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RHEUMATOID ARTHRITIS

Karen Inger Maijer

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RHEUMATOID ARTHRITIS:

FROM THE AT RISK PHASES ALL THE WAY UP TO THE DEVELOPMENT OF THE DISEASE

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aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

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ten overstaan van een door het College voor Promoties ingestelde commissie,
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op vrijdag 12 mei 2017, te 10:00 uur

door

Karen Inger Maijer
geboren te Zeist

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	prof. dr. B. Keymeulen	Universitair Ziekenhuis Brussel
	dr. R.J.E.M. Dolhain	Erasmus Medisch Centrum

Faculteit der Geneeskunde

CONTENTS

1

GENERAL INTRODUCTION

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by synovial inflammation in multiple joints, affecting ~1% of the population worldwide. Patients present with symptoms of pain, joint swelling and stiffness. RA is associated with excessive turnover of connective tissues of the joints. Ultimately, the disease can lead to joint damage and disability, with considerable socioeconomic costs [1, 2].

Pathogenesis of RA

The etiology of RA, though largely unknown, is considered multifactorial and genetic factors as well as various environmental risk factors are considered to be involved. In combination, these factors create a condition in which tolerance can be broken and an autoimmune reaction can be initiated. Analyzing the synovium of RA patients has been important in providing a deeper understanding of the disease. RA synovial tissue (ST) is hypertrophic and edematous and is characterized by marked intimal lining hyperplasia and by accumulation of T lymphocytes, B lymphocytes, plasma cells, mast cells, neutrophils, macrophages, natural killer cells and dendritic cells in the synovial sublining [1]. Furthermore, angiogenesis can be observed in the ST already in the earliest phase of the disease. The number of blood vessels is already significantly increased in patients with early disease, and the vasculature is clearly activated as shown by increased expression of adhesion molecules [2, 3]. Also, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) in patients with early arthritis demonstrates increased vascularity and suggests that angiogenesis plays a key role in the pathogenesis of RA [4].

In RA, inflammation leads to excessive remodelling and tissue turnover. Tissue destruction of the extracellular matrix (ECM) in RA is mediated by enzymatic cleavage by several proteases, though predominantly by matrix metalloproteinases (MMPs). MMPs have been shown to be highly up-regulated in RA [5, 6]. The ECM of cartilage mainly consist of type II collagen, while type III collagen is the main protein of soft tissue (like the synovium and entheses) [7]. Examining the turnover of these and other collagens may aid the understanding of RA pathogenesis. Moreover, these protein-degradation products may be specific for the tissue of origin and for the involved enzymes, and may therefore be used as diagnostic and prognostic biomarker [8].

Although the synovium is the most important site of pathology in the established phase of RA, it is most probably not the site where the disease is initiated [9]. Other sites that have been suggested to play a role in disease initiation and to be involved before signs and symptoms of arthritis become apparent include the lung, periodontium, gut, lmph nodes and bone marrow [10-14]. The analysis of these tissues from articular as well as from extra-articular sites obtained from individuals at risk of RA, may aid understanding of the processes leading to synovial tissue inflammation and the detrimental autonomous disease progression.

Individuals at risk of developing RA

To facilitate research in the earliest phases of RA, the Study Group for Risk Factors for RA, established by the European League Against Rheumatism (EULAR) Standing Committee on Investigative Rheumatology, has defined nomenclature for the different phases up to the development of RA [15]. This study group recommended that, in prospective studies, individuals at risk of developing RA are described as having: genetic risk factors for RA (phase a); environmental risk factors for RA (phase b); systemic autoimmunity associated with RA (phase c); symptoms without clinical arthritis (phase d); unclassified arthritis (phase e); the descriptions may be used in a combinatorial manner. Insights into the initiation of the disease may help to more accurately define individuals at high risk of developing RA and may lead to the discovery of targets for preventive therapy.

Multiple genes determine RA disease susceptibility [16]. First degree relatives of RA patients share at least some genetic and environmental risk factors with RA patients and may therefore provide an opportunity to enrich the population at risk of developing RA.

As the prevalence of obesity has increased dramatically, obesity may be an example of an important life style risk factor in the development of RA [17]. The most abundant cell type in the adipose tissue is the adipocyte, which are known to secrete several bioactive peptides called adipo(cyto)kines [18]. In RA patients it has been shown that serum levels of adipokines are higher compared to healthy controls and non-RA controls and are related to disease activity [19-22]. Also in the synovial fluid and synovial tissue of RA patients adipokines are increased compared to non-RA controls [22-24]. Taken together, these observations suggest that adipokines may play a role in the disease process of RA.

ACPAs, as part of systemic autoimmunity associated with RA, are found in the majority of RA patients and are associated with more aggressive disease [25]. Together with IgM rheumatoid factor (IgM-RF), they can be present years before clinical symptoms of RA appear [25]. Individuals with arthralgias who are positive for ACPA have a chance of ~30% of developing RA within 1 year [26]. Moreover, in the presence of ACPA the risk for arthritis development is enhanced by IgM-RF: about 40% of ACPA-positive and IgM-RF-positive individuals with arthralgia develop arthritis after 2 years [26]. Thus, the presence of ACPA and/or IgM-RF may assist in the identification of individuals at risk of developing RA, which may facilitate pathogenetic studies as well as research on preventive strategies during the preclinical window of opportunity [27].

Early arthritis

Since RA is known to be a potentially destructive disease, and early treatment has been shown to efficiently decrease or prevent joint damage, and improve functional outcome, it is important to diagnose RA early and start treatment accordingly [3-5]. In 2010, the American College of Rheumatology (ACR) and the European League Against

Rheumatism (EULAR) jointly developed new criteria to classify RA [6, 7]. These criteria enabled earlier classification of RA in research cohorts and clinical trials; some patients classified as 'unclassified arthritis' (UA) might still fulfill classification criteria for RA after follow up [4, 5]. Using the ACR/EULAR 2010 classification criteria for RA, the disease appears on average less severe and more frequently self-limiting compared to when the 1987 ACR criteria were used.[28, 29]. Clearly, it is important to identify patients with potentially persistent, destructive disease compared to self-limiting disease in an early phase of the disease.

AIM AND OUTLINE OF THIS THESIS

In this thesis we studied the earliest phases of RA in order to understand the processes underlying the development of RA from a phase in which individuals are at risk of developing RA to having clinically evident disease. In **chapter 2** we review data from studies on various tissues collected during the at risk phases leading up to the development of RA. For future studies on tissues we recommend the use of standardised definitions of the different stages of the disease of RA.

It is known that the presence of circulating autoantibodies, namely IgM-RF and/or ACPA, may precede the development of RA by several years. In **chapter 3** we describe ACPA and IgM-RF positivity in a large cohort of unaffected first degree relatives of RA patients. These results are important for research programs aimed at the identification of individuals at risk of developing RA.

Obesity has been suggested as risk factor for the development of RA. We have previously found that overweight increases the risk of developing RA in individuals already at increased risk by the presence of systemic autoimmunity associated with RA. Therefore, we hypothesized that active adipose tissues producing various bioactive peptides have a pivotal role in the development of RA. In **chapter 4** we explored this further by examining the expression of adipokines in serum and synovial tissue of these individuals.

Recently, we have shown that NF-κB-inducing kinase (NIK) is a key regulator of inflammation-induced angiogenesis in RA ST. In **chapter 5**, we investigated synovial NIK expression in autoantibody-positive individuals at risk for developing RA and in patients with early arthritis.

The analysis of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) using pharmacokinetic modeling (PKM) provides quantitative measures that mirror microvessel integrity and can be used as objective markers of the amount of synovial

inflammation and as a non-invasive marker of synovial angiogenesis. In **chapter 6** we investigated the PKM parameters K^{trans}, k_{ep} and v_e in a prospective cohort of disease-modifying antirheumatic drug (DMARD) naïve patients with early arthritis.

Early and aggressive treatment in RA has been shown to improve the disease course. We hypothesized that serum biomarkers might improve the current diagnostic and/or prognostic process in patients with early arthritis. In **chapters 7 and 8** we studied whether specific biomarkers (the Multi-biomarker Disease Activity (MBDA) score, C1M (which is a product of MMP-cleavage of type I collagen and a biomarker of soft tissue destruction), C2M (a MMP-generated neo-epitope of type II collagen), C3M (a biomarker of soft tissue turnover associated with inflammation), VICM (which evaluates citrullinated and MMP-degraded vimentin), and CRPM (the MMP-depended degradation product of C-reactive protein)) can be used as diagnostic or prognostic markers in patients with early arthritis.

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2

WHERE DOES RHEUMATOID ARTHRITIS START? A SYSTEMATIC REVIEW OF STUDIES OF SYNOVIUM, LYMPH NODE, GINGIVA AND LUNG

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Submitted for publication

ABSTRACT

Studies of tissues potentially involved in the aetiology of rheumatoid arthritis (RA), such as synovium, lymph node, gingiva and lung, are critical to understand the development from a healthy to a diseased state. In this systematic review data from studies on various tissues collected during at risk phases leading up to the development of RA are examined. For future studies on tissues we recommend the use of standardised definitions of the different stages of the disease together with an accurate description of the duration of symptoms to facilitate communication between researchers and comparisons between studies in order to start to understand the processes underlying the development of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by pain, swelling and stiffness of the joints due to synovial inflammation. Despite growing insights into the involvement of specific molecules and pathways in synovial tissue inflammation in established RA, the local processes leading to initiation of the disease have not yet been elucidated. Although the synovium is the most important site of pathology in the established phase of RA, it is most probably not the site where the disease is initiated [1]. Other sites that have been suggested to play a role in disease initiation and to be involved before signs and symptoms of arthritis become apparent are the lymph nodes, periodontium, lungs, gut and bone marrow [2-6]. The analysis of these tissues from articular as well as from extra-articular sites obtained from individuals at risk of RA, may aid understanding of the processes leading to synovial tissue inflammation and the detrimental autonomous disease progression.

To facilitate research in the earliest phases of RA, the Study Group for Risk Factors for RA, established by the European League Against Rheumatism (EULAR) Standing Committee on Investigative Rheumatology, has defined nomenclature for the different phases up to the development of RA [7]. This study group recommended that, in prospective studies, individuals at risk of developing RA are described as having: genetic risk factors for RA (phase a); environmental risk factors for RA (phase b); systemic autoimmunity associated with RA (phase c); symptoms without clinical arthritis (phase d); unclassified arthritis (phase e); the descriptions may be used in a combinatorial manner (see Table 1).

Table 1. Nomenclature for the different phases up to the development of rheumatoid arthritis (RA) defined by the Study Group for Risk Factors for Rheumatoid Arthritis, established by the European League Against Rheumatism (EULAR) Standing Committee on Investigative Rheumatology [7]

Phase	**Description**
(a)	Genetic risk factors for RA
(b)	Environmental risk factors for RA
(c)	Systemic autoimmunity associated with RA
(d)	Symptoms without clinical arthritis*
(e)	Unclassified arthritis*
(f)	RA

* The term “arthritis” is used to denote clinically apparent soft tissue swelling or fluid (not bony overgrowth alone)

Insights into the initiation of the disease may help to more accurately define individuals at high risk of developing RA and may lead to the discovery of targets for preventive therapy. The aim of the current study is to systematically review the availability of

and data from studies on the analysis of tissues in all phases of the disease up to the development of RA. This overview will help to define future research projects on tissue analysis in the earliest phases of RA.

METHODS

Search methods for identification of studies

A systematic literature search was performed searching MEDLINE/PubMed and EMBASE (1947 to March Week 13 2014) for articles with specific medical subject headings (MeSH), such as “synovial membrane”, “mucous membrane”, “lymphoid”, “periodontal ligament”, “lung”, “tissues”, “biopsy” and “rheumatoid arthritis”, and additional keywords, such as “pre-clinical”, “at risk”, “overweight”, “smoker” and “undifferentiated arthritis”. These search terms include specific well-known environmental risk factors for RA, such as periodontitis, overweight and smoking. The complete search strategies for the database searches are provided in Tables 2 and 3 (MEDLINE/PubMed search strategy Table 2, EMBASE search strategy Table 3).

Table 2. MEDLINE/PubMed search strategy

#	Subject headings (MeSH) and additional keywords
1	"Synovial Membrane"[Mesh] OR syno*[tiab] OR "Tissues"[Mesh] OR tissue*[tiab] OR "Mucous Membrane"[Mesh] OR mucous[tiab] OR mucosa*[tiab] OR lymphoid[tiab] OR lymphatic[tiab] OR "Periodontal Ligament"[Mesh] OR (periodontal[tiab] AND tissue*[tiab]) OR alveolodental[tiab] OR epitheli*[tiab] OR “lung”[Mesh] OR lung[tiab]
2	"Biopsy"[Mesh] OR biops*[tiab] OR biopt*[tiab]
3	#1 OR #2
4	pre-clinical*[tiab] OR preclinical*[tiab] OR at risk[tiab] OR pre-rheumatoid[tiab] OR undifferentiated arthritis[tiab] OR unclassified arthritis[tiab] OR “overweight”[Mesh] OR obes*[tiab] OR “tobacco use”[Mesh] OR tobacco use* [tiab] OR smoker*[tiab] OR smoking[tiab] OR “periodontal diseases”[Mesh:NoExp] OR “periodontitis[Mesh] OR periodont*[tiab] OR “arthralgia”[Mesh:NoExp] OR arthralgia[tiab]
5	Arthritis, Rheumatoid"[Mesh:NoExp] OR rheumatoid arthritis[tiab] OR (RA[tiab] AND arthritis[tiab])
6	((#4) AND #5) AND #3
7	#6 NOT (Animals[Mesh] NOT Humans[Mesh])

Articles describing analysis of tissue samples obtained from subjects with an increased risk of developing RA, defined as phase a, b, c, d or e according to the recommendations by the EULAR Study Group for Risk Factors for RA [7], were eligible for inclusion. There were no restrictions with regard to the kind of tissue analysed. Articles

describing analysis of synovial fluid or bronchoalveolar lavage were excluded. Non-English language articles were eligible for selection to reduce the risk of language bias. Translation of non-English articles was performed by native speakers. Articles that did not describe an original study, such as reviews, were excluded. Additional reports were identified from expert knowledge.

Data collection and extraction

The above search strategy identified a set of potentially relevant articles (title/abstracts), which were assessed independently by two reviewers (KIM, IYC), based on the previously mentioned selection criteria. Full articles were obtained when suitability of inclusion could not be judged from the title/abstract or when consensus could not be reached.

Next, the full articles of the selected studies were obtained and assessed independently by two reviewers (KIM, IYC) to determine eligibility for final inclusion in the analysis. Differences in assessment between the two reviewers were resolved by consensus.

Information about study population characteristics, phase according to the EULAR Study Group for Risk Factors for RA [7],symptom duration, type of tissue and the main findings of the studies were independently extracted from the final included studies by both reviewers, who then met to compare the data and reach consensus.

