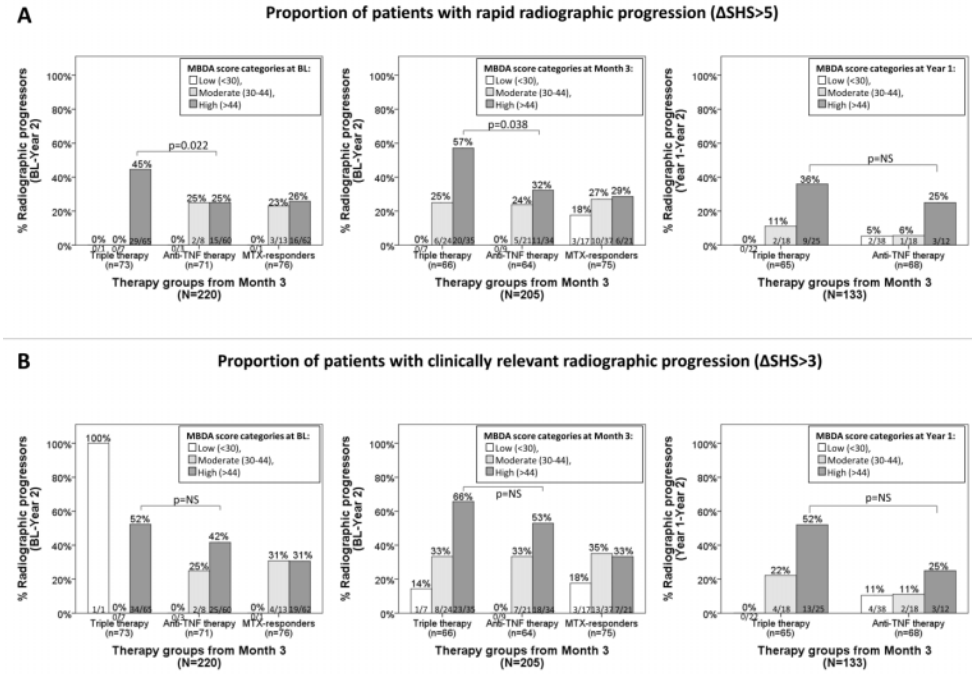

Figure 7. Radiographic progression among 3 therapy groups (triple therapy group, anti-TNF treatment group and MTX-responders) stratified by MBDA categories at multiple time-points. Proportion of patients with radiographic progression defined as ΔSHS>5 (A) and ΔSHS>3 (B). Left and middle bar-graphs represent proportion of patients with 2-year radiographic progression stratified by the MBDA score at BL and Month 3, respectively. Right bar-graph shows radiographic progression from Year 1 to Year 2 among patients stratified by the MBDA score at Year 1.

4.2 STUDY II (PAPER III)

4.2.1 Association of validated categories of the MBDA score with treatment outcome

It has been shown previously that of patients randomised to MTX+IFX therapy, significantly more proportion achieved EULAR good response than from patients on TT (39% vs 25%, respectively; p=0.016) (96). Even being statistically significant, the difference is not very large from clinical point of view. In MTX-incomplete responders, the MBDA score categories at the time of treatment randomisation yielded clinically meaningful difference and moreover, it identified a subgroup that had a higher chance of achievement of LDA on TT compared with patients on IFX (Figure 8). Namely, of patients with low MBDA score, more patients

achieved LDA if treated with TT than with IFX (88% vs 18%, p=0.006) and reverse was true for patients with high MBDA score (35% vs 58%, p=0.040).

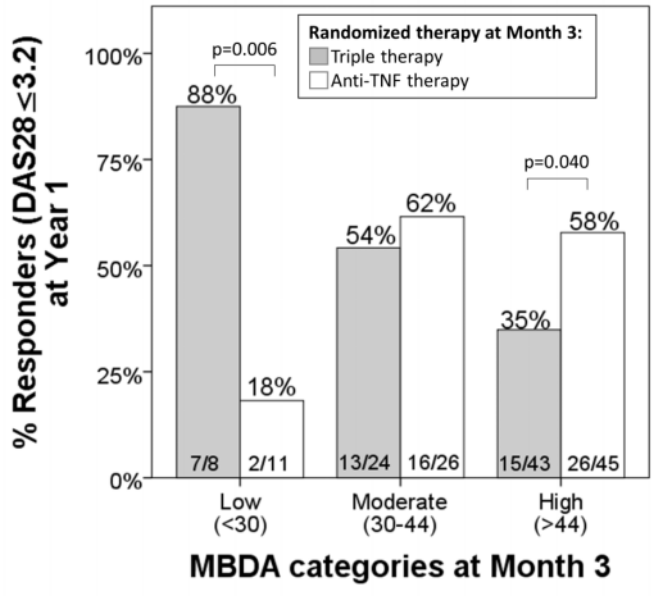

Figure 8. Proportion of patients with response (DAS28≤3.2) to second-line therapy at Year 1 stratified by conventional cut-offs of MBDA score at the start of treatment intensification. Proportion of responders at Year 1 to triple therapy (grey bars) or anti-TNF therapy (white bars) stratified by low (<30), moderate (30-44), and high (>44) MBDA categories at Month 3.