Table 3. EMBASE search strategy

#	Subject headings (MeSH) and additional keywords
1	exp rheumatoid arthritis/ or (rheumatoid arthritis or (RA and arthritis)).ti,ab.
2	exp *obesity/ or exp *"tobacco use"/ or *periodontal disease/ or exp *periodontitis/ or *arthralgia/ or (pre-clinical* or preclinical* or at risk or pre-rheumatoid or undifferentiated arthritis or unclassified arthritis or overweight or obes* or tobacco use* or smoker* or smoking or periodont* or arthralgia).ti,ab.
3	synovium/ or exp tissues/ or exp mucosa/ or periodontal ligament/ or exp lung/ or exp biopsy/ or (syno* or tissue* or mucous or mucosa* or lymphoid or lymphatic or (periodontal and tissue*) or alveolodental or epitheli* or lung or biops* or biopt*).ti,ab.
4	1 and 2 and 3
5	not ((exp animal/ or animal*.hw.) not human/)

RESULTS

Results of the search

The search of electronic databases (performed in Week 13 2014) resulted in 2868 records, of which 809 were duplicate records (Figure 1). Of the remaining 2059 records included in the screening process, 1975 records were excluded based on title or abstract screening, leaving 84 records for detailed review. We excluded 25 articles after full text reviewing. Two articles were excluded because the study population did

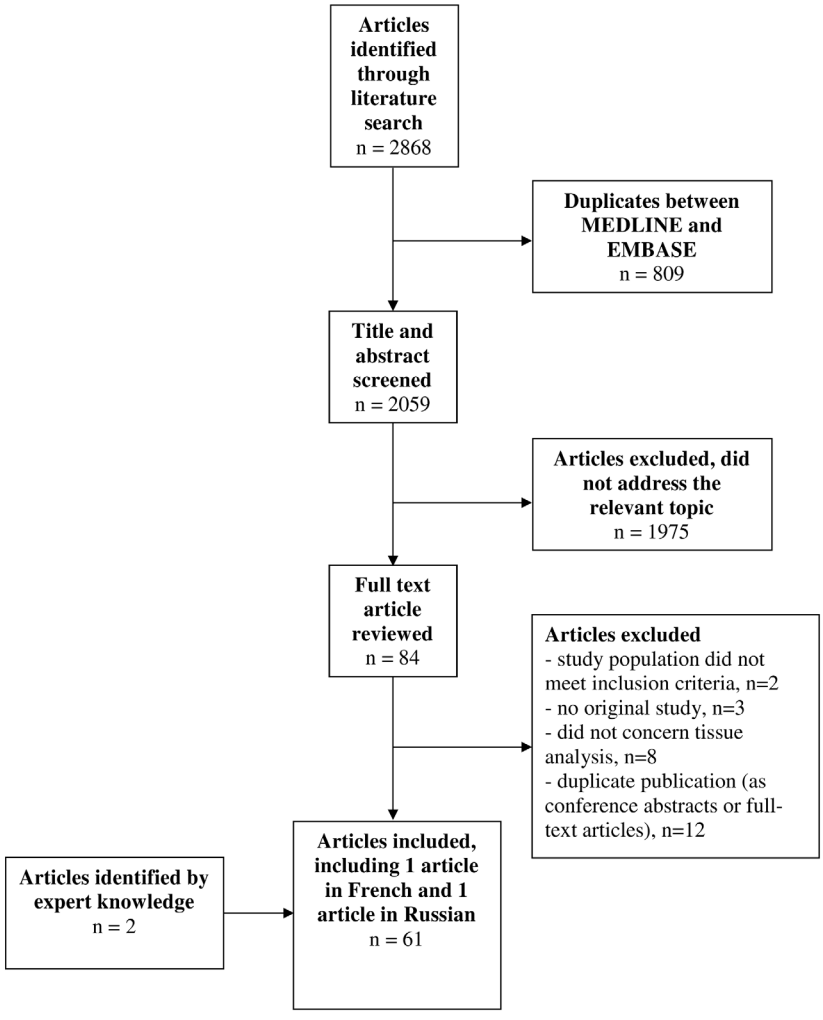

Figure 1. Study Selection Flow Diagram

MEDLINE/PubMed and EMBASE were searched for studies meeting inclusion criteria. The search process is explained in the methods section of the text.

not meet the inclusion criteria [8, 9].Three articles were excluded because they did not describe an original study [10-12]. Eight more articles were excluded since they did not describe tissue analysis [13-20].Additionally, 12 conference abstracts that were reporting the same results or that were subsequently published as articles were excluded. Two articles were identified from expert knowledge and were also included in this review [21, 22].

Studies included in the final analysis

A total of 61 articles and conference abstracts were ultimately included for this review. Forty of these selected studies were published as full text articles (Balaji 2010 [23]; Beffa 2013 [24]; Brennan 2001 [25]; Cunnane 1999 [26]; Cunnane 2001 [27];De Hair 2014 [28]; De Launay 2012 [29]; Fang 1999 [30]; Fischer 2012[21]; Goldbach-Mansky 2000 [31];Hakala 1986 [32]; Hammer 1992 [33]; Hartgring 2009 [34]; Harvey 2013 [35]; Jahangier 2006 [36]; Jarnbring 2002 [37]; Karateev 2003 [38]; Kempsell 2001 [39]; Kotake 1997 [40]; Kraan 1999 [41]; Kuipers 2009 [42]; Liu 2011 [43]; Makrygiannakis 2008 [44]; Nanbara 2012 [45]; Nesse 2012 [46]; Ogawa 1989 [47]; O'Hara 2004 [48]; Ouhara 2012 [49]; Pando 2000 [50]; Peltier 1977 [51]; Prochorec-Sobieszek 2008 [52]; Schmid 2007 [53]; Schumacher 1972 [22]; Siala 2008 [54]; Stahl 2000 [55]; Van Baarsen 2013 [5]; Van de Sande 2012 [56]; Van de Sande 2011 [1]; Van de Sande 2013 [57]; Van der Heijden 1999 [58]); The remaining 21 studies were available as conference abstracts (Bingham 2010 [59]; Bugatti 2013 [60]; Cheng 2013 [61]; Choi2012 [62]; De Hair 2011 [63]; DiCicco 2012 [64]; Focant 2011 [65]; Hähnlein 2013 [66]; Hähnlein 2014 [67]; Lugli 2013 [68]; Maijer 2013 [69]; Nesse 2009 [70]; Nile 2012 [71]; Noort 2012 [72]; Oliver-Bell 2013 [73]; Ramwadhdoebe 2014 [74]; Ramwadhdoebe 2014 [75]; Tak 2011 [76]; Totaro 2012 [77]; Van de Sande 2009 [78];Van Roon 2009 [79]). The relevant findings of the tissue analysis regarding the at risk phases leading up to the development of RA up for each article are listed in table 4, together with other details of the studies.

Study population and types of tissue being studied

In 28 studies, individuals without arthritis were included. In 16 of these studies, individuals could be classified as being in phase b according to the EULAR Study Group for Risk Factors for RA, because of having periodontitis (Balaji 2010 [23]; Bingham 2010 [59]; Cheng 2013 [61]; Harvey 2013 [35]; Jarnbring 2002 [37]; Liu 2011 [43]; Nanbara 2012 [45]; Nesse 2009 [70]; Nesse 2012 [46]; Nile 2012 [71]; Ogawa 1989 [47]; Oliver-Bell 2013 [73]; Ouhara 2012 [49]) or because of being a smoker (Hakala 1986 [32]; Lugli 2013 [68]; Makrygiannakis 2008 [44]). In 3 studies the individuals could be classified as being in phase c because of positivity for IgM-RF and/or ACPA status (Fischer 2012 [21]; Maijer 2013 [69]; Tak 2011 [76]). In 9 studies the individuals could be classified as being in phase c + d because of positivity for IgM-RF and/or ACPA and the presence of arthralgia (Choi 2012 [62]; De Hair 2011 [63]; Hähnlein 2013 [66]; Hähnlein 2014 [67];

Table 4. Characteristics of studies included (alphabetical order of first author)

Author Year	RA phase description (according to the EULAR Study Group for Risk Factors for Rheumatoid Arthritis)	Duration of symptoms (in case of unclassified arthritis)	Origin of tissue
Main relevant findings of each article			
Balaji et al. [23] 2010	Phase b (periodontitis)	Not applicable	Periodontium
Telomerase can be used as a marker to assess the severity of inflammation in chronic periodontitis.			
Beffa et al. [24] 2013	Phase e (unclassified arthritis)	Not described	Synovial tissue
Using the absolute densities (e.g. cells/mm2 of subintimal tissue) in immunohistochemical stained synovial tissue when comparing early undifferentiated arthritis with other chronic arthritis or non-inflammatory arthropathies is superior to the relative method, using the inflammatory cell's percentage of the total inflammatory cell population, which is the total sum of 5 major inflammatory cell types in synovial infiltrates.			
Bingham et al. [59] 2010	Phase b (periodontitis)	Not applicable	Periodontium, buccal mucosa and tonsil
Citrullination is present in multiple oral locations and is not limited to inflamed periodontium. Similarly, PAD enzymes 2, 3, 4 and 6 are localized in oral tissue in the presence or absence of inflammation. This study indicates that there are multiple potential pathways responsible for posttranslational citrullination in the oral mucosa.			
Brennan et al. [25] 2001	Phase e (unclassified arthritis)	"Symptoms of synovitis < 1 year"	labial salivary glands
Patients with synovitis of recent onset have a higher than expected frequency of focal sialadenitis.			
Bugatti et al. [60] 2013	Phase e (unclassified arthritis)	"Symptoms of synovitis < 1 year"	Synovial tissue
The presence of high degree of synovial B cell infiltration and aggregation are most frequently observed in early RA and UA patients who are at need for methotrexate initiation.			
Cheng et al. [61] 2013	Phase b (periodontitis)	Not applicable	Periodontium
IL-17 producing CD4+ T cells are present in gingival tissue from periodontitis lesions. Furthermore, P. gingivalis can activate monocytes resulting in subsequent induction of IL-17 responses in human CD4+ T cells.			
Choi et al. [62] 2012	Phase c (IgM-RF and/ or ACPA positive) + phase d (arthralgia) Phase e (unclassified arthritis)	Not applicable "Inflammatory arthritis of < 1 year disease duration".	Synovial tissue
Stromal cell markers CD248, gp38, PDI and CD55 are all expressed in the earliest stages of clinically manifest arthritis, independent of the diagnosis and outcome after follow up. Compared to early RA, the expression of gp38 might be lower in individuals at risk for RA.			
Cunnane et al. [26] 1999	Phase e (unclassified arthritis)	"Early arthritis with symptom duration < 18 months"	Synovial tissue

Matrix metalloproteinase and the cysteine proteases, cathepsin B and cathepsin L were expressed in early arthritis patients, though no differences were seen between the disease categories. The enzymes were detected both in the lining and sublining layers, as well as in the perivascular and endothelial cells. The detection of these proteases in the synovium shortly after symptom onset implies that the potential for joint destruction exists at a very early stage in the disease.

Cunnane et al. [27] 2001	Phase e (unclassified arthritis)	"Early inflammatory arthritis with symptom duration < 18 months at time of first presentation"	Synovial tissue

Matrix metalloproteinase 1 (MMP-1), cathepsin B and cathepsin L are all detected in the synovium soon after the onset of arthritis symptoms. High levels of MMP-1 mRNA expression in the lining layer distinguished patients with more rapidly progressive erosive disease (erosion after 1 year).

De Hair et al. [28] 2014	Phase c (IgM-RF and/or ACPA positive), with/without phase a (first degree relative of RA patient), with/without phase d (arthralgia)	Not applicable	Synovial tissue

CD3+ T cell numbers (in particular, expression of both CD3 and CD8 T cells) in synovial tissue of individuals at risk for RA (IgM-RF and/or ACPA positive) showed a borderline association with subsequent development of clinically manifest arthritis. Combination of CD3 expression in synovium with ACPA positivity (defined as recognition of more than 1 citrullinated peptide) resulted in an increased association with arthritis development, as compared with expression of CD3 in synovium alone.

De Hair et al. [63] 2011	Phase c (IgM-RF and/or ACPA positive) + phase d (arthralgia)	Not applicable	Synovial tissue

Local expression of prostaglandins does not seem to be involved in the pain sensation in individuals at risk for RA.

De Launay et al. [29] 2012	Phase e (unclassified arthritis)	"Early arthritis with disease duration < 1 year, as measured from the first clinical signs of arthritis"	Synovial tissue

Activation of ERK and JNK, members of the mitogen-activated protein kinase (MAPK) family, was enhanced in early arthritis patients meeting RA criteria compared to UA and SpA. JNK activation was enhanced in UA-RA vs. UA-UA (1987 criteria) and is also predictive for erosive disease development, suggesting JNK may represent an attractive target in treating RA early in the disease process.

DiCicco et al. [64] 2012	Phase e (unclassified arthritis)	"Early inflammatory arthritis with < 12 months duration"	Synovial tissue

The presence of lymphocytic aggregates is more frequent in early RA vs. early UA and early PsA.

Fang et al. [30] 1999	Phase e (unclassified arthritis)	"Early arthritis with synovitis < 3 years duration"	Synovial tissue

In early seronegative spondyloarthropathy and UA, the T cell receptor (TCR) heterogeneity/repertoire seems greater compared to healthy synovium and RA. The TCR repertoire also tended to narrow with time in RA and SpA.

Fischer et al. [21] 2012	Phase c (ACPA positive)	Not applicable	Lung tissue

The lung phenotypic characteristics of a cohort of patients with ACPA positivity and lung disease (in the absence of existing RA) resemble those of established RA.A few of these patients developed articular RA after follow-up.

Focant et al. [65] 2011	Phase e (unclassified arthritis)	Not described	Synovial tissue

The combination of gene expression data and clinical symptoms can be useful in making a diagnosis.

Goldbach-Mansky et al. [31] 2000	Phase e (unclassified arthritis)	"Early peripheral joint synovitis of > 6 weeks and < 12 months duration"	Synovial tissue

In early RA versus early SpA and UA, the tissue expression of matrix metalloproteinase (MMP)-14, the activator for pro-MMP-2, was significantly higher; the expression of tissue inhibitor of metalloproteinase TIMP-2, an inhibitor of MMP-2, was lower. High synovial tissue level of MMP-2 activity were significantly correlated with the presence of early erosions in early RA patients (none of the early SpA and UA patients included in this study developed erosions).

Hähnlein et al. [66] 2013	Phase c (IgM-RF and/ or ACPA positive) + phase d (arthralgia)	Not applicable	Lymph node tissue

Cultured human lymph node stromal cells (LNSCs) express typical stromal cell markers and are responsive for toll-like receptor 3 triggering. The LNSCs express the transcriptional regulator Deaf1 which may indicate peripheral tissue antigen expression by LNSCs.

Hähnlein et al. [67] 2014	Phase c (IgM-RF and/ or ACPA positive) + phase d (arthralgia) Phase e (unclassified arthritis)	Not applicable Not described	Lymph node tissue

Cultured human lymph node stromal cells (LNSCs) express Aire and these data suggest that human fibroblastic reticular cells (FRC: gp38+ , CD 31-) as well as double negative cells express it. This may indicate tissue-specific self-antigen expression by LNSCs pointing towards a role for the lymph node stromal environment in peripheral tolerance and autoimmunity.

Hakala et al. [32] 1986	Phase b (smoking)	Not applicable	Lung tissue

Smoking alone does not explain the lesions of the small airways found in connective tissue disorder (CTD) patients and bronchiolitis may be specifically associated with the basic disorder.

Hammer et al. [33] 1992	Phase e (unclassified arthritis)	Not described	Synovial tissue

Only small numbers of Chlamydiae may be present in inflamed joints. Chlamydial rRNA was found in 3 synovial fluid samples of 24 UA patients. Of these patients, 11 synovial tissue samples were available, but in none of these samples was Chlamydial rRNA detected.

Hartgring et al. [34] 2009	Phase e (unclassified arthritis)	"Arthritis ≥ 4 weeks despite at least 2 intra-articular injections of gluco-corticoids"	Synovial tissue

There is an increased expression of interleukin-7 receptor α-chain (IL-7Rα), which mediate the IL-7 activity (a potent immunoregulatory cytokine), in the synovial tissue of patients with RA and persistent UA as compared with patients with OA.

Harvey et al. [35] 2013	Phase b (periodontitis)	Not applicable	Periodontium

PAD enzymes 2 and 4 (protein and mRNA) as well as citrullinated proteins are present in inflamed gingiva, and ACPAs can be detected in the gingival crevicular fluid of some patients. Tissue expression of citrullinated proteins and PAD increase with severity of inflammation.

Jahangier et al. [36] 2006	Phase e (unclassified arthritis)	"Persistent arthritis despite at least 2 intra-articular gluco-corticoid injections in an outpatient setting and ongoing for ≥ 4 weeks after the last gluco-corticoid injection"	Synovial tissue

Intra-articular treatment either with yttrium-90 and glucocorticoids or with glucocorticoids alone is especially successful in patients with marked synovial inflammation, independent of the diagnosis (RA or non-RA, like UA and PsA)

Jarnbring et al. [37] 2002	Phase b (periodontitis)	Not applicable	Periodontium

Five to 12 percent of the keratinocytes in the basal layers of the epithelium proliferate in patients with gingivitis and patients with periodontitis. Only in the most apical part of the sulcus the number of apoptotic keratinocytes exceeds the number of proliferative ones in patients with periodontitis.

Karateev et al. [38] 2003	Phase e (unclassified arthritis)	"Early arthritis with symptoms < 1 year"	Synovial tissue

Accumulation of large amounts of macrophages and lymphocytes in the infiltrate was significantly more often detected in synovial tissue of RA patients compared to synovial tissue of non-RA patients.

Kempsell et al. [39] 2001	Phase e (unclassified arthritis)	Not described	Synovial tissue

M. tuberculosis group organism RNA sequences were not found in healthy synovial fluid or tissue of UA patients.

Kotake et al. [40] 1997	Phase e (unclassified arthritis)	"Early synovitis: oligoarthritis with duration ≤ 12 months"	Synovial tissue

Cytokine mRNA profiles in patients with early RA, reactive arthritis, UA are skewed toward pro-inflammatory macrophage-derived and type 1 cytokines. IL-10 (not IL-4 or IL-13) mRNA appears to be the major anti-inflammatory cytokine mRNA.

Kraan et al. [41] 1999	Phase e (unclassified arthritis)	"Disease duration of < 1 year, as measured from the first clinical signs of arthritis regardless of which joint was initially affected"	Synovial tissue

Marked infiltration by plasma cells, B cells and macrophages in the synovial sublining differs between early RA and other forms of early arthritis, specifically OA, SpA, PsA and UA.

Kuipers et al. [42] 2009	Phase e (unclassified arthritis)	Not described	Synovial tissue

The rate of Chlamydia trachomatis-PCR positivity in synovial fluid and tissue was low in patients with reactive arthritis (none out of 8) and UA patients (3 out of 23).

Liu et al. [43] 2011	Phase b (periodontitis)	Not applicable	Periodontium

Excessive inflammatory cytokine levels, miR-17, and Smurf1 are all involved in a coherent feed-forward loop. In this circuit, inflammatory cytokines led to direct activation of Smurf1 and downregulation of miR-17, thereby increasing degradation of Smurf1-mediated osteoblast-specific factors.

Lugli et al. [68] 2013	Phase b (smoking)	Not applicable	Lung tissue

There is widespread citrullination of proteins in lung tissue of never smokers and there is a modest increase with smoking and chronic obstructive pulmonary disease. This pattern of expression corresponds to that of PAD 2.

Maijer et al. [69] 2013	Phase c (IgM-RF and/ or ACPA positive) Phase e (unclassified arthritis)	Not applicable "Early arthritis with arthritis duration < 1 year"	Synovial tissue

Synovial NF-B-inducing kinase (NIK), a key regulator of inflammation-induced angiogenesis in RA, expression is associated both with systemic markers of disease activity (ESR and CRP) and with local disease activity in early arthritis patients, independent of the diagnosis (early RA/UA/CA/OA/SpA). In autoantibody-positive individuals, synovial NIK expression is not associated with development of arthritis.

Makrygiannakis et al. [44] 2008	Phase b (smoking)	Not applicable	Lung tissue

Smoking enhances PAD 2 expression in the bronchial mucosal and alveolar compartment, with consequent generation of citrullinated proteins in the latter.

Nanbara et al. [45] 2012	Phase b (periodontitis)	Not applicable	Periodontium

The modulation of wingless protein Wnt5a expression by P. gingivalis may play a role in the periodontal inflammatory process and serve as a target for the development of new therapies.

Nesse et al. [70] 2009	Phase b (periodontitis)	Not applicable	Periodontium

Citrullination occurs in vivo in the periodontium. Furthermore, human cartilage glycoprotein 39/HLAc are present in periodontitis tissue.

Nesse et al. [46] 2012	Phase b (periodontitis)	Not applicable	Periodontium

Within the periodontal stroma, citrullination is an inflammation-depended process. In periodontal epithelium, citrullination is a physiological process. Additional citrullinated proteins are formed in periodontitis, apparently similar to those formed in RA-affected synovial tissue.

Nile et al. [71] 2012	Phase b (periodontitis)	Not applicable	Periodontium

IL-17E (IL-25) derived from endothelial cells and invading leucocytes play a role in the pathogenesis of periodontal disease as a negative regulator of oral immunity. IL-17E can down regulate the expression of key neutrophil chemo-attractants and therefore possibly inhibit neutrophil chemotaxis into the periodontium.

Noort et al. [72] 2012	Phase e (unclassified arthritis)	Not described	Synovial tissue

AIRE, a transcription factor that is suggested to play a complementary role in tolerance induction, is more expressed in RA compared to UA.

Ogawa et al. [47] 1989	Phase b (periodontitis)	Not applicable	Periodontium

Significant numbers of viable plasma cells/Ig-secreting cells can be isolated from inflamed gingival tissues. Further, IgG subclass responses in gingiva are similar to those found in synovia of RA patients, and in stimulated PBMC and spleen. However, the number of IgG4- and IgA2-secreting cells increased in the advanced stage of periodontal disease.

O'Hara et al. [48] 2004	Phase e (unclassified arthritis)	Not described	Synovial tissue

Acute phase serum amyloid A (A-SAA) gene expression was detected by real time-PCR in synovial tissue from PsA, RA, UA and sarcoid arthritis patients. Immunohistochemical analysis showed expression of A-SAA protein production by several synovial cell populations, and immunofluorescence analysis confirmed A-SAA colocalization with the macrophage marker CD68.

Oliver-Bell et al. [73] 2013	Phase b (periodontitis)	Not applicable	Periodontium

The proportion of B1a cells is not significantly altered in the gingiva of human periodontitis (PD) patients, or systemically in mouse models of PD. However, B1a cells express more receptor activator of nuclear factor kappa-B ligand (RANKL) than other mature B cell subsets in the gingiva and draining lymph nodes of mice with PD.

Ouhara et al. [49] 2012	Phase b (periodontitis)	Not applicable	Periodontium

IL-32 is constitutively produced by human gingival fibroblasts which can be suppressed by P. gingivalis and may play a role in the downregulation of inflammatory responses, such as IL-8 production, in periodontal tissue.

Pando et al. [50] 2000	Phase e (unclassified arthritis)	"Symptomatic disease duration of ≤ 1 year"	Synovial tissue

Most of the early RA and UA patients had histologic evidence of synovitis (angiogenesis, proliferation, activation of synovial lining cell, and presence of at least moderate infiltrates) in the asymptomatic joint, while those with early reactive arthritis did not.

Peltier et al. [51] 1977	Phase e (unclassified arthritis)	Not described	Synovial tissue

The presence of cells with fluorescent cytoplasm during immunofluorescent examination of the synovial membrane may be regarded as an additional criterion supporting the diagnosis of RA.

Prochorec-Sobieszek et al. [52] 2008	Phase e (unclassified arthritis)	Not described	Bone marrow

RA and neutropenia patients represent a continuous spectrum of T-cell large granular lymphocyte (T-LGL) proliferations, although monoclonal expansions were most frequently observed. The histopathological pattern and immunophenotype of bone marrow infiltration as well as molecular characteristics were similar in T-LGL leukemia patients with and without arthritis.

Ramwadhdoebe et al. [74] 2014	Phase c (IgM-RF and/ or ACPA positive), with phase d (arthralgia) Phase e (unclassified arthritis)	Not applicable "Early arthritis with disease duration < 1 year"	Lymph node tissue

Pro-inflammatory as well as regulatory cytokines and T-cell subsets are increased in peripheral lymphoid tissue during the earliest phases of arthritis, suggesting an early change in the immunoregulatory balance.

Ramwadhdoebe et al. [75] 2014	Phase c (IgM-RF and/ or ACPA positive), with phase d (arthralgia)	Not applicable	Lymph node tissue

Dendritic cell (DC) subsets are present in lymph nodes (LN) during the earliest phases of arthritis. As CD1c+DCs are the main DC subset present in early arthritis and the only subset related with ACPA status. This study supports the notion that in addition to the well-known capacity of CD1c+DCs to activate naïve T cells, CD1+DC might also contribute to B cell responses.

Schmid et al. [53] 2007	Phase e (unclassified arthritis)	Not described	Synovial tissue

The presence of parvovirus B19 DNA in synovial tissue of patients with joint inflammation (UA) does not allow the diagnosis of parvovirus induces arthritis.

Schumacher et al. [22] 1972	Phase e (unclassified arthritis)	"Arthritis ≤ 1 month"	Synovial tissue

Synovial membrane inflammation was comparable between early RA and early UA patients.

Siala et al. [54] 2008	Phase e (unclassified arthritis)	Not described	Synovial tissue

Bacterial DNA found by 16S rRNA PCR, cloning and sequencing of synovial tissue is comparable in reactive arthritis and UA.

Stahl et al. [55] 2000	Phase e (unclassified arthritis)	"Disease duration < 1 year"	Synovial tissue

One or more viruses (found by polymerase chain reaction analysis of viral DNA material in synovial fluid and tissue) can be detected in the synovium of patients with early arthritis, irrespective of the clinical diagnosis of UA, RA, SpA, CA, OA, septic arthritis or trauma. This indicates that detection of viral DNA in joint samples has limited diagnostic potential.

Tak. [76] 2011	Phase c (IgM-RF and/ or ACPA positive)	Not applicable	Synovial tissue

MRI findings (maximal enhancement, rate of enhancement, synovial volume and enhancement shape curve distribution) and immunohistochemical analysis of synovial tissue (phenotypic markers, adhesion molecules, and vascularity) were comparable between autoantibody positive individuals and healthy controls.

Totaro et al.[77] 2012	Phase e (unclassified arthritis	Not described	Synovial tissue

UA and RA patients carrying HLA DRB1*04 allele showed a higher positivity for P. gingivalis DNA in the synovial tissue compared to patients negative for the allele.

Van Baarsen et al. [5] 2013	Phase c (IgM-RF and/ or ACPA positive), with/without phase a (first degree relative of RA patient), with/ without phase d (arthralgia) Phase e (unclassified arthritis)	Not applicable "Early arthritis with arthritis duration ≤ 6 months"	Lymph node tissue

There is increased immune activation with lymph nodes of early arthritis patients as well as in autoantibody-positive individuals at risk of developing RA.

Van de Sande et al. [56] 2012	Phase e (unclassified arthritis)	"Early arthritis with disease duration < 1 year, as measured from the first clinical evidence of joint swelling"	Synovial tissue

The features of synovial inflammation in patients with UA are the same in those who eventually develop RA as classified according to only 2010 criteria and in those who develop RA classified according to 1987 criteria

Van de Sande et al. [1] 2011	Phase c (IgM-RF and/ or ACPA positive), with/without phase a (first degree relative of RA patient), with/ without phase d (arthralgia)	Not applicable	Synovial tissue

Synovial tissue of IgM-RF and/or ACPA positive individuals showed very low scores for phenotype markers, adhesion molecules and vascularity, all in the same range as those in normal controls.

Van de Sande et al. [57] 2013	Phase e (unclassified arthritis)	"Early arthritis with disease duration < 1 year, as measured from the first clinical evidence of joint swelling"	Synovial tissue

Expression of angiopoietin 1 (Ang-1) was comparable between patients with early RA at baseline and patients with early UA who fulfilled the criteria for RA over time.

Van de Sande et al. [78] 2009	Phase e (unclassified arthritis)	"Early arthritis with < 1 year disease duration"	Synovial tissue

Baseline synovial tissue of UA patients showed no differences in the presence and size of lymphoid aggregates between patients who fulfill RA criteria after 2 years of follow-up and who stays classified as non-RA. Also, the presence of lymphoid aggregates is not correlated with the prognostic outcome (self-limiting or persistent erosive/ non erosive) in UA patients.

Van der Heijden et al. [58] 1999	Phase e (unclassified arthritis)	"Disease duration was measured from the first clinical signs of arthritis"	Synovial tissue

Mycobacterial genus-specific PCR applied on DNA extracts isolated directly from synovial fluid or synovial tissue, followed by sequence analysis of the PCR product, did not provide evidence for a pathogenic role of mycobacteria in SpA, UA or RA.

Van Roon et al. [79] 2009	Phase e (unclassified arthritis)	Not described	Synovial tissue

Increased expression of IL-7Ralpha was found in synovial tissue of RA and UA patients compared to OA, and it is correlated with CD3 and IL-7 (a potent T cell stimulatory cytokine) expression in the synovial tissue.

ACPA: Anti–citrullinated protein antibodies; CA: Crystal arthritis; CRP : C-reactive protein; ESR: erythrocyte sedimentation rate; IL: interleukin; OA: osteoarthritis; PAD: peptidyl arginine deiminase; PCR: polymerase chain reaction; PsA: psoriatic arthritis; RA: rheumatoid arthritis; RF: rheumatoid factor; SpA: spondyloathropathy; UA: unclassified arthritis.

Ramwadhdoebe 2014 [74]; Ramwadhdoebe 2014 [75]), with or without also being in phase a because of having an RA patient as a first degree relative (De Hair 2014 [28]; Van de Sande 2011[1]; Van Baarsen 2013[5]).

Synovial tissue was analysed in 6 studies (Choi 2012 [62]; De Hair 2014 [28]; De Hair 2011 [63]; Maijer 2013 [69]; Tak 2011 [76]; Van de Sande 2011 [1]) and lymph node tissue was analysed in 5 studies (Hähnlein 2013 [66]; Hähnlein 2014 [67]; Ramwadhdoebe 2014 [74]; Ramwadhdoebe 2014 [75]; Van Baarsen 2013 [5]) in individuals without arthritis. Periodontium was analysed in 13 studies including individuals having periodontitis. In 1 of these studies, buccal mucosa and tonsil were also obtained for analyses (Bingham 2010 [59]). Lung tissue was analysed in 4 studies. Three of these studies included smokers (Hakala 1986 [32]; Lugli 2013 [68]; Makrygiannakis 2008 [44]) and 1 study included individuals being positive for ACPA (Fischer 2012 [21]).

In five of the aforementioned studies, both individuals without arthritis and individuals with unclassified arthritis were included (Choi 2012 [62]; Hähnlein 2014 [67]; Maijer 2013 [69]; Ramwadhdoebe 2014 [74]; Van Baarsen 2013 [5]). In total, 38 studies included individuals with unclassified arthritis. These patients were classified as being in phase e according to the EULAR Study Group for Risk Factors for RA. In this phase of the disease, the main tissue analysed was synovium. In three studies lymph node tissue was analysed (Hähnlein 2014 [67]; Ramwadhdoebe 2014 [74]; Van Baarsen 2013 [5]). In one study bone marrow was analysed (Prochorec-Sobieszek 2008 [52]) and in one study labial salivary gland biopsies were analysed (Brennan 2001 [25]).

Symptom duration in patients with unclassified arthritis

The duration of symptoms varied in patients with unclassified arthritis (phase e). From the 38 studies that included patients with unclassified arthritis, 24 studies described patients as having “early arthritis” or the duration of symptoms was defined (Brennan 2001 [25]; Bugatti 2013 [60]; Choi 2012 [62]; Cunnane 1999 [26]; Cunnane 2001 [27]; De Launay 2012 [29]; DiCicco 2012 [64]; Fang 1999 [30]; Goldbach-Mansky 2000 [31]; Hartgring 2009 [34]; Jahangier 2006 [36]; Karateev 2003 [38]; Kotake 1997 [40]; Kraan 1999 [41]; Maijer 2013 [69]; Pando 2000 [50]; Ramwadhdoebe 2014 [74]; Schumacher 1972 [22]; Stahl 2000 [55]; Van Baarsen 2013 [5]; Van de Sande 2012 [56]; Van de Sande 2013 [57]; Van de Sande 2009 [78]; Van der Heijden 1999 [58]). The definition of symptom duration varied in these 24 studies from symptom duration of ‘one month or less’, ‘less than 1 year’, ‘less than 18 months’, ‘less than 3 years’ or ‘ persistent arthritis for ≥ 4 weeks after glucocorticoid injection’. In the remaining 14 studies (Beffa 2013 [24]; Focant 2011 [65]; Hähnlein 2014 [67]; Hammer 1992 [33]; Kempsell 2001 [39]; Kuipers 2009 [42]; Noort 2012 [72]; O’Hara 2004 [48]; Peltier 1977 [51]; Prochorec-Sobieszek 2008 [52]; Schmid 2007 [53]; Siala 2008 [54]; Totaro 2012 [77]; Van Roon 2009 [79]) the duration of symptoms of patients with unclassified arthritis was not specified.

Tissue analyses

The selected studies in our review mainly analysed synovial tissue, lymph nodes, periodontium and lungs in the phases leading up to the development of RA (phase a – e). Next, a short overview of the main findings per tissue is listed.

Synovial tissue

When looking at the various infiltrating inflammatory cells using immunohistological techniques to analyze the synovium, the extent of synovial inflammation more in general (including angiogenic processes) is comparable between UA patients and RA patients [22, 56, 57, 60, 69]. Differences in the number of macrophages and lymphocytes could be found depending on the disease activity [38, 41, 51, 62, 80]. Furthermore, various proteins that are increased in the serum of RA patients could also be demonstrated in the synovium of UA patients, such as IL-7 [34], pro-inflammatory macrophage-derived type 1 cytokines [40], and acute phase serum amyloid A (A-SAA) [48]. A-SAA is known to induce matrix metalloproteinase (MMP) production, involved in the process of joint destruction, and when evaluated in the synovial tissue of UA compared to RA patients, conflicting results are reported [26, 27, 31].