4.2.2 New threshold of the MBDA score for prediction of treatment outcome

ROC curve analysis yielded a cut-off of 38 for dichotomisation of patients into higher and lower categories, which illustrated similar associations (79% vs 44%, respectively, p=0.019 and 36% vs 58%, respectively, p=0.018; Figure 9). Other inflammatory markers (CRP, ESR and DAS28) at 3 months did not illustrate or showed a weaker association.

Our findings support the results from the RACAT trial demonstrating that of patients not responding to MTX+etanercept, some switchers to TT responded, while some of non-responders on TT who switched to MTX+etanercept also achieved better clinical outcome (94). The MBDA score compared with DAS28, ESR and CRP is a multi-component molecular marker which probably indicates changes much earlier than the clinical or mono-

component molecular markers can do. Thus, validation of these results could lead to reduction of unnecessary use of biological drugs for a subgroup of patients, while indicator for their successful use for another subset of patients.

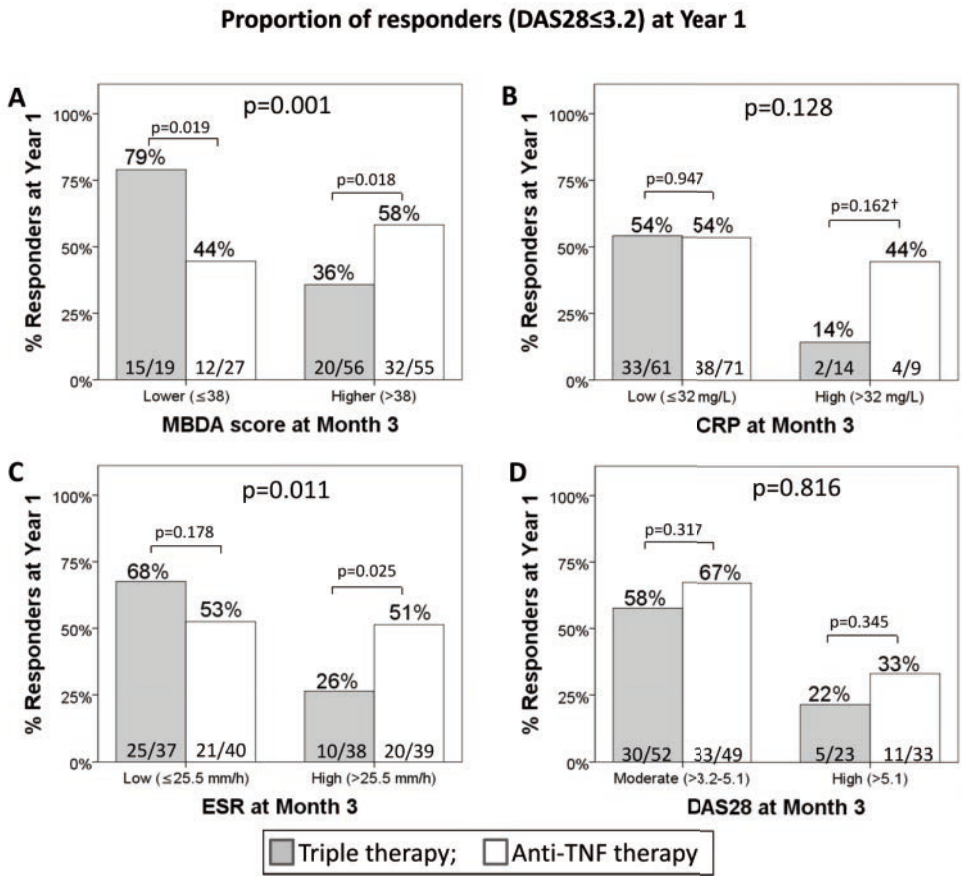

Figure 9. Proportion of clinical responders (DAS28≤3.2) to second-line therapy at Year 1 stratified by ROC-based cut-offs of disease activity measures at Month 3. Proportion of responders at Year 1 to triple therapy (grey bars) or anti-TNF therapy (white bars) stratified by MBDA score (A), CRP (B), ESR (C) and DAS28 (D). The overall p-values for 4 groups are calculated by Breslow-Day test, and for triple therapy versus anti-TNF therapy groups – by χ^2 test, unless otherwise stated.

†p-value was calculated using Fisher's Exact test.

4.3 STUDY III (PAPER IV AND V)

In early RA patients treated with MTX, baseline serum levels of biomarkers were analysed for associations with achievement of treatment outcome (LDA or EULAR good response) at 3 months follow-up.

4.3.1 Study IIIa (paper IV)

In the paper IV, the 12 component proteins of the MBDA score were analysed as potential predictors of response to MTX.

4.3.1.1 Identification of potential predictors

Of 298 analysed patients 104 achieved LDA. Among baseline parameters, as it has been previously published (98), sex, age and some clinical parameters were significantly different between patients achieving and not achieving LDA at 3 months (Table 6).

Table 6. Baseline characteristics of early RA patients from the SWEFOT trial

Baseline Characteristics Median (±IQR)	Stratified by disease activity (DAS28) at 3 months follow-up		
	DAS28 ≤ 3.2 (N=104)[a]	DAS28 > 3.2 (N=194)[b]	P-value
Female: N (%)	60 (58)	154 (79)	<0.001
Age: years	61 (51-69)	55 (44-62)	0.001
Smoking: N (%)	19 (24)	47 (26)	0.780
Symptom Duration: months	5 (3-8)	5 (4-8)	0.283
Anti-CCP Positive: N (%)	63 (64)	107 (58)	0.292
RF Positive: N (%)	73 (71)	124 (65)	0.274
Prednisolone use: N (%)	15 (14)	20 (10)	0.293
28 Swollen Joint Count	9 (7-13)	11 (7-15)	0.087
28 Tender Joint Count	6 (4-10)	10 (6-15)	<0.001
ESR (mm/h)	28 (15-42)	36 (24-62)	0.001
CRP (mg/L)	15 (9-33)	20 (9-53)	0.033
PatG (VAS 0-100mm)	50 (29-67)	63 (43-77)	0.001
Pain (VAS 0-100mm)	49 (33-64)	62 (46-74)	<0.001
HAQ	1.0 (0.50-1.38)	1.25 (0.88-1.75)	<0.001
DAS28	5.2 (4.6-5.9)	5.9 (5.3-6.4)	<0.001

IQR – interquartile range, anti-CCP - anti-cyclic citrullinated peptide, RF – rheumatoid factor, ESR – erythrocyte sedimentation rate, CRP – C-reactive protein, PatG – Patient's Global Assessment of Disease Activity score, VAS – visual analogue scale, HAQ, - health assessment questionnaire, DAS – disease activity score.

[a] Missing patients from DAS28≤3.2 column: Smoking (n=25), Anti-CCP (n=6) and RF (n=1).

[b] Missing patients from DAS28>3.2 column: Smoking (n=11), Anti-CCP (n=9), RF, PatG and Pain (n=2), HAQ (n=4) and DAS28 (n=3).

The MBDA score did not differ between these 2 groups of patients. In CAMERA trial, on the other hand, the MBDA score was associated with DAS28-CRP and discriminated patients between remission/low and moderate/high disease activity levels (287). In a univariate analysis, four of the 12 proteins differed at a level of $p<0.05$ (Table 7).

Table 7. Baseline biomarkers of early RA patients from the SWEFOT trial

Baseline Biomarkers Median (±IQR)	Stratified by disease activity (DAS28) at 3 months follow-up		
	DAS28 ≤ 3.2 (N=104)[a]	DAS28 > 3.2 (N=194)[b]	P-value
VCAM-1 (mg/L)	0.70 (0.60-0.86)	0.64 (0.56-0.77)	0.005
TNF-RI (µg/L)	1.9 (1.6-2.4)	1.7 (1.4-2.2)	0.005
IL-6 (ng/L)	49 (22-97)	67 (29-150)	0.044
CRP (mg/L)	20 (8-43)	27 (10-110)	0.045
MMP-1 (µg/L)	9.35 (5.87-15)	11 (6.48-18)	0.067
MMP-3 (µg/L)	48 (28-82)	56 (29-123)	0.105
Leptin (µg/L)	9.25 (3.58-14)	10.50 (4.60-20.25)	0.108
VEFG (ng/L)	405 (265-600)	390 (250-590)	0.719
Resistin (µg/L)	6.60 (5.35-8.78)	6.80 (5.10-8.68)	0.857
YLK-40 (µg/L)	85 (54-120)	79 (57-130)	0.862
EGF (ng/L)	160 (103-258)	170 (100-253)	0.903
SAA (mg/L)	20 (7-46)	17 (4-73)	0.990
MBDA score	59 (47-66)	59 (49-74)	0.143

IQR – interquartile range, DAS – disease activity score, VCAM-1 - vascular cell adhesion molecule-1, TNF-RI - tumour nectosis factor receptor-1, IL-6 - interleukin-6, CRP – C-reactive protein, MMP - matrix metalloproteinase, VEGF - vascular endothelial growth factor, YKL-40 - human cartilage glycoprotein-39, EGF - epidermal growth factor, SAA - serum amyloid A, MBDA – multi-biomarker disease activity.

Biomarkers that had p-value <0.2 (n=7), were entered into a multivariate logistic regression model for identification of LDA at 3 months as a dependent outcome. Four of these biomarkers were independently associated with LDA (increase in concentrations of VCAM-1 and TNF-RI, as well as decrease in CRP and leptin were associated with LDA, Table 8). There is no clear data regarding these four biomarkers illustrating predictive capacity for response to MTX. Controversial results are published by different researchers regarding CRP, some illustrating association of its low level with response (110) and some failing to do so (114, 318-320). The association of high baseline leptin levels with high DAS28 at 6 months in RA patients treated with non-biological DMARDs was illustrated by a research group (262). Being associated with BMI, we could not find any relation of BMI with treatment outcome in our study (data not shown). Increased sTNF-RI might indicate increased sTNF, since cleavage of membrane-bond TNF-RI is considered as negative regulation of increased production of TNF and inflammation (decoy receptor) (200, 201). Considering this fact and since MTX is known to decrease TNF level (321, 322), higher levels of TNF-RI could mean a disease

driven by TNF, where MTX could be a proper option for treatment. Indeed, a study has demonstrated that patients with high sTNF-RI had better response to MTX (161).