No clear differences in the presence of lymphocyte aggregates were found in the synovium of UA patients who could be classified as RA after a follow-up of 2 years and those who remain unclassified after follow-up [78], suggesting that the presence of lymphocyte aggregates at baseline is not specific for RA. Interestingly, synovial tissue of both early RA and early UA patients demonstrates similar histological changes in the symptomatic and asymptomatic joints within one patient [50], indicating that inflammatory changes in the synovium may be subclinical. However, features of the synovium of individuals with arthralgia who were IgM-RF and/or ACPA positive, were not different to those from healthy controls, even for the at risk individuals who eventually developed RA [1, 28], suggesting that subclinical inflammation of the synovium does not coincide with the appearance of serum autoantibodies and that synovial infiltration by inflammatory cells occurs only relatively shortly (not more than weeks to months) before the onset of clinically apparent joint swelling. The findings also support the hypothesis that systemic autoimmunity exists years before the development of (subclinical) synovitis and that a second hit is needed for the arthritis development. This might, for example, be joint trauma or a viral infection, leading to expression of citrullinated antigens in the joint which could trigger autonomous disease progression in the presence of pre-existing ACPA [1]. Some studies have been performed trying to find evidence for this hypothesis, looking for signs of viral or bacterial infection in synovium [6, 33, 39, 42, 53-55, 58]. A variety of bacterial and viral fragments have indeed been detected in rheumatoid synovial tissue, as well as in other forms of arthritis. Interestingly, tissue analysis has also shown the presence of P. gingivalis, one of the common pathogens in periodontitis, in the synovial tissue of arthritis patients, including UA patients [77].

Lymph nodes

The recruitment of activated immune cells to the site of inflammation is initiated after informing a nearby lymph node of a danger signal. The selected studies in our review show that increased immune cell activation within lymph nodes of early arthritis patients as well as autoantibody-positive individuals at risk of developing RA can be found [5]. Pro-inflammatory as well as regulatory cytokines and T-cell subsets are increased in peripheral lymphoid tissue during the earliest phases of arthritis [74]. Furthermore, it has been shown that cultured human lymph node stromal cells (LNSCs) express Aire [67], a transcription factor that regulates the expression of peripheral tissue-specific antigens and may play a role in peripheral tolerance induction [81-83].

Periodontium

The periodontal tissue shows remarkable similarities in the pathologic processes of RA. Synovial tissue analyses suggested, for instance, that the pro-inflammatory cytokine IL-17 contributes to the active, pro-inflammatory state of RA [84]. Also in periodontitis, it has been shown that IL-17 producing cells are present in gingival tissue [61]. Additionally, the production of autoantibodies, such as ACPA, are thought to play a role in the pathogenesis of both diseases [85-87]. The results from our review of tissue studies analyzing periodontal and gingival tissue, respectively, show that potential pathways responsible for citrullination in the oral mucosa include endogenous PAD expression, upregulated PADs at inflammatory foci, and exogenous PAD sources [35, 59]. It is proposed that oral citrullination is an inflammation-depended process leading to a break in tolerance with consequent ACPA formation in RA [46].

Lung tissue

The association of smoking with the development of ACPA positive RA has led to interest in the lungs as the potential initial site of inflammation [44, 88]. The studies included in our review support this notion, showing that lung abnormalities can be present in ACPA positive individuals without clinical arthritis [21]. Furthermore, they suggest that the lung is a site for priming the ACPA response by showing widespread citrullination of proteins in lung tissue [68]. Citrullinated protein formation in the lung alveolar compartment is associated with smoking and with higher expression of PAD2 enzyme in different lung compartments [44, 68].

DISCUSSION

This systematic review provides an overview of available studies reporting the changes in various tissues in the different phases up to the development of RA, an active research field of considerable interest.

Analyzing the synovium of patients at risk for RA has been important in providing a deeper understanding as to where the disease starts and when the synovial tissue gets involved. Various studies have focused on synovial tissue analysis comparing UA patients with RA patients as well as autoantibody-positive individuals at risk for developing RA compared to healthy controls. Most studies have shown that the synovium is most likely not the initial site involved in the disease onset and that systemic autoimmune activation may precede synovial inflammation by many years. In individuals at risk of developing RA, inflammatory changes or citrullinated proteins may be found in compartments other than the synovium before the onset of synovitis. Therefore, analysing tissues of other areas of interest, such as lymph nodes, periodontium and lungs, may give additional insight into the aetiology and the timing of events in the development from a healthy individual to a patient with RA.

Many studies have reported on the analysis of tissues obtained during the phase of unclassified arthritis (phase e). In most of these studies, the subsequent diagnosis of RA was based on the 1987 ACR criteria for RA [89]. Studies comparing the 1987 ACR and the 2010 ACR/EULAR classification criteria for RA have shown that a large proportion of patients who were classified in such studies as UA according to the 1987 ACR criteria could already be classified as RA when the 2010 ACR/EULAR criteria were applied [90-92]. When applying the 2010 ACR/EULAR classification criteria similar inflammatory changes in the synovial tissue are observed compared to the 1987 ACR criteria for RA [56]. However, using the 2010 ACR/EULAR classification criteria for RA, the disease is less severe and more frequently self-limiting [90, 91]. It will therefore be important to keep in mind which classification criteria are used when comparing results of studies on tissues of patients in phase e.

Frequently described risk factors for RA (phase b) are smoking [93-95], obesity [95-98], and periodontitis [3, 99]. In our search, we have included search terms specifically describing these environmental risk factors in combination with RA. A limitation of our review is therefore that we might have excluded papers focusing on other less frequently described risk factors as well as papers describing smoking, obesity and periodontitis not specifically marked as an environmental risk factor for RA.

Of note, studies describing cells in body fluids were not included in our search. However, analysis of cells in fluids, for instance synovial fluid and bronchoalveolar lavage, may

also be important for understanding the pathogenesis of RA since signs of inflammation are present in these fluids [100, 101].

We found inconsistencies in reporting of data related to symptom duration between different studies focusing on UA patients (phase e). A clear description of the duration of symptoms is necessary in order to study the well-established 'window of opportunity' and it will facilitate better comparison of studies conducted by different research groups [102]. Ultimately, a better understanding of the molecular changes in different tissues during various stages of the disease could lead to the development of preventive strategies for subjects at risk of developing RA. In addition, information on symptom duration in patients in phase d was virtually never presented and a systematic approach to capturing this information is needed in future studies.

Taken together, our systematic review shows that tissue analyses have been performed in the phases before the clinical features of RA become apparent. Most analyses have focused on the synovium but analyses of compartments other than the synovium may provide additional pivotal insights into the aetiology and pathogenesis of RA. To allow comparison of different studies, it will be important to describe in a detailed and consistent way the phase the subjects were in at the time of study. Furthermore, it is important to understand whether pathological processes change following the onset of symptomatic disease (phase c onwards), as an evolution of pathological processes may suggest that treatments would need to be tailored depending on symptom duration. It is pivotal that the duration and description of symptoms of which duration is timed from is mentioned more explicit in studies trying to elucidate the pathogenesis of RA.

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3

PREVALENCE OF ANTI-CITRULLINATED PROTEIN ANTIBODIES AND IGM RHEUMATOID FACTOR IN FIRST-DEGREE RELATIVES OF DUTCH RHEUMATOID ARTHRITIS PATIENTS

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In the preclinical phase of rheumatoid arthritis (RA), the risk of arthritis development is enhanced by the presence of anti-citrullinated protein antibodies (ACPAs) and further enhanced by the presence of IgM rheumatoid factor (IgM-RF): about 40% of individuals with arthralgia who are positive for both ACPA and RF develop arthritis within 2 years of detection of these antibodies [1]. Thus, the presence of ACPA and/or IgM-RF may assist in identification of individuals who are at risk of developing RA. Furthermore, multiple genes [2], as well as lifestyle and environmental factors [3, 4], contribute to RA susceptibility. First-degree relatives (FDRs) of RA patients share at least some genetic and environmental risk factors with patients and have an increased risk of developing RA compared to individuals without a family history [5]. This may provide an opportunity to enrich identification of the population at risk of RA development, for research purposes. Therefore, we investigated the prevalence of ACPA and IgM-RF in FDRs of a European population of patients with RA.

Five hundred seventy-seven FDRs of RA patients were enrolled between February 2010 and April 2012. To ascertain relatives for enrollment, RA patients at the outpatient clinic of the Division of Clinical Immunology and Rheumatology, Academic Medical Center (AMC), University of Amsterdam were asked whether they had FDRs who might be interested in participating in the study, and the FDRs were then contacted to determine eligibility. In addition, FDRs were recruited at public fairs across The Netherlands: the proband diagnosis of RA was determined by questioning of the FDR by a trained physician who attended the fair. FDRs included parents, offspring, and siblings. The subjects had no current evidence of arthritis and no history of arthritis. Sera from the FDRs were tested for ACPA by second-generation anti-cyclic citrullinated peptide enzyme-linked immunosorbent assay (ELISA) [6] and for IgM-RF by ELISA. Cutoffs for positivity were designated according to the standard criteria established at each of the participating laboratories. Data on sex, age, smoking history (never, ever, pack years), and self-reported joint symptoms were collected by questionnaire. Questions related to joint symptoms are shown in Supplementary Figure 1. Symptoms suggestive of arthritis included arthralgia (not further defined), morning stiffness, and joint swelling. FDRs who tested positive for ACPA and/or IgM-RF subsequently visited the outpatient clinic for confirmation, by an experienced rheumatologist, of the absence of arthritis. If there were swollen joints (1 or more), the subject was excluded from the study.

The study was approved by the Medical Ethics Review Board of the Faculty of Medicine, Academic Medical Center. All study subjects provided written informed consent.

Baseline characteristics of the subjects are shown in Table 1. Of the 462 FDRs of RA patients with available data on the presence or absence of arthralgia, arthralgia was present in 345 (75%). Most of the subjects were female (475 (82%)). The median age was 51 years (interquartile range (IQR) 41-57 years). Of the 460 subjects with available

Table 1. Baseline characteristics of the 577 first-degree relatives of patients with RA.

	FDRs n = 577
Gender, female (n (%))	475 (82,3)
Age, years (median (IQR))[a]	51 (41-57)
Smoking history, ever (n (%))[b]	131 (28,5)
Pack years (median (IQR))[c]	0 (0-10)
Self-reported arthralgia (n %))[d]	345 (74,7)
Self-reported morning stiffness (n (%))[e]	284 (62,3)
Self-reported joint swelling (n (%))[f]	164 (35,8)
ACPA positive (n (%))	11 (1,9)
IgM-RF positive (n (%))[g]	36 (6,6)
Both ACPA and IgM-RF positive (n (%))[h]	6 (1,1)

Parameters are described as number (n (%)) or median (interquartile range), as appropriate.
FDRs RA = first degree relatives of rheumatoid arthritis patients
ACPA = anti-citrullinated protein antibody
IgM-RF = IgM rheumatoid factor
Smoking history = never smoked: 0 pack-years; ever smoked: >0 pack-years.
[a] missing for 25 individuals, [b] missing for 117 individuals, [c] missing for 145 individuals, [d] missing for 115 individuals, [e] missing for 119 individuals, [f] missing for 119 individuals, [g+h] missing for 30 individuals

data on smoking status, 29% were current or past smokers. Of the 458 FDRs with available data on self-reported morning stiffness and joint swelling, more than half (284 (62%)) reported morning stiffness and approximately one-third (164 (36%)) reported swelling of the joints, although these were never confirmed by a physician.

Eleven of the 577 FDRs (1.9%) tested positive for ACPA. Among ACPA-positive FDRs, there was a significantly higher percentage of ever smokers compared to the percentages among ACPA-negative FDRs (6 (60%) versus 125 (28%); p=0.026). Other characteristics were similar between the 2 groups (Table 2). IgM-RF was analyzed in 547 out of the 577 FDRs, of whom 36 (6.6%) tested positive. The median age was significantly higher in the group of IgM-RF-positive FDRs compared to IgM-RF-negative FDRs (median 56 years (IQR 47-62) versus 50 years (IQR 41-57); p=0.018). No other baseline characteristics differed significantly between the IgM-RF-positive and IgM-RF-negative relatives (Table 2). Six individuals (1.1%) tested positive for both ACPA and IgM-RF. Among these double-positive FDRs, the proportion with self-reported joint swelling was significantly higher compared to the proportion among the other subjects (5 (83%) versus 178 (37%); p=0.019); double-positive subjects did not differ from the other subjects for any other baseline parameter measured (data not shown).

Table 2. Baseline characteristics of the first-degree relatives of patients with RA, according to ACPA and IgM-RF status

	ACPA positive FDRs n=11	ACPA negative FDRs n=566		IgM-RF positive FDRs n=36	IgM-RF negative FDRs n=511	
Gender, female (n (%))	9 (82)	466 (82)	p=0.965	32 (89)	418 (82)	p=0.282
Age, years (median (IQR))[a]	51 (40-56)	51 (41-58)	p=0.846	56 (47-62)	50 (41-57)	**p=0.018**
Smoking history, ever (n (%))[b]	6 (60)	125 (28)	**p=0.026**	11 (32)	114 (28)	p=0.627
Pack years (median (IQR))[c]	4 (0-18)	0 (0-10)	p=0.485	0 (0-5)	0 (0-10)	p=0.952
Self reported arthralgia (n %))[d]	9 (82)	336 (75)	p=0.581	28 (80)	297 (74)	p=0.426
Self-reported morning stiffness (n (%))[e]	7 (64)	277 (62)	p=0.925	20 (57)	245 (62)	p=0.582
Self-reported joint swelling (n (%))[f]	6 (55)	158 (28)	p=0.189	12 (34)	143 (36)	p=0.846

Parameters are described as number (n (%)) or median (interquartile range), as appropriate.
FDRs RA = first degree relatives of rheumatoid arthritis patients
ACPA = anti-citrullinated protein antibody
IgM-RF = IgM rheumatoid factor
Smoking history = never smoked: 0 pack-years; ever smoked: >0 pack-years.

[a] missing for 25 ACPA-, 1 IgM-RF+ and 23 IgM-RF- tested individuals [b] missing for 1 ACPA+, 116 ACPA-, 2 IgM-RF+ and 110 IgM-RF- tested individuals, [c] missing for 3 ACPA+, 142ACPA-, 7 IgM-RF+ and 133 IgM-RF- tested individuals, [d] missing for 115 ACPA-, 1 IgM-RF+ and 109 IgM-RF-tested individuals, [e]missing for 121 ACPA-, 1 IgM-RF+ and 115 IgM-RF-tested individuals, [f]missing for 119 ACPA-, 1 IgM-RF+ and 113 IgM-RF-tested individuals

Studying the preclinical RA state is currently a hot topic in rheumatology research. ACPA and RF may be present years before the appearance of clinical signs and symptoms of RA [7], and this finding provides an opportunity for application of screening approaches that may help to identify individuals at risk of developing RA. It can be expected that the prediction models will further improve by identification of novel molecular biomarkers, as well as life style factors, that contribute to the models [3]. The ultimate objective is to obtain a deeper understanding of the etiology of RA and to evaluate preventive strategies during the preclinical phase. An example of the latter is the currently ongoing Prevention of Rheumatoid Arthritis by Rituximab study (Netherlands Trial Register no. NTR1969) (http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=1969).

One way to investigate the preclinical phase of RA may be to study unaffected FDRs

at risk of developing RA. In this study, the prevalence of ACPA positivity and IgM-RF positivity in FDRs of Dutch RA patients was 1.9% and 6.6%, respectively. Further, 1.1% of the FDRs tested positive for both ACPA and IgM-RF. This is the largest European cohort of FDRs with data on ACPA and IgM-RF status, although serologic risk markers in FDRs have previously been described in a relatively small Swedish cohort [8]. Our results are in accordance with those of a large study from the US [9] and comparable with those of a Canadian study in a mixed urban and rural population [10]. However, the prevalence was markedly higher in Canadian native tribes; in these relatively genetically isolated people, high population frequencies of the shared epitope (SE) correlate with high rates of RA [11].

Current or past smoking was more common among ACPA-positive compared to ACPA-negative FDRs. This is consistent with other studies in which ACPA positivity was more frequent among FDRs with a history of smoking [10]. Indeed, smoking is a major known risk factor for RA. It has been suggested that smoking, in the context of HLA-DR SE genes, may trigger citrullination in the lungs and thereby provide a substrate for immune activation [12].

The median age among IgM-RF-positive FDRs was significantly higher as compared to the IgM-RF-negative subjects. This is consistent with previously reported studies showing that the presence of IgM-RF increases with age [13].

The percentage of subjects who reported joint swelling was significantly higher in the group who were positive for both ACPA and IgM-RF. Of note, there were no signs or symptoms of arthritis at the time of enrollment in the study and no confirmed previous diagnosis of arthritis by a physician. However, the possibility that previous synovial swelling was present in some of the subjects cannot be excluded.

In conclusion, we investigated ACPA and IgM-RF positivity in a large cohort of 577 unaffected FDRs of RA patients and found that 1.9% were positive for ACPA and 6.6% were positive for IgM-RF. These results are important with regard to research aimed at the identification of individuals at risk of developing RA.

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SUPPLEMENTARY INFORMATION

Do you suffer from pain in the joints?

❑ yes

❑ no

Do you have complaints of swelling of the joints?

❑ yes

❑ no

Do you experience stiffness of the joints in the morning?

❑ yes

❑ no

How often do you have one of these symptoms?

❑ daily

❑ weekly

❑ rarely

Which joints are mainly involved?

……

Supplementary figure 1. Questionnaire regarding self-reported joint symptoms. Symptoms suggestive of arthritis included arthralgia (not further defined), the presence of morning stiffness and joint swelling. The questionnaire was taken under the guidance of a trained physician.

4

SERUM VASPIN LEVELS ARE ASSOCIATED WITH THE DEVELOPMENT OF CLINICALLY MANIFEST ARTHRITIS IN AUTOANTIBODY-POSITIVE INDIVIDUALS

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ABSTRACT

Objectives

We have previously shown that overweight may increase the risk of developing rheumatoid arthritis (RA) in autoantibody-positive individuals. Adipose tissue could contribute to the development of RA by production of various bioactive peptides. Therefore, we examined levels of adipokines in serum and synovial tissue of subjects at risk of RA.

Methods

Fifty-one individuals positive for immunoglobulin M rheumatoid factor (IgM-RF) and/or anti-citrullinated protein antibodies (ACPA), without arthritis, were included in this prospective study. Levels of adiponectin, vaspin, resistin, leptin, chemerin and omentin were determined in baseline fasting serum samples (n=27). Synovial tissue was obtained by arthroscopy at baseline and we examined the expression of adiponectin, resistin and visfatin by immunohistochemistry.

Results

The development of clinically manifest arthritis after follow-up was associated with baseline serum vaspin levels (HR1.5 (95% CI 1.1 to 2.2); p=0.020), also after adjustment for overweight (HR1.7 (95% CI 1.1 to 2.5); p=0.016). This association was not seen for other adipokines. Various serum adipokine levels correlated with BMI (adiponectin r=-0.538, leptin r=0.664; chemerin r=0.529) and systemic markers of inflammation such as CRP levels at baseline (adiponectin r=-0.449, omentin r=-0.557, leptin r=0.635, chemerin r=0.619, resistin r=0.520) and ESR (leptin r=0.512, chemerin r=0.708), p-value<0.05. Synovial expression of adiponectin, resistin and visfatin was not associated with development of clinically manifest arthritis.

Conclusions

In this exploratory study, serum adipokines were associated with an increased inflammatory state in autoantibody-positive individuals at risk of developing RA. Furthermore, serum vaspin levels may assist in predicting the development of arthritis in these individuals.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by synovial inflammation in multiple joints leading to joint damage and disability. The etiology of RA, though not completely understood yet, is considered multifactorial and genetic factors as well as various environmental and life style risk factors are considered to be involved. During recent years the incidence of RA has increased [1, 2]. The cause of this increase is not known, but it appears likely that environmental or life style factors account for this increase in a relatively short period of time. As the prevalence of obesity has increased dramatically, obesity may be an important life style risk factor in the development of RA [3]. However, the reporting of the potential influence of obesity on the development of RA has shown inconsistencies in cross-sectional studies [4-6]. We found in a prospective study in autoantibody-positive subjects at risk of developing RA that after a median of 27 months follow up the overall arthritis risk was increased from 28% to 60% in individuals with a smoking history combined with overweight [7]. In contrast, the risk of developing arthritis in never smokers with normal weight was only 2%. The identification of obesity as a risk factor for the development of RA was supported by a larger prospective study [8].

Obesity is associated with a chronic inflammatory state. The most abundant cell type in adipose tissue is the adipocyte, but it also contains endothelial cells, fibroblasts, leucocytes and macrophages, which may highly infiltrate the adipose tissue in case of obesity. Adipocytes are known to secrete several bioactive peptides called adipo(cyto) kines [9]. These peptides include, amongst others, adiponectin, leptin, resistin, vaspin and visfatin. It is important to note that these peptides are not exclusively derived from adipose tissue, but may also be produced by for example macrophages at other sites. Furthermore, many other cytokines, such as tumour-necrosis factor (TNF), interleukin 1 (IL-1), IL-6 and monocyte chemotactic protein 1 (MCP-1) can be produced by the adipose tissue.

Serum levels of adipokines are higher in RA patients compared to healthy controls and non-RA controls and are related to disease activity [10-13]. Also in the synovial fluid and synovial tissue of RA patients adipokines are increased compared to non-RA controls [13-15]. Interestingly, adipose tissue obtained from the joint of RA patients can produce both pro- and anti-inflammatory cytokines as well as adipokines. Factors secreted by the RA articular adipose tissue can also stimulate fibroblast like synoviocytes (FLS) to produce pro-inflammatory cytokines [16]. Taken together, these observations suggest that adipokines produced by adipose tissue may play a role in the disease process in RA.

We hypothesized that adipokines may have a role in the development of RA during the preclinical stage of the disease. In this exploratory study, we examined serum levels and synovial expression of adipokines in autoantibody-positive individuals at risk of

developing RA and evaluated their association with the subsequent development of RA.

PATIENTS AND METHODS

Study subjects

Between June 2005 and September 2012 we included 51 individuals who were positive for immunoglobulin M rheumatoid factor (IgM-RF) and/or anti-citrullinated protein antibody (ACPA) and had either arthralgia and/or a positive family history for RA, but who did not present with arthritis (as determined by an experienced rheumatologist) [7]. Furthermore, they can be classified as having phase c (systemic autoimmunity associated with RA) with or without phase a (genetic risk factors for RA) and phase d (symptoms without clinical arthritis) in the development up to RA according to the recommendations of the EULAR Study Group for Risk Factors for RA [17]. Individuals were excluded if they had a history of arthritis, or if they had used disease-modifying antirheumatic drug (DMARD) therapy or corticosteroids for inflammatory joint complaints. In the current study, only individuals were included of whom fasting serum samples and/or synovial tissue was available. The study was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam (AMC) and performed according to the Declaration of Helsinki. All patients gave written informed consent.

Study design

At baseline, demographics were collected and the following clinical and laboratory parameters were obtained: 68 tender joint count (TJC-68); 66 swollen joint count (SJC-66); patient's visual analogue scale (VAS) for pain (scale 0-100mm); body mass index (BMI) (overweight was defined as a BMI $\geq$25kg/m^2 according to the World Health Association (fact sheet n°311)); IgM-RF levels using IgM-RF ELISA (Sanquin, Amsterdam, the Netherlands (upper limit of normal (ULN) 12.5 IU/ml)) until December 2009 and thereafter using IgM-RF ELISA (Hycor Biomedical, Indianapolis, IN (ULN 49 IU/ml)); ACPA using anti-citrullinated cyclic peptide (CCP)2 ELISA CCPlus (Eurodiagnostica, Nijmegen, the Netherlands (ULN 25 kAU/l)); erythrocyte sedimentation rate (ESR (mm/hr)); serum levels of C-reactive protein (CRP (mg/L)). Yearly study visits were performed and for individuals who were suspected of having developed arthritis an additional visit was performed at which the presence of arthritis (clinically manifest joint swelling) was independently assessed by two investigators (MH and DG or IC and DG).

Serum adipokines

A baseline serum sample after overnight fasting was taken in a subset of the autoantibody-positive individuals (n=27) and stored at -80°C until further use. These samples were used to determine the levels of the following adipokines, using different comercially available ELISA kits; adiponectin (BioVendor, Human Adiponectin ELISA, High Sensitivity Sandwich ELISA (RD191023100)), leptin (R&D Systems, human Leptin Quantikin ELISA (DLP00)), chemerin (BioVendor, human Chemerin Sandwich ELISA (RD191136200R)), resistin (BioVendor, Human Resistin Sandwich ELISA (RD191016100)), omentin (Biovendor, human Omentin-1 Sandwich ELISA (RD191100200R)), and vaspin (AdipoGen, human Vaspin ELISA Kit 1a (AG-45A-0017)). Adipokine levels were measured in ng/mL.

Mini-arthroscopic synovial tissue sampling

At baseline, all study subjects underwent mini-arthroscopic synovial tissue sampling of a knee joint as previously described [18, 19]. At least six specimens were collected for immunohistochemistry as described before to correct for sampling error [20]. The synovial tissue was snap-frozen en bloc in Tissue-Tek OCT (Sakura Finetek Europe B.V., Alphen aan de Rijn, the Netherlands) immediately after collection. Cryostat sections were cut (5 µm each) and mounted on Star Frost adhesive glass slides (Knittelglass, Braunschweig, Germany). Sealed slides were stored at -80°C until further use.

Immunohistochemistry

Synovial tissue sections were stained using the following antibodies: goat polyclonal anti-adiponectin (Acrp30; R&D Systems, Minneapolis, MN), mouse monoclonal anti-resistin (184305; R&D Systems, Minneapolis, MN) and mouse monoclonal anti-visfatin (P4D5AT; Enzo Life Sciences, Farmingdale, NY). Staining of adiponectin and resistin was performed using a two-step immunoperoxidase method. Staining of visfatin was performed using a three-step immunoperoxidase method, as previously described [21]. As a negative control, isotype–matched immunoglobulins were applied to the sections instead of the primary antibody.

The expression of synovial adipokines was analyzed by semi-quantitative analysis (SQA) by two independent observers (KM and AD). The expression of adipokines in synovial tissue was scored on a 5-point scale (range 0-4), as previously described [22]. A score of 0 represented minimal expression, while a score of 4 represented high expression. Minor differences between observers were resolved by mutual agreement.

The tissue sections were also stained for CD68 to detect macrophages, CD3 to detect T cells, and CD55 to detect FLS and analyzed by SQA, as described before [23].

Statistical analysis

Categorical data were depicted as number (%) and continuous variables as median (interquartile range, IQR). To compare baseline characteristics between the individuals who did develop arthritis after follow-up and those who did not, Chi-square test or Mann-Whitney U test was used as appropriate. Bivariate correlations were analyzed using Spearman's rank correlation test. Cox's proportional hazard regression analysis was used to evaluate the association of serum adipokines (assessed as continuous variable) and synovial tissue adipokines (assessed as categorical variable 0-4) with arthritis development. Follow-up duration was defined as the time between inclusion in the cohort and the onset of clinically manifest arthritis, or between inclusion and April 2014 (censored). We performed both univariate analyses, and analyses adjusted for overweight.

All statistical analyses were performed using SPSS v19.0 software (IBM Corp., Armonk, NY). A P-value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics are depicted in Table 1.

Of the 51 included individuals, 18 (35%) individuals were solely IgM-RF positive, 22 (43%) were solely ACPA-positive and 11 (22%) were positive for both autoantibodies. Twelve of the 51 (24%) individuals developed arthritis after a median follow up time of 22 (IQR 12-36) months. Of these 12 individuals who developed arthritis, 8 patients fulfilled the 2010 American College of Rheumatology and the European League Against Rheumatism (ACR/EULAR) criteria for RA at arthritis onset [24], 3 patients were initially classified as having unclassified arthritis but later on fulfilled the RA classification criteria, and 1 patient fulfilled the ACR classification criteria for osteoarthritis (OA) of the hand, but not for RA [25]. The median follow up time of the 39 (76%) individuals who did not develop arthritis was 26 (IQR 17-46) months (*classified as autoantibody-positive individuals at risk of developing RA).*

In this cohort of autoantibody-positive individuals overweight was borderline significantly associated with development of arthritis (p=0.083).

Serum adiponectin, leptin and chemerin levels correlate with body mass index

Fasting baseline serum samples were available from 27 autoantibody-positive individuals (n=9 developed arthritis, n=18 did not develop arthritis). The median (IQR) concentrations of the different adipokines, expressed in ng/mL, were as follows:

Table 1. Baseline demographic and clinical characteristics for individuals who did not develop arthritis after follow-up and individuals who did

Characteristics	No arthritis N= 39	Arthritis N=12	**P-value**
Sex, female (n (%))	27 (69)	8 (67)	0.868
Age, years (median (IQR))	48 (35-53)	48 (42-56)	0.386
CRP, mg/L (median (IQR))	2.0 (1.0-5.7)	3.4 (1.3-11.0)	0.208
ESR, mm/hr (median (IQR))	9 (2-15)	7 (5-14)	0.678
Patient VAS pain, mm (median (IQR))	28 (4-66)	52 (10-78)	0.298
TJC-68 (median (IQR))	2 (0-6)	2 (0-8)	0.856
Arthralgia present (n (%))	36 (92)	11 (92)	0.943
SJC-66 (median (IQR))	0	0	1.000
IgM-RF + (n (%))	21 (54)	8 (67)	0.433
ACPA + (n (%))	24 (62)	9 (75)	0.393
IgM-RF and ACPA + (n (%))	6 (15)	5 (42)	0.053
Smoking history, ever (n (%))	23 (59)	11 (92)	0.036
BMI (median (IQR))	24.6 (22.8-28.6)	27.6 (24.9-29.7)	0.053
BMI ≥25 kg/m^2 (n (%))	18 (46)	9 (75)	0.083

Parameters are described as number (n (%)) or median (interquartile range, IQR) as appropiate. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; VAS= visual analogue scale; TJC= tender joint count; SJC= swollen joint count; IgM-RF = immunoglobulin M rheumatoid factor; ACPA = anti-citrullinated protein antibody; Smoking history = never smoked: 0 pack-years; ever smoked: >0 pack-years; BMI = body mass index

P-values < 0.05 in bold.

adiponectin 12792.0 (9399.8-17695.0), leptin 13538.0 (8932.9-25025.5), chemerin 152.7 (131.9-183.1), resistin 4.6 (3.9-6.1), omentin 720.0 (605.0-994.0) and vaspin 0.9 (0.4-1.6).

Serum adiponectin levels negatively correlated with BMI (r=-0.538; p=0.004). Serum levels of leptin (r=0.664; p<0.001) and chemerin (r=0.529; p=0.005) positively correlated with BMI. Serum levels of resistin (r=0.189; p=0.346), omentin (r=-0.305; p=0.122) and vaspin (r=0.082; p=0.689) did not significantly correlate with BMI (Figure 1).

Serum vaspin levels at baseline are associated with the development of arthritis after follow up

Serum vaspin levels were associated with the development of clinically manifest arthritis after follow up (HR1.5 (95% CI 1.1 to 2.2); p=0.020), even after adjustment for overweight (HR1.7 (95% CI 1.1 to 2.5); p=0.016) (Table 2). We did not observe an association between serum levels of adiponectin, leptin, chemerin, resistin, or omentin and the subsequent development of clinically manifest arthritis (see Table 2).

Table 2. Cox's proportional hazard regression analysis for the association between serum adipokine levels and arthritis development

Variables in model Univariable	**Hazard ratio (95% confidence interval)**	**P-value**
Vaspin	1.5 (1.1 to 2.2)	0.020
Adiponectin	1.0 (1.0 to 1.0)	0.352
Leptin	1.0 (1.0 to 1.0)	0.142
Chemerin	1.0 (1.0 to 1.0)	0.281
Resistin	1.2 (0.9 to 1.6)	0.155
Omentin	1.0 (1.0 to 1.0)	0.353
Variables in model Multivariable	**Hazard ratio (95% confidence interval)**	**P-value**
Vaspin	1.7 (1.1-2.5)	0.016
BMI (≥25 vs ≤25 kg/m^2)	2.3 (0.5-10.7)	*0.296*

BMI = body mass index

Univariate analysis for the association between serum adipokines and arthritis development.

Multivariate analysis for the association between serum vaspin and arthritis development, adjusted for overweight.

P-values < 0.05 in bold.

Hazard ratio per unit increase in adipokine level, or for BMI ≥25 vs BMI ≤25 kg/m^2

Serum adipokine levels correlate with systemic markers of inflammation

Serum adiponectin (r=-0.449; p=0.019) and omentin (r=-0.557; p=0.003) levels negatively correlated with CRP levels while serum leptin (r=0.635; p=<0.001), chemerin (r=0.619; p=0.001), and resistin (r=0.520; p=0.005) levels positively correlated with CRP levels. Serum levels of vaspin (r=0.317; p=0.115) did not correlate significantly with CRP levels (see supplementary data, figure 1).

Serum levels of leptin (r=0.512; p=0.006) and chemerin (r=0.708; p<0.001) correlated positively with ESR. Serum adiponectin (r=-0.141; p=0.482), resistin (r=-0.047; p=0.818), omentin (r=-0.079; p=0.694) and vaspin (r=0.063; p=0.761) levels did not correlate significantly with ESR (see supplementary data, figure 2).