Table 8. Multivariate logistic regression of protein biomarkers at baseline for prediction of low DAS28 at 3 months

Biomarkers	OR	95% CI
VCAM-1 (per mg/L increase)	**6.70**	1.17-38.62
TNF-RI (per µg/L increase)	**3.10**	1.70-5.71
IL-6 (per ng/L increase)	0.99	0.99-1.00
CRP (per mg/L increase)	**0.99**	0.98-0.99
MMP-1 (per µg/L increase)	0.98	0.94-1.01
MMP-3 (per µg/L increase)	1.00	0.99-1.00
Leptin (per µg/L increase)	**0.97**	0.95-0.99
Results after step-wise logistic regression analysis		
VCAM-1 (per mg/L increase)	**8.19**	1.47-45.65
TNF-RI (per µg/L increase)	**2.48**	1.43-4.31
CRP (per mg/L increase)	**0.99**	0.98-0.99
Leptin (per µg/L increase)	**0.97**	0.95-0.99

VCAM - vascular cell adhesion molecule, TNF-RI - tumour necrosis factor receptor 1, IL-6 - interleukin-6, CRP - C-reactive protein, MMP - matrix metalloproteinase, OR - odds ratio, CI - confidence interval

4.3.1.2 Dichotomisation of biomarker levels

ROC curve analyses identified cut-off levels based on highest sum of sensitivity and specificity generating low and high categories for these biomarkers: CRP: ≤51.5 and >51.5 mg/ml, leptin: ≤14.5 and >14.5 µg/ml, TNF-RI: ≤1.75 and >1.75 µg/ml and VCAM-1: ≤0.605 and >0.605 mg/ml. There were significantly higher proportions of patients achieving LDA among subjects with low CRP and leptin, and high TNF-RI and VCAM-1 (Figure 10).

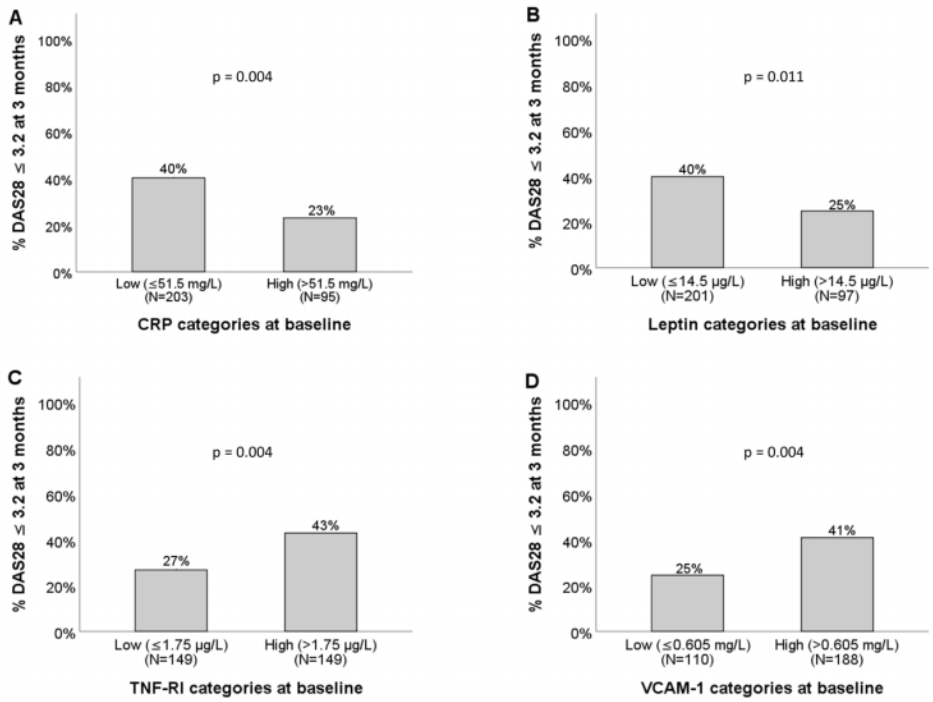

Figure 10. Proportions of eRA patients achieving low disease activity after 3 months of MTX monotherapy, stratified for different biomarkers. Proportions of patients with low DAS28 in patients dichotomised according to CRP (A), leptin (B), TNF-RI (C) and VCAM-1 (D). DAS28 – disease activity score based on 28 joints, CRP – C-reactive protein, DAS28 – 28-joint disease activity score, MTX – methotrexate, TNF-RI – tumour necrosis factor receptor-1, VCAM-1 – vascular cell adhesion molecule-1.

4.3.1.3 Combined biomarker score

The combined biomarker score that was based on the four proteins, was independently associated with treatment outcome (odds ratio adjusted for RF, ACPA, sex, age and current smoking OR=0.44, 95CI=0.30-0.65). There was a gradual decrease in proportion of patients in LDA or EULAR good response with increase of the combined biomarker score (Figure 11).