Synovial expression of adipokines is not associated with the development of arthritis after follow up

The synovial tissue of a range of 29 to 39 individuals could be included in the analyses for expression of the various markers by immunohistochemistry. This selection of individuals was based on the presence of sufficient quality of the tissue sections according to the strict quality control system based on the presence of an intimal lining layer.

Expression of adiponectin, resistin and visfatin in the synovium was not only observed

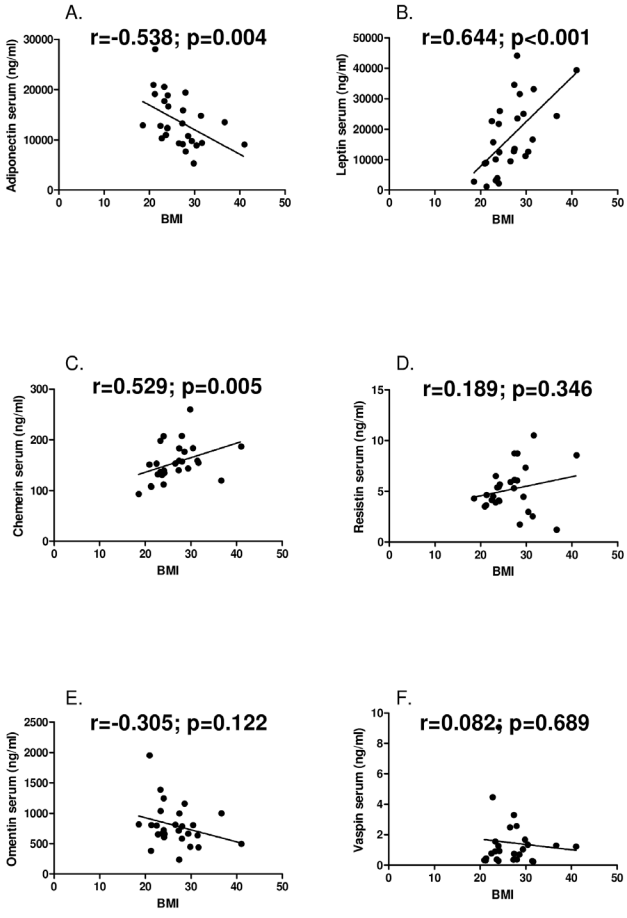

Figure 1. Correlations of serum levels of adipokines and body mass index.

A, adiponectin **B,** leptin **C,** chemerin **D,** resistin **E,** omentin **F,** vaspin.

in individuals who developed arthritis after follow-up, but also in those who did not. Expression of adiponectin was observed predominantly in the synovial sublining vasculature as well as in the surrounding sublining layers and to a much lesser extent in the intimal lining. Resistin expression was more observed in the synovial sublining layers compared to the intimal lining. Visfatin was expressed both in the intimal lining and the sublining layers, including some expression in the sublining vasculature (Figure 2). Synovial expression of adiponectin (df=4; p=0.567), resistin (df=4; p=0.924) and visfatin (df=4; p=0.706) was not associated with the development of clinically manifest arthritis.

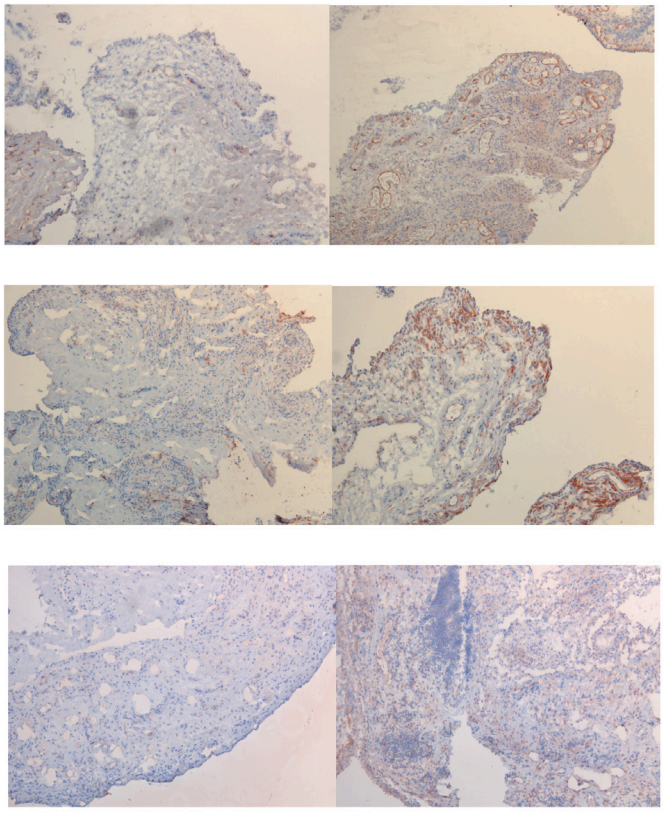

Figure 2. Baseline synovial expression of adipokines in autoantibody-positive individuals.

In **A**, representative immunohistochemical staining of low expression of adiponectin (left) and of high expression of adiponectin (right). In **B**, representative immunohistochemical staining of low expression of resistin (left) and of high expression of resistin (right). In **C**, representative immunohistochemical staining of low expression of visfatin (left) and of high expression of visfatin (right). Magnification 100x.

Association between serum and synovial levels of adipokines

Only for adiponectin and resistin were levels determined in serum as well as in synovial tissue. Serum levels of adipokines were not significantly related to synovial expression of these adipokines, consistent with the notion that the synovial tissue from clinically non-inflamed joints are not a major source of adipokines.

Association between synovial adipokines and synovial markers of inflammation

There was not clear cut relationship between adipokine expression in the synovium and synovial markers of inflammation (data not shown). Of note, there was no question of arthritis in these joints, and there were only few inflammatory cells in the synovial tissue.

Association between serum adipokines and synovial markers of inflammation

Previous work has suggested a positive relationship between synovial inflammatory cells and serum levels of adipokines in OA patients [26]. In our cohort of autoantibody-positive individuals at risk for RA we found a statistically significant relation between synovial inflammatory CD3 cells and serum leptin levels (p=0.008), but otherwise we did not find a significant relationship between the presence of inflammatory cells in the synovium and serum levels of adipokines (data not shown).

DISCUSSION

Overweight appears to be a risk factor for the development of RA [7, 8] and is characterized by an increased volume of adipose tissue able to produce adipokines. This is the first study investigating serum levels and synovial expression of adipokines in autoantibody-positive individuals at risk of developing RA. In this exploratory study, we found a statistically significant association between serum levels of vaspin and the development of arthritis after follow up, also after adjustment for overweight. For the other serum adipokines examined we did not observe a relationship with arthritis development. Also, serum adipokine levels were correlated with BMI and systemic markers of inflammation, such as CRP and ESR. There was no clear cut relationship between adipokine serum levels and features of the synovium during the preclinical stage of the disease, supporting the view that in this phase of the disease joints are not a major source of adipokines.

The finding of vaspin levels in serum being associated with subsequent arthritis development may suggest a role for vaspin in the development of arthritis in these individuals at risk of RA. Apart from the fact that overweight appears to be a risk factor for RA, and vaspin is produced by adipose tissue, it is not immediately clear what the role of vaspin could be in the disease process. Various adipokines and cytokines produced by adipose tissue may play different and even opposing roles. It has been suggested that vaspin may have anti-inflammatory effects in the context of obesity-associated inflammation and cardiovascular disease [27-29]. Thus, vaspin could in fact play a role in the (failed) mechanisms aimed at resolution of inflammation [30]. Only very few studies investigated the role of vaspin in RA, describing elevated vaspin levels in serum and synovial fluid of RA patients when compared to healthy controls and OA

patients, respectively [10, 31], but its role remains to be elucidated [32-34]. Consistent with our data, previous work showed no correlation between serum levels of vaspin on the one hand and levels of adipo(cyto)kines, acute-phase reactants and disease activity indices on the other hand in RA patients [10].

No association between serum adiponectin, leptin, chemerin, resistin, and omentin levels and the development of arthritis could be found, despite the fact that these adipokines are known to have anti- or pro- inflammatory effects in RA. Adiponectin, for instance, is the most abundant adipokine secreted from adipose tissue and exists in various isoforms with counteracting functions [32, 34]. Adiponectin can reduce TNF-induced monocyte adhesion as well as the expression of adhesion molecules and can therefore function as anti-inflammatory adipocytokine [35]. In RA, adiponectin expression has been found at higher levels in synovial fluid and synovial tissue compared to OA patients [14, 15]. Furthermore, serum adiponectin concentrations are higher in RA patients compared to healthy controls [12].

Another well studied adipokine is leptin and it is known to have various functions, including metabolic and immunoregulatory functions [9, 36-38]. In RA, serum levels of leptin were shown to be elevated compared to healthy controls [39].

The pro-inflammatory effects of resistin and chemerin have also been described [40, 41]. Resistin has been detected in RA synovial fluid at higher levels compared to non-inflammatory control patients [14] and expression of resistin and chemerin were higher in synovial tissue of RA patients compared to OA patients [13, 41].

Overall, the complex picture of several adipokines and their isoforms with different and even opposing effects could explain the fact that we did not observe a relationship between serum levels of the other adipokines examined (adiponectin, leptin, chemerin, resistin, and omentin) and subsequent development of arthritis. Adipokine levels are elevated in the synovial compartment of established RA patients, which suggests local production in the inflamed joint. We did not observe a relationship between synovial expression of adipokines and the subsequent development of arthritis. This can be explained by the fact that the joints in these individuals are not a major source of adipokines. The synovial tissue shows only minimal inflammatory cell infiltration during this stage of the disease [23].

We did find a positive correlation between serum levels of leptin, chemerin and resistin on the one hand and systemic markers for inflammation, such as ESR and CRP, on the other, which is in agreement with other reports in RA and metabolic syndrome I diseases [42-44]. Furthermore, leptin and chemerin also correlated positively with BMI, in line with other studies [45, 46]. In contrast, adiponectin and omentin levels correlated negatively with CRP and/or BMI in our study, which is consistent with the anti-inflammatory effects that

have been described for adiponectin and with previous studies in obese and/or diabetic patients [47, 48]. Collectively, these data support the notion that overweight can lead to the secretion of both pro- and anti-inflammatory adipokines, collectively attributing to an altered inflammatory state.

There were some limitations of this study. The first limitation is the relatively small sample size; of 51 individuals fasting serum samples and/or synovial tissue was available, and only 12 (24%) individuals developed arthritis after follow up. Therefore, extensive multivariable analyses could not be performed. However, the results observed in our study provide the rationale for larger studies to build on these initial findings. The second limitation is that this study identified overweight using BMI, which is based on body weight regardless of its composition. Waist circumference and waist-to-hip ratio may reflect body fat more accurately.[49] Yet, these measures were not determined in our study.

In conclusion, this is the first, exploratory study in which serum adipokines have been associated with an increased inflammatory state and with overweight in autoantibody-positive individuals at risk of developing RA. In these individuals serum vaspin levels were associated with subsequent arthritis development, suggesting a role for vaspin in the development of arthritis in these individuals at risk of RA.

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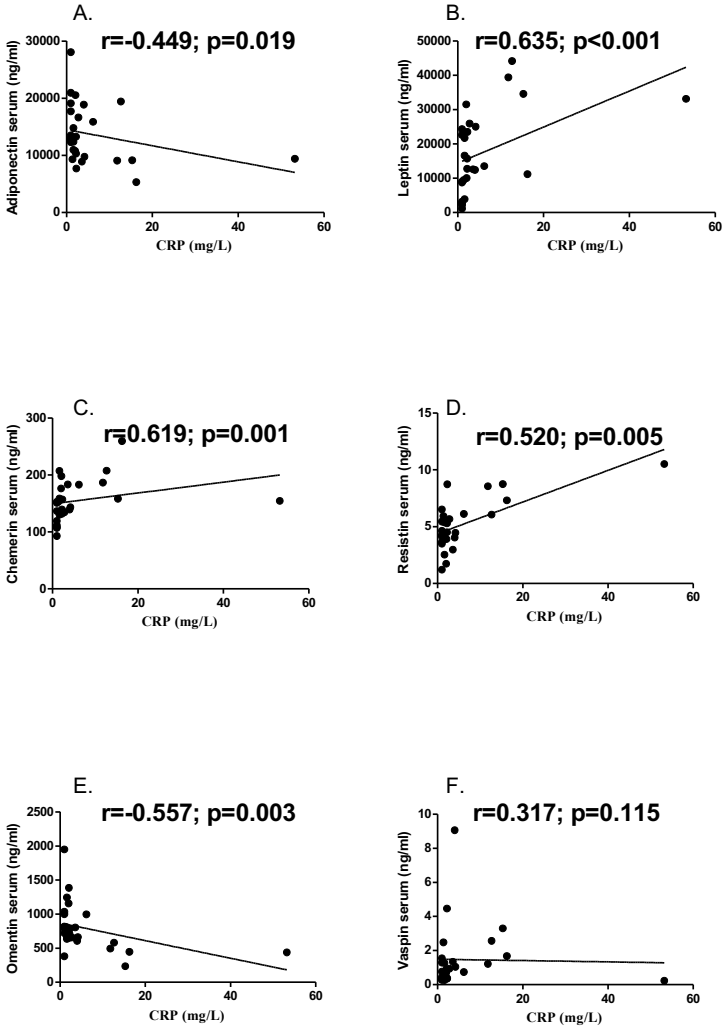
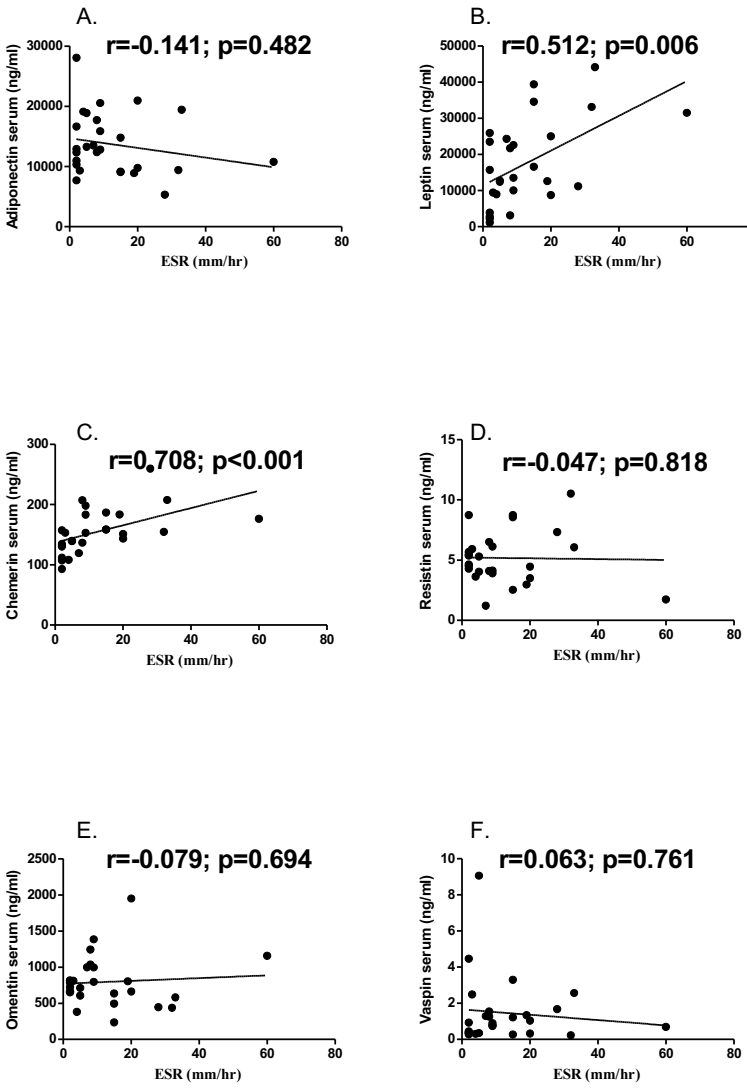
SUPPLEMENTARY INFORMATION



Supplementary figure 1. Correlations of serum levels of adipokines and C-reactive protein (CRP).

A, adiponectin **B,** leptin **C,** chemerin **D,** resistin **E,** omentin **F,** vaspin.



Supplementary figure 2. Correlations of serum levels of adipokines and erythrocyte sedimentation rate (ESR).

A, adiponectin **B,** leptin **C,** chemerin **D,** resistin **E,** omentin **F,** vaspin.

5

NUCLEAR FACTOR-κB-INDUCING KINASE IS EXPRESSED IN SYNOVIAL ENDOTHELIAL CELLS IN PATIENTS WITH EARLY ARTHRITIS AND CORRELATES WITH MARKERS OF INFLAMMATION: A PROSPECTIVE COHORT STUDY

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ABSTRACT

Objectives

The nuclear factor-κB (NF-κB) family of transcription factors is strongly involved in synovial inflammation. We have previously shown that NF-κB-inducing kinase (NIK) is a key regulator of inflammation-induced angiogenesis in rheumatoid arthritis (RA) synovial tissue (ST). Here, we investigated synovial NIK expression in patients with early arthritis and in autoantibody-positive individuals at risk of developing RA.

Methods

ST biopsies were obtained by arthroscopy from 154 patients with early arthritis (duration <1 year) with various diagnoses and 54 IgM rheumatoid factor-positive and/or anticitrullinated protein antibodies-positive individuals without evidence of arthritis. ST was stained for NIK and endothelial cell (EC) markers. Additionally, measures of disease activity were collected and contrast-enhanced magnetic resonance imaging (MRI) was performed in a subset of these patients.

Results

In patients with early arthritis, NIK was predominantly expressed in EC of small blood vessels. Further, NIK expression correlated with erythrocyte sedimentation rate ($r=0.184$, $p=0.024$), C-reactive protein ($r=0.194$, $p=0.017$), joint swelling ($r=0.297$, $p<0.001$), synovial immune cell markers (lining $r=0.585$, $p<0.001$; sublining macrophages $r=0.728$, $p<0.001$; T cells $r=0.733$, $p<0.001$; and B cells $r=0.264$, $p=0.040$), MRI effusion ($r=0.665$, $p<0.001$), MRI synovitis ($r=0.632$, $p<0.001$), and MRI total score ($r=0.569$, $p<0.001$). In 18.5% of autoantibody-positive individuals ST NIK^+EC were present, but this was not predictive of the development of arthritis.

Conclusions

NIK^+EC are present in the earliest phase of synovial inflammation and may be indicative of high angiogenic activity in the inflamed ST. Therefore, NIK^+EC may play an important role in the persistence of synovitis. Collectively, our data underscore the importance of angiogenesis in synovial inflammation and identify NIK as a potential therapeutic target in arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial inflammation that may lead to joint destruction. In RA synovial tissue (ST), angiogenesis can already be observed in the earliest phase of disease. Angiogenesis is defined as the formation of new blood vessels from the preexisting vasculature [1]. The number of blood vessels is already significantly increased in patients with early disease, and the vasculature is clearly activated as shown by an increased expression of adhesion molecules [2,3]. Also, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) in patients with early arthritis demonstrates increased vascularity and suggests that angiogenesis plays a key role in the pathogenesis of RA [4]. Interestingly, anti-tumor necrosis factor (TNF) therapy and other antirheumatic therapies result in the deactivation of vascular endothelium, providing indirect experimental evidence that targeting vascular endothelium may lead to decreased cell trafficking toward the synovial compartment [5,6]. This notion is supported by experimental studies in animal models: angiogenesis is involved in the switch from acute to chronic synovial inflammation and specific targeting of neovasculature results in reduced synovial inflammation [7,8]. Angiogenesis is considered an important factor in the pathogenesis of RA and may be a good target [1,9].

The nuclear factor-κB (NF-κB) family of transcription factors is crucially important in the development and perpetuation of (synovial) inflammation [10,11]. NF-κB can be activated by two signal transduction pathways that have distinct roles [12]. The canonical NF-κB pathway is activated in response to proinflammatory stimuli. In this pathway, inhibitor of κB kinase (IKK) β is essential for NF-κB activation, whereas IKKα is dispensable. In contrast, the noncanonical pathway is strictly dependent on NF-κB-inducing kinase (NIK) and IKKα homodimers, and can be activated through the triggering of TNF-receptor superfamily members, such as the lymphotoxin ß receptor (LTßR), B-cell activating factor belonging to the TNF family (BAFF)-receptor, and CD40. Activation of the noncanonical pathway results in stabilization of NIK, the most important activating kinase of this pathway that results in the activation of IKKα, followed by nuclear translocation of mainly p52-RelB dimers that target specific genes. In RA ST, the noncanonical NF-κB stimuli LTβ and LIGHT (homologous to Lymphotoxins, exhibits inducible expression, and competes with HSV Glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T-lymphocytes), both ligands of the LTβR and CD40L, are widely expressed, mainly by B cells and T cells [13-15]. We have recently demonstrated that noncanonical NF-κB signaling in endothelial cells (EC) regulates pathological angiogenesis in (pre)clinical models of arthritis, independent of vascular endothelial growth factor (VEGF) [16].

Currently, little is known about the contribution of noncanonical NF-κB signaling to the

onset and perpetuation of RA. The function of NIK and downstream noncanonical NF-κB signaling is to a large extent cell-type-specific: in synovial fibroblasts, osteoclasts, EC, and B cells, noncanonical NF-κB signaling contributes to the inflammatory process, whereas in macrophages, dendritic cells and T cells, this pathway probably has a regulatory role [17]. Detailed knowledge of the role of noncanonical NF-κB signaling in angiogenesis and its contribution to chronic inflammation may provide more insight into the pathogenesis of RA and could lead to the development of novel-targeted therapeutic interventions. Therefore, we investigated synovial NIK$^+$EC in the earliest phases of RA compared to other forms of arthritis in a prospective cohort of disease-modifying antirheumatic drug (DMARD)-naïve patients with early arthritis, as well as in a cohort of autoantibody-positive individuals at risk of developing RA.

MATERIALS AND METHODS

Study subjects

First, 154 patients with early arthritis who were included in the prospective early arthritis cohort 'Synoviomics' at the Academic Medical Center (AMC)/University of Amsterdam, the Netherlands, were enrolled [18]. At inclusion, all patients had < 1 year of disease duration, as measured from the first clinical evidence of joint swelling. All patients had an active arthritis of at least a wrist, ankle or knee joint and were DMARD-naive. These patients are collectively referred to as "patients with early arthritis". The second group consisted of 54 individuals from the prospective observational cohort 'PreSynoviomics' at the AMC. This cohort included individuals with either arthralgia and/or a positive family history for RA, but without (a history of) arthritis (as determined by an experienced rheumatologist), who were positive for IgM rheumatoid factor (IgM-RF) and/or anticitrullinated protein antibodies (ACPA). These individuals can be classified as phase c (systemic autoimmunity associated with RA) with/without phase a (genetic risk factors for RA) and/or phase d (symptoms without clinical arthritis) according to the European League Against Rheumatism (EULAR) Study Group for Risk Factors for Rheumatoid Arthritis [19], and are collectively referred to as "autoantibody-positive individuals". Both studies were approved by the Medical Ethics Committee of the AMC and performed according to the Declaration of Helsinki. All patients gave written informed consent.

Study design

At baseline, demographics were collected and the following clinical and laboratory variables were obtained: patient's visual analogue scale ((VAS) range 0-100 mm) for

pain in the biopsied joint; Disease Activity Score in 28 joints (DAS(28)); the severity of swelling of the biopsied joint as assessed by the investigator (range of 0 (no swelling) to 3 (severe swelling)) [19]; IgM-RF levels using IgM-RF ELISA (Sanquin, Amsterdam, the Netherlands (upper limit of normal (ULN) 12.5 IU/ml)) until December 2009 and thereafter using IgM-RF ELISA (Hycor Biomedical, Indianapolis, IN (ULN 49 IU/ml)); ACPA using anti-citrullinated cyclic peptide (CCP)2 ELISA CCPlus (Eurodiagnostica, Nijmegen, the Netherlands (ULN 25 kAU/l)); erythrocyte sedimentation rate (ESR); and serum levels of C-reactive protein (CRP). Radiographs were taken of hands and feet.

For the patients with early arthritis, a diagnosis was made at baseline, and after followup, patients were reclassified based on the 2-year clinical diagnosis (cumulative) founded on fulfillment of standard classification criteria for established rheumatic diseases or the absence of these criteria (unclassified arthritis/UA, phase e according to the EULAR Study Group for Risk Factors for RA) [19]. Further, patients with UA at baseline were categorised as 'UA-RA' if they converted to RA during this followup, or as 'UA-UA' if their arthritis remained unclassified. All patients diagnosed with RA at baseline were categorized as 'RA-RA'.

Finally, after 2 years of followup, patients with early arthritis fulfilling the 2010 American College of Rheumatology (ACR)/EULAR criteria for RA were classified for arthritis outcome: *self-limiting disease*, defined as no arthritis on examination and no use of DMARD or steroids in the preceding 3 months; *persistent nonerosive disease*, defined as presence of arthritis in at least 1 joint and/or requirement of DMARD or steroids in the preceding 3 months; or *erosive disease*, defined as presence of joint erosions on radiographs of the hands and/or feet [20].

In the autoantibody-positive individuals, yearly study visits were performed, and for individuals who developed arthritis, an additional visit was performed at which the presence of arthritis was independently assessed by 2 investigators.

Synovial tissue biopsy sampling, stainings for immunohistochemistry and immunofluorescence, and quantification

At baseline, all study subjects underwent arthroscopic ST biopsy sampling as described [21-23]. In patients with early arthritis, ST biopsy sampling was performed in inflamed wrist, ankle, knee or other joints (metacarpophalangeal or metatarsophalangeal). Autoantibody-positive individuals underwent ST biopsy sampling from a knee joint [23]. ST of all patients was stained using an anti-NIK monoclonal mouse antibody (sc-8417, Santa Cruz Biotechnology, Santa Cruz, CA) and/or a monoclonal anti-von Willebrand factor antibody (vWF; F8/86; DAKO) as described [24].

In a random subset of the patients with early arthritis (based on availability), sections

were stained for CD68 to detect macrophages (n=51), CD3 to detect T cells (n=51), and CD22 to detect B cells (n=61), and analyzed by semiquantitative analysis, as described [24]. In 10 randomly selected patients with early arthritis from this subset, we performed double-immunofluorescence stainings on NIK (sc-8417, Santa Cruz Biotechnology) with vWF (0082, DAKO). See supplementary methods for staining protocols and quantification.

Magnetic Resonance Imaging

At baseline, prior to the arthroscopy, a contrast-enhanced MRI (CE-MRI) of the knee was performed in a subset of both patients with early arthritis (n=36) and autoantibody-positive individuals (n=40) [4]. Subsets were selected based on presentation with a knee arthritis (for patients with early arthritis only), the absence of contraindications for CE-MRI, such as claustrophobia, metal implants, or elevated serum creatinine, and was only performed during a specific timespan in these studies. Images were acquired on a 1.5 T MRI scanner (GE Signa Horizon Echospeed, LX9.0, General Electric Medical Systems, Milwaukee, WI), as described [4]. The MRI was scored for effusion in 4 compartments (lateral, medial, central, and suprapatellar), a Baker's cyst was scored as an extra compartment. Synovitis was scored in 4 compartments. For effusion and synovitis, a score of 0 (normal) to 3 (large volume) for each compartment was given. Edema, cartilage defects, and erosions were scored as being present (1) or absent (0) in 6 joint compartments (lateral/medial femoral surface, lateral/medial tibial plateau, patellar surface and trochlea). A total MRI score was calculated (0–45). Scoring was done by two musculoskeletal radiologists (CvdL and MM), who were blinded to the patients' diagnoses.

Statistical analysis

Categorical data were depicted as number (%) and differences between study groups were analyzed using chi-square test. Variables not normally distributed were depicted as median (interquartile range, IQR). To compare baseline characteristics and NIK expression between the different classification groups, the Kruskal-Wallis test was used when more than 2 groups were compared: subsequently the Mann-Whitney U test was used to compare differences between 2 subgroups. Bivariate correlations of not normally distributed variables were analyzed using Spearman rank correlation test. All statistical analyses were performed using SPSS v19.0 software (IBM Corp., Armonk, NY). A p value of <0.05 was considered statistically significant.

RESULTS

Patients with early arthritis

Baseline characteristics of the patients with early arthritis are shown in Table 1.

Table 1. Baseline characteristics of patients with early arthritis

	Patients with Early Arthritis
Characteristic	N = 154
Sex, female (n (%))	90 (58)
Age, years (mean (SD))	49 (14)
IgM-RF-positive (n (%))*	39 (25)
Anti-CCP-positive (n (%)) **	35 (23)
IgM-RF and anti-CCP both positive (n (%))	28 (18)
ESR, mm/hr (median (IQR))	25 (11-43)
CRP, mg/L (median (IQR))	9.3 (3.0-28.3)
VAS pain biopsied joint, mm (median (IQR))	59 (27-81)
Swelling biopsied joint, score 0-3 (median (IQR))	2 (1-2)
DAS28 (median (IQR))	4.4 (3.2-5.4)
Disease duration months (median (IQR))	3 (1-8)

Values are described as number (n (%)), mean (standard deviation) or median (interquartile range) as appropiate. IgM-RF = immunoglobulin M rheumatoid factor; anti-CCP = anti-cyclic citrullinated peptide; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analoque scale (range 0-100 mm); DAS28 = disease activity score at 28 joints

* missing for 2 patients ** missing for 3 patients

Of the 154 included patients with early arthritis, 64 were classified as having RA at baseline, 61 as UA, 11 as crystal arthropathy (CA), 4 as inflammatory osteoarthritis (OA), and 14 as spondyloarthritis (SpA). Overall median (IQR) disease duration at baseline was 3 (1-8) months. Of the 61 patients who were initially classified as UA, 18 fulfilled the 2010 ACR/EULAR criteria after 2 years of followup (UA-RA), 31 remained unclassified after followup (UA-UA), and 12 were lost to followup and therefore excluded from the diagnostic outcome analysis. Of the 82 patients fulfilling the RA criteria after 2 years of followup, 12 had *self-limiting disease*, 38 had *persistent nonerosive disease*, and 11 had *erosive disease.* For 21 patients the arthritis outcome data were not available and therefore those patients were excluded from these analyses.

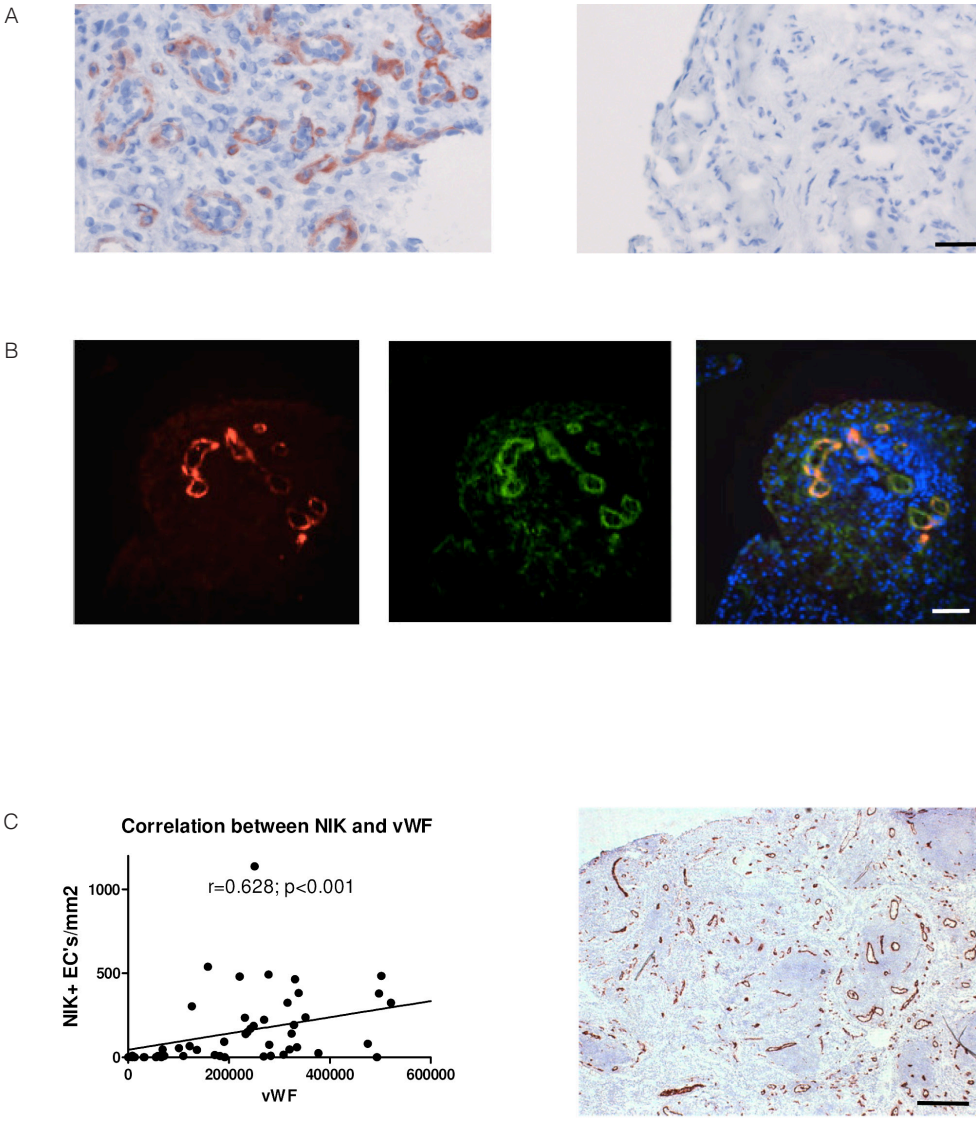

Figure 1. Baseline synovial NIK expression in patients with early arthritis.