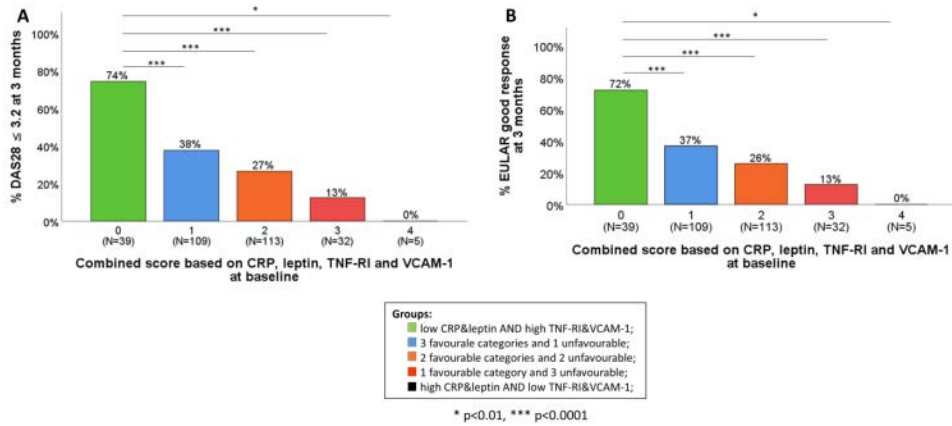

Figure 11. Proportions of early RA patients achieving low DAS28 or EULAR good response after 3 months of MTX monotherapy, stratified for the combined biomarker score. Proportions of patients with low DAS28 (A) or EULAR good response (B) within subsets based on the combined biomarker score: combined score = 0 (green bars), combined score = 1 (blue bars), combined score = 2 (orange bars), combined scores = 3 (red bars) and combined score = 4 (black bars).

The biomarker score was tested in combination with previously published (98) predictors (sex and age) for identification of patients achieving treatment outcome. Even though one of the components of the combined biomarker score has a co-linearity with sex (females have higher leptin level compared with males), the combined prediction matrix identified subset of patients with very high predictability of response. For example, among older male patients with the lowest combined biomarker score (n=14), 13 achieved LDA and 12 EULAR good response, while among younger female patients with the combined biomarker score of 3 and 4 (n=22), only one achieved LDA or EULAR good response (Figure 12).

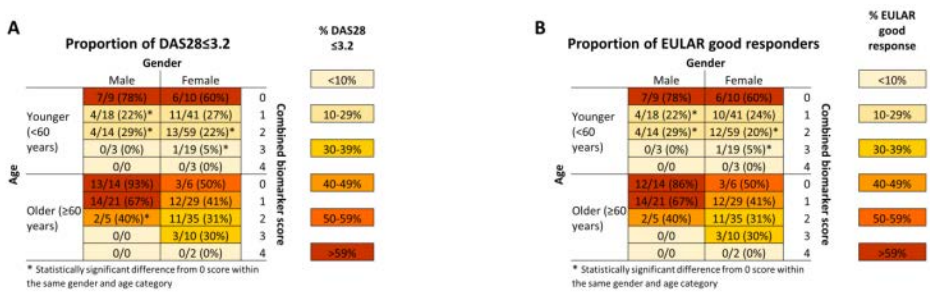

Figure 12. Matrix of prediction of response to MTX in early RA patients, based on age, gender and combined biomarker score. Proportion of patients achieving low DAS28 (A) or EULAR good response (B).

The main limitation of the study is the limited number of patients in some subgroups which emphasises the need for validation in other study populations. The SWEFOT trial, on the other hand, is designed with low bias, due to the few exclusion criteria and routine-care based recruitment, and all patients were treated with MTX, which is the recomendet first-line therapy today.

4.3.2 Study IIIb (paper V)

4.3.2.1 Identification of predictive proteins

The 135 patients included in this study had more often RF than the remaining patients from the SWEFOT population (72% and 58%, respectively, p=0.002). Of screened 177 serum proteins at baseline, in univariate analysis, eight had different level of MFI (at the level of p<0.001) among patients achieving and not achieving LDA at 3 months. Of the eight candidate biomarkers, two: MMP-7 and α-chain of fibrinogen (FGA) were independently associated with treatment outcome. The ROC curve analysis resulted in area under the curve of 0.692 and 0.699 for MMP-7 and FGA, respectively (p<0.001, Figure 13A). Low levels of the two biomarkers indicated significantly higher proportion of patients in LDA at 3 months (Figure 13B).

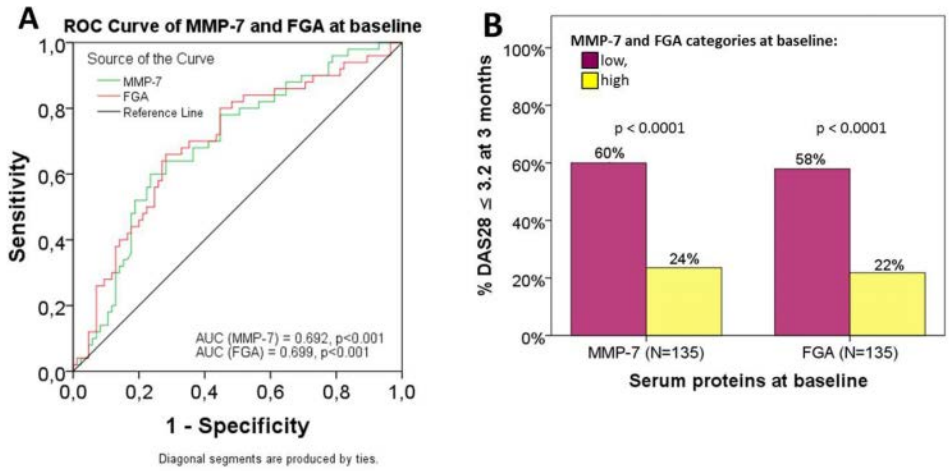

Figure 13. Assessment of baseline levels of MMP-7 and FGA as predictors of LDA at 3 months. Receiver operating characteristic curve analysis and area under the curve (A) of MMP-7 (green line), FGA (red line); Proportion of patients achieving low DAS28 at 3 months among groups dichotomised by MMP-7 or FGA (B) low levels – purple bars and high levels – yellow bars.

When considering both biomarkers simultaneously, of patients with low levels for both biomarkers, 79% had LDA and EULAR good response, while of those with high levels for both biomarkers, 18% and 15%, respectively achieved LDA or EULAR good response ($p<0.001$, Figure 14).

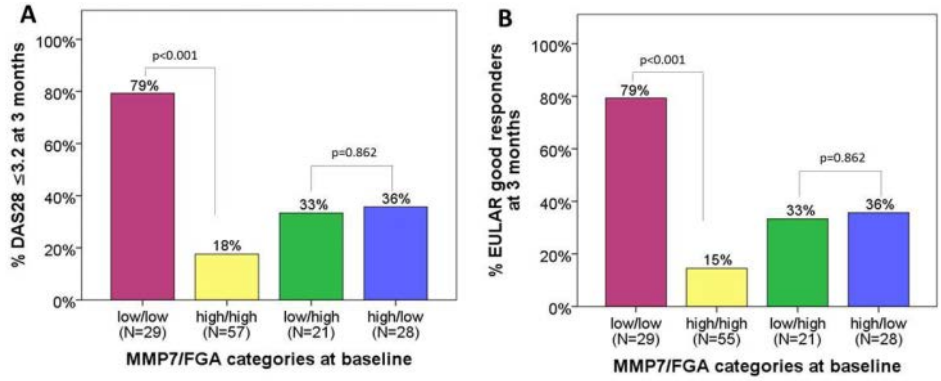

Figure 14. Assessment of combined baseline levels of MMP-7 and FGA as predictors of outcome. Proportion of patients in LDA (A) and EULAR good response (B) among patients with low/low (purple bar), high/high (yellow bar), low/high (green bar) and high/low (blue bar) categories of MMP-7/FGA.

4.3.2.2 An attempt of replication of the results for MMP-7 in the COMBINE cohort

Neither MMP-7 nor FGA have been shown to predict response to MTX. Validation of results for MMP-7 in another RA cohort (COMBINE, N=74) did not confirm the finding. We found three differences between the SWEFOT and COMBINE cohorts: method of measuring MMP-7, significantly lower glucocorticoid use and significantly higher DAS28 at baseline and at 3 months among patients from SWEFOT compared with COMBINE cohort. When considering only patients treated without glucocorticoids, we still could not confirm the association of baseline low MMP-7 with LDA at 3 months in COMBINE cohort. Therefore, we assume that method of measurement of MMP-7 and baseline DAS28 could contribute to the bias.

Confirmation of these results in another similar RA cohort with untreated early RA patients using the same method of measurement, might increase efficiency of MTX monotherapy choice in treatment of RA.

4.4 STUDY IV (PAPER VI)

4.4.1 Distribution of data

Immunogenicity of biological medications has been established as a challenge for achievement of optimal treatment outcome (310, 323). Of 289 available serum samples (from all 3 time-points together), we observed a very low sIFX level (<0.2 μg/ml) in 64 samples, and of those, 47 (73.4%) were ADA-positive. Of 101 patients analysed in this study, 34 patients were ever ADA-positive. There were no baseline parameters significantly different between ever and never ADA-positive patients. However, there were more women among ever (85%) compared with never (67%) ADA-positive patients, respectively (p=0.052, Table 9) with similar trend for RF-positivity (79% vs 62%, p=0.0079).

Table 9. Characteristics of SWEFOT participants at the time of randomization to IFX stratified into ever and never anti-drug antibody positive patients

Patient Characteris, Median (IQR)[a]	MTX-incomplete responders randomized to IFX therapy (N=101)			
	All (N=101)[b]	Ever ADA-positive (N=34)[c]	Never ADA-positive (N=67)[d]	P-value[e]
Female: N (%)	74 (73)	29 (85)	45 (67)	0.052
Age (years)	55 (43-62)	50 (42-59)	55 (44-63)	0.246
BMI	24.2 (22.2-26.4)	23.8 (22.2-27.0)	24.4 (22.3-26.2)	0.740
Current Smokers: N (%)	27 (27)	10 (29)	17 (25)	0.665
Symptom Duration (months)	6 (4-8)	6 (3-9)	5 (4-8)	0.610
Anti-CCP Positive: N (%)	62 (67)	22 (71)	40 (64)	0.534
RF Positive: N (%)	68 (68)	27 (79)	41 (62)	0.079
28 Swollen Joint Count	6 (3-10)	4 (3-9)	6(3-10)	0.483
28 Tender Joint Count	6 (4-10)	7 (4-12)	6 (3-9)	0.134
ESR (mm/h)	23 (12-37)	24 (14-37)	21 (11-34)	0.446
CRP Level (mg/L)	9 (4-17)	9 (4-15)	9 (4-18)	0.853
PatG (VAS 0-100mm) Score	49 (35-67)	50 (36-67)	49 (34-68)	0.979
Pain VAS	47 (30-64)	44 (30-66)	49 (30-63)	0.740
PhysG	2 (2-2)	2 (2-2)	2 (2-2)	0.392
HAQ	0.9 (0.63-1.38)	0.9 (0.38-1.25)	1.0 (0.63-1.38)	0.081
DAS28	4.9 (4.1-5.6)	5.0 (4.3-5.8)	4.8 (4.1-5.5)	0.328

ADA – anti-drug antibody, IQR – interquartile range, IFX – infliximan, BMI – body mass index, anti-CCP - anti-cyclic citrullinated peptide, RF – rheumatoid factor, ESR – erythrocyte sedimentation rate, CRP – C-reactive protein, PatG – Patient's Global Assessment of Disease Activity, VAS – visual analogue scale, PhysG – Physician Global Assessment of Disease Activity, HAQ – health assessment questionnaire, DAS – disease activity score.

a Data for Female, Age, Current Smoking, Symptom Duration, Anti-CCP and RF are presented from diagnosis and trial recruitment date.

b Number of missing patients for "101" column: BMI (n=27), Anti-CCP (n=8), RF (n=1), 28 Swollen Joint Count, 28 Tender Joint Count, CRP, PatG VAS (n=3), ESR, Pain VAS, PhysG and HAQ (n=4).

c Number of missing patients for IFX-Responders column: BMI (n=7), Anti-CCP (n=3), 28 Swollen Joint Count, 28 Tender Joint Count, ESR, CRP, PatG VAS, Pain VAS, PhysG and HAQ (n=1).
d Number of missing patients for IFX-non-responders column: BMI (n=20), RF (n=1), Anti-CCP (n=5), 28 Tender Joint Count, 28 Swollen Joint Count, CRP and PatG VAS (n=2), ESR, Pain VAS, PhysG and HAQ (n=3).
e Comparing ever and never ADA-positive patients.

4.4.2 Association of sIFX levels and ADA status with treatment outcome

Higher proportion of patients in LDA or remission was observed among patients with sIFX >0.2 µg/ml compared with ADA-positive patients, and the difference was significant at the study cessation (at 21 months, Figure 15A and B). Similarly higher sIFX level was associated with better clinical outcome (Figure 15C and D). These results are in concordance with previous studies (303, 305, 310, 324).

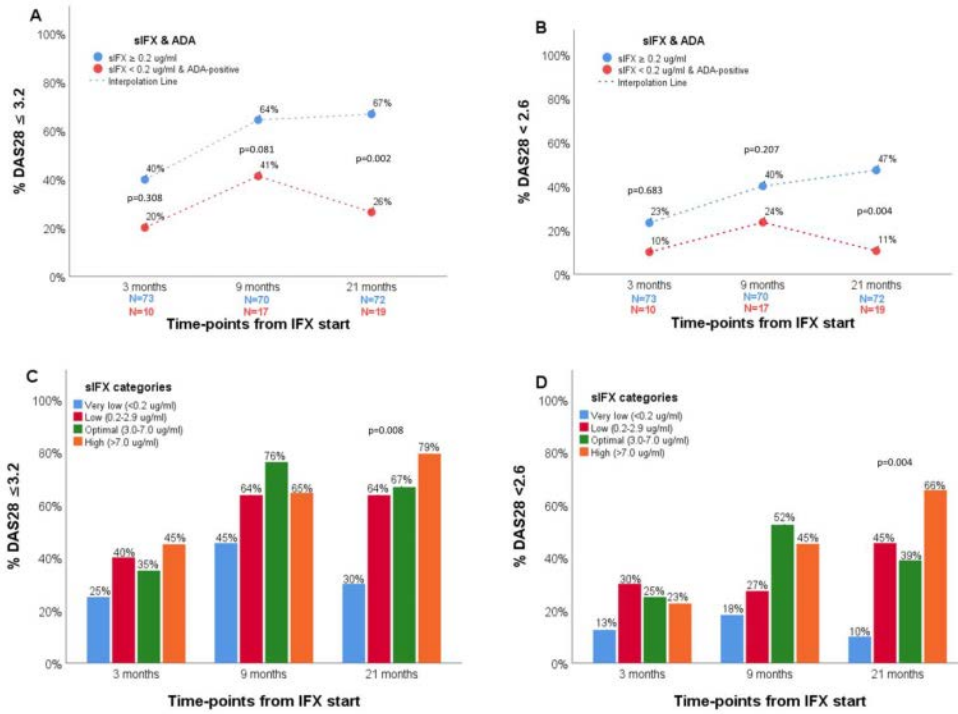

Figure 15. Clinical outcome of patients at 3, 9 and 21 months stratified for sIFX and ADA status. Proportion of patients in LDA (A) and remission (B) among patients with sIFX level ≥0.2 µg/ml (blue dots) and ADA-positive patients with very low sIFX levels (red dots). Proportion of patients in LDA (C) and remission (D) among four strata of patients according to sIFX levels: very low sIFX level – blue bars, 0.2-2.9 µg/ml – red bars, 3.0-7.0 µg/ml – green bars, and >7.0 µg/ml – orange bars.

4.4.3 Prediction of low sIFX or ADA

Gender and RF status among other baseline parameters showed borderline significnat associations with ever ADA-positivity. In multivariate analyses, female gender and RF-positivity remained borderline significantly associated with development of ADA. Higher frequency of ADA-positivity among women compared with men could be explained by the fact that B cells in females have higher capacity for antibody production (325). However, there are controversial data regarding associations of RF and gender with ADA status (324, 326). Unlike SWEFOT, patients from these studies have significantly higher disease duration (6-14 years) and lower incidence of ADA-positive patients, which indicates more suppressed immune system by long-lasting DMARD therapy, leading to reduced capacity of antibody production. In addition, RF may interfere with ELISA kit for ADA measurement and give false-positive results. However, we also observed a trend of RF-positivity with low sIFX level, and the trend became stronger at 21 months (34% vs 16%, p=0.059, Figure 16). Similar trend was observed when comparing female versus male patients with significant difference at 21 months (35% vs 7%, respectively, p=0.006, Figure 16).

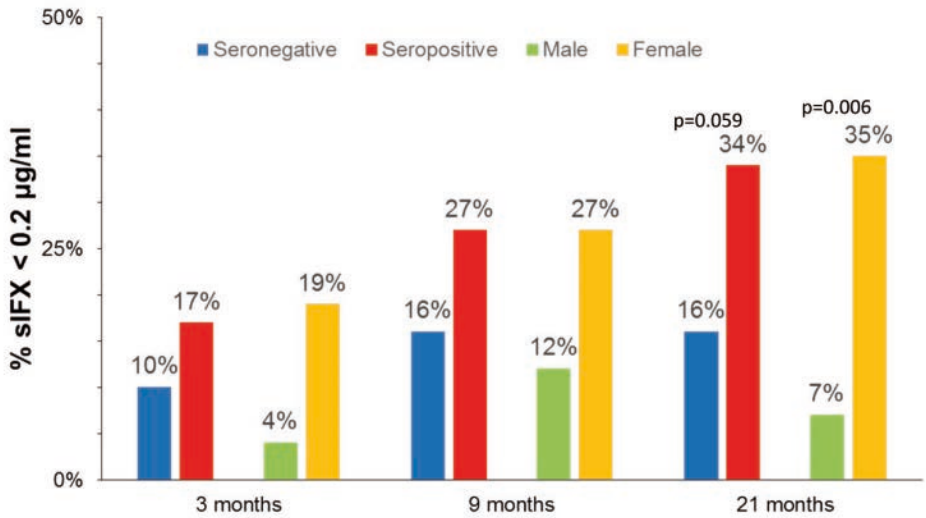

Figure 16. Proportion of patients with very low sIFX level at 3, 9 and 21 months stratified for RF and gender. Blue bars represent proportion among RF-negative and red bars – among RF-positive subset. Green bars represent proportion among males and orange bars –among females.

It is important to note that sIFX levels analysed in this study were from samples taken at follow-up visits and not just before next infusion (i.e. trough levels), which is the biggest limitation. However, confirmation of associations of the sIFX levels and ADA-positivity with treatment outcome at later time-point, found by others researchers at earlier time-points indicates that the trend associations observed in our study could be strengthened if trough levels are used. Therefore, further investigations of prediction of response to IFX using sIFX levels, ADA and other baseline parameters might help identify patients at higher risk and improve decision-making for the switch of biological therapy.

5 CONCLUSION

In the presence of different treatment options for RA and the heterogeneity of the disease, there is a huge need for predictive tools to help chose the optimal treatment for each individual patient. This thesis project overall, tried to address this question via exploratory analyses of serum proteins, as potential predictors of treatment outcome.

In paper I and II we showed predictive capacity of the MBDA score at baseline and follow-ups for RP during subsequent one or two years. Apart from confirming the association of low MBDA score with very low risk of RP and superiority of the MBDA score compared to CRP, ESR and DAS28, we also showed for the first time that patients with high MBDA score would benefit more from MTX+IFX therapy than from TT to lower RP.

In paper III, the MBDA score identified a subset of patients that benefited significantly more from TT compared with biological IFX treatment, a finding that yielded much attention since TT is much cheaper, and has now been supported by results from O'Dell et al (94).

Paper IV and V highlighted some protein biomarkers at baseline for prediction of response to MTX monotherapy. As in all biomarker studies, those findings need to be validated since these molecules can be potential key players in the pathology of some RA patients.

In paper VI we confirmed previously published results on association of low sIFX levels and ADA-positivity with poorer treatment response to IFX, but also found that RF and female gender might be risk factors for immunogenicity and low sIFX levels.

In summary, through investigations of serum proteins related to inflammation we identified potential predictors of RP and clinical outcome, which may help to understand pathology behind RA and aid therapy choice. For biological treatment, studies of immunogenicity and blood trough level of the drug support routine monitoring of sIFX and ADA in the clinic and serve as basis for development of an algorithm when to switch to other treatment options.

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