A, representative immunohistochemical staining of a NIK-positive patient with early arthritis (left) and of a NIK-negative patient with early arthritis (right). Magnification 400x, scale bar 50µm. **B,** immunofluorescence staining of NIK (red) with vWF (green) and nuclei (blue) in a NIK-positive patient with early arthritis. Magnification 250x, scale bar 100µm. **C**, the correlation between NIK expression and vWF (n=52) (left) as analyzed using Spearman rank correlation test and a representative immunohistochemical staining of a vWF-positive patient with early arthritis (right), scale bar 200µm.

NIK: nuclear factor-κB-inducing kinase, EC: endothelial cell, vWF: anti- von Willebrand factor antibody

NIK is expressed in synovial blood vessel endothelial cells in patients with early arthritis

In this exploratory study, we found that NIK was almost exclusively expressed in EC of synovial blood vessels of patients with early arthritis (Figure 1A, B). This is substantiated by the significant positive correlation between NIK and vWF in these patients (r=0.628; p<0.001) (Figure 1C). This is in line with previous results, showing that about 70% of EC in established RA ST were NIK-positive [16]. NIK was expressed in all different diagnostic groups (RA, UA, CA, OA, and SpA) and no significant difference was observed in the number of NIK^+EC between these different diagnoses at baseline (Figure 2A). When patients were re-classified based on the 2-year clinical diagnosis, the number of NIK^+EC at baseline was significantly higher in UA patients compared to RA patients (p=0.003) (Figure 2B). In patients with UA that were re-classified as RA after 2 years of followup, the number of NIK^+EC at baseline was in the same range as patients with RA who fulfilled the 2010 ACR/EULAR criteria for RA already at baseline (Figure 2C).

Interestingly, NIK was significantly differentially expressed in the various biopsied joints (p<0.001). NIK expression (median (IQR)) was highest in the knee joint (154.9 (24.6-444.9); n=100), lowest in the wrist joint (0.0 (0.0-18.7); n=19) and intermediate in the ankle joint (4.7 (0.0-56.0); n=33) and in the other joints (32.4 (30.2-34.5); n=2) (Supplementary table 1).

Baseline expression of NIK is not related to arthritis outcome

We next investigated synovial NIK expression in relationship to arthritis outcome (*self-limiting, persistent nonerosive* or *erosive disease*) in patients fulfilling the 2010 ACR/EULAR criteria for RA after 2 years of followup. However, NIK expression (median (IQR)) did not differ among the different arthritis outcome groups (*self-limiting*: 14.7 (1.0-69.9), *persistent nonerosive disease*: 11.6 (0.0-203.9), and *erosive disease*: 11.9 (0.0-59.3); p=0.850).

NIK expression correlates with markers of disease activity in patients with early arthritis

NIK expression correlated positively with systemic markers of disease activity, such as ESR (r=0.184; p=0.024) and CRP (r=0.194; p=0.017), and with local assessment of swelling of the biopsied joint (r=0.297; p<0.001) (Figure 3A), but not with the VAS pain for the biopsied joint and DAS28 (data not shown).

NIK expression also correlated positively with cellular markers of inflammation in the ST; NIK correlated highly significantly with $CD68^+$ lining and sublining macrophages (r=0.585; p<0.001, and r=0.728; p<0.001, respectively), $CD3^+$ T cells (r=0.733; p<0.001), and B cells (r=0.264; p=0.040).

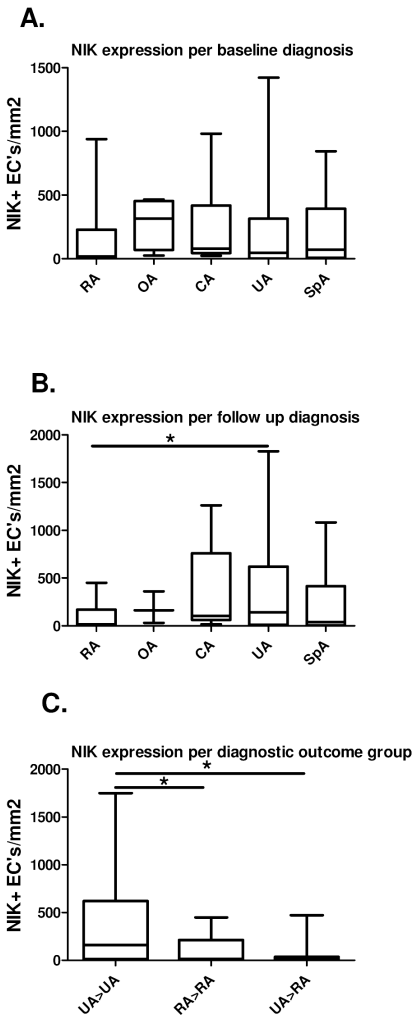

Figure 2. Synovial tissue NIK expression in relation to different classification groups.

A, NIK ST expression of patients with early arthritis diagnosed at baseline with rheumatoid arthritis (RA; n=64), inflammatory osteoarthritis (OA; n=4), crystal arthropathy (CA; n=11), unclassified arthritis (UA; n=61), and spondyloarthritis (SpA; n=14). **B,** NIK ST expression after reclassifying patients based on the 2-year clinical diagnosis with RA (n=82), OA (n=4), CA (n=11), UA (n=31) and SpA (n=14). **C,** Patients with UA at baseline were categorized as 'UA-RA' (n=18) if they converted to RA during followup, or as 'UA-UA' (n=31) if their arthritis remained unclassified. Patients with RA at baseline were categorized as 'RA-RA' (n=64). The Kruskal-Wallis test was used and data are presented as box plots (25th to 75th percentiles), lines within the box mark the median value, lines outside the boxes denote the 10th and 90th percentiles. Lines connecting datasets indicate statistically significant differences between groups. Twelve patients with UA were lost to follow-up after 2 years. *p<0.01.

ST: synovial tissue, NIK: nuclear factor-κB-inducing kinase, EC: endothelial cell

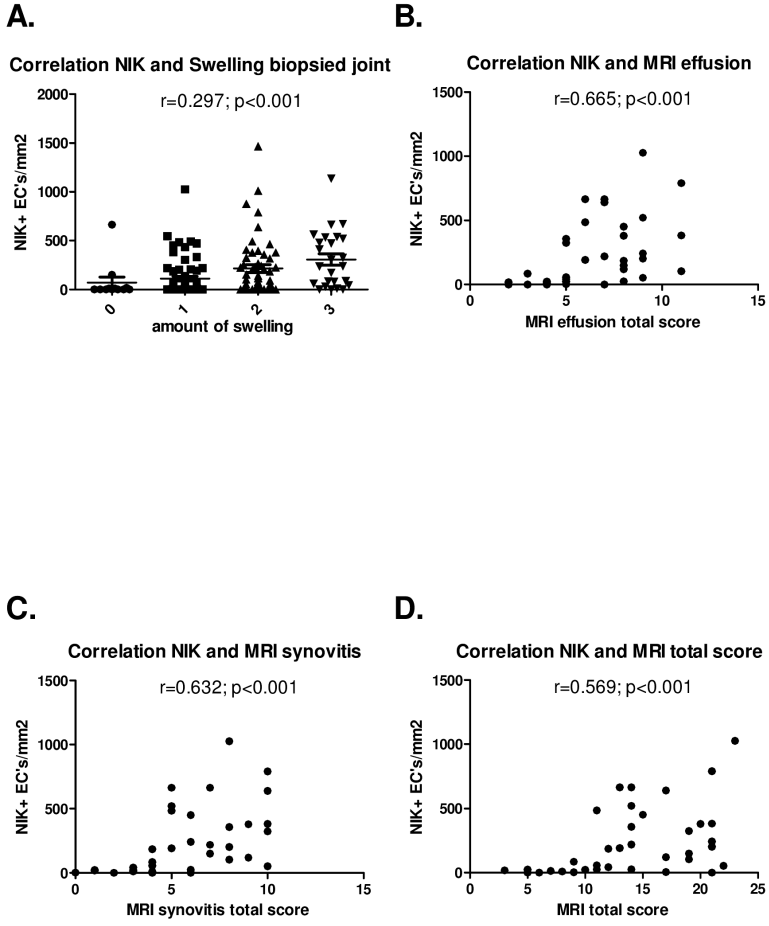

Figure 3. Correlations between baseline synovial NIK expression and local markers for disease activity in patients with early arthritis.

A, comparison of NIK expression and the amount of swelling of the biopsied joint as assessed by the doctor (score of 0 (no swelling) – 3 (severe swelling))(n=145). The MRI (n=36) was scored for effusion in 5 compartments, for synovitis in 4 compartments and for edema, cartilage degeneration, and erosions in 6 compartments. A total MRI score was calculated (0-45). **B**, the correlation between NIK expression and MRI effusion. **C**, the correlation between NIK expression and MRI synovitis. **D**, the correlation between NIK expression and total MRI score. Spearman rank correlation test was used to analyze all correlations.

NIK: nuclear factor-κB-inducing kinase, EC: endothelial cell, MRI: magnetic resonance imaging

NIK expression also correlated significantly with MRI effusion (r=0.665; p<0.001), MRI synovitis (r=0.632; p<0.001), and MRI total score (r=0.569; p<0.001) in a subset of the patients with early arthritis (n=36) (Figure 3B-D). NIK expression did not significantly correlate with MRI edema (r=-0.012; p=0.946), MRI cartilage damage (r=-0.032; p=0.855), or MRI erosion scores (r=-0.026; p=0.882).

Subjects at risk of developing RA

Baseline characteristics of the 54 autoantibody-positive individuals are depicted in Table 2.

Twenty individuals were solely IgM-RF-positive, 22 were solely ACPA-positive, and 12

Table 2. Baseline characteristics of autoantibody-positive individuals

Characteristic	Autoantibody-positive individuals		p Value
	No arthritis developed N= 41	Arthritis developed N=13	
Sex, female (n (%))	29 (71)	8 (62)	0.538
Arthralgia (n (%))	30 (91)*	12 (92)	0.880
Age, years (median (IQR))	48 (35-53)	48 (43-55)	0.341
IgM RF-positive (n (%))	23 (56)	9 (69)	0.401
Anti-CCP-positive (n (%))	24 (59)	10 (77)	0.232
IgM-RF and anti-CCP both positive (n (%))	6 (15)	6 (46)	0.017
ESR, mm/hr (median (IQR))	9 (2-17)	7 (5-12)	0.660
CRP, mg/L (median (IQR))	2.0 (1.0-5.9)	2.8 (1.4-9.4)	0.474
VAS pain biopsied joint, mm (median (IQR))	9 (0-39)**	4 (0-28)	0.507
Follow-up time months (median (IQR))	42 (18-59)	18 (6-40)	0.030

Parameters are described as number (n (%)) or median (interquartile range) as appropiate. IgM-RF = immunoglobulin M rheumatoid factor; anti-CCP = anti-cyclic citrullinated peptide; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analoque scale (range 0-100 mm). * missing for 8 individuals ** missing for 4 individuals

were positive for both autoantibodies. Thirteen of the 54 individuals (24%) developed arthritis during followup after a median duration of 18 (IQR 6-40) months. Of the 13 patients who developed arthritis, 9 fulfilled the 2010 ACR/EULAR criteria for RA [25] at arthritis onset and 3 were initially diagnosed as having UA but fulfilled the RA classification criteria later on. One patient fulfilled the ACR classification criteria for OA of the hand, but not for RA [26].

The median follow-up time of the 41 (76%) individuals who did not develop an arthritis was 42 (IQR 18-59) months. In the individuals who developed arthritis after followup, significantly more individuals were both IgM-RF- and ACPA-positive.

NIK is expressed in synovial EC in certain autoantibody-positive individuals, but this is not predictive of the development of arthritis

In autoantibody-positive individuals, NIK expression was very low in general and NIK$^+$EC were only present in 10 out of the 54 autoantibody-positive individuals (Figure 4 and Supplementary table 2).

Synovial NIK expression in the 13 patients who developed arthritis was in the same range as in the autoantibody-positive individuals who did not develop arthritis during

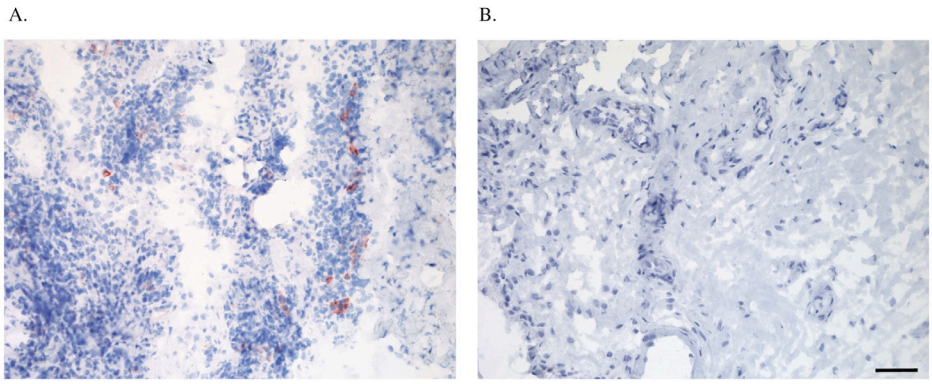

Figure 4. Baseline synovial NIK expression in auto-antibody positive individuals.

A, representative immunohistochemical staining of a NIK-positive individual and **B**, of a NIK-negative individual. Magnification 400x, scale bar 50μm. See Supplementary data table 2 for quantification of these data.

NIK: nuclear factor-κB-inducing kinase

followup (Supplementary table 2). Therefore, the presence of NIK$^+$EC is not predictive of the development of arthritis in autoantibody-positive individuals.

NIK expression is not associated with markers of inflammation in autoantibody-positive individuals

The autoantibody-positive individuals had normal inflammatory variables (median (IQR)), such as ESR (8 (2-15) and CRP (2.1 (1.0-7.5). Synovial NIK expression was not associated with the systemic markers of inflammation (ESR (p=0.544) and CRP (p=0.227)), or with the VAS pain of the biopsied joint (p=0.526). Previously, we demonstrated that the ST in these autoantibody-positive individuals contains very low numbers of immune cells, comparable with healthy controls [27]. In line with this, we did not observe a correlation between NIK$^+$EC and CD68$^+$ macrophages, CD3$^+$ T cells, or B cells in these tissues (data not shown). Further, the presence of NIK$^+$EC also did not correlate with the MRI data (MRI effusion (p=0.881), MRI synovitis (p=0.475), MRI cartilage damage (p=0.293), MRI edema (p=0.415) and MRI erosion scores (p=0.804)) in autoantibody-positive individuals (data not shown).

DISCUSSION

We demonstrated that NIK is highly expressed in synovial blood vessels of patients with various forms of early arthritis. Additionally, we showed that synovial NIK expression is associated with systemic and local markers of disease activity in patients with early arthritis. Interestingly, NIK$^+$EC could also be found in some individuals at risk of developing RA. We demonstrate that NIK is expressed in synovial blood vessels already in the earliest phases of inflammatory joint disease.

NIK was not differentially expressed between the different diagnoses at baseline. Nevertheless, reclassification of patients based on the 2-year clinical diagnosis showed that ST from patients with UA contained significantly more NIK$^+$EC at baseline compared with the other types of arthritis. Although this could be an intrinsic feature of UA, another perhaps more likely explanation for this is that the mean swelling of the biopsied joint was also higher in this group (data not shown). Also, the variation in NIK expression was high, which makes it unsuitable in clinical practice as a prognostic marker for individual patients with UA. Therefore, we do not advocate the routine use of synovial biopsy to establish the final diagnosis in patients with UA, except in specific cases to rule out infectious causes, crystal-induced arthritis, or neoplasms, or in early drug-development studies [28].

We also observed differential NIK expression in the various joint types. Although this could be a specific characteristic of the individual joints, a more likely explanation is again the increased mean swelling and cellular inflammation scores in the joints that contained the highest NIK expression irrespective of the joint type (Supplementary table 1). Nevertheless, we cannot completely rule out that joint-specific features, such as positional gene expression patterns, can affect synovial NIK expression or predispose joints to development of arthritis [29].

The presence of NIK$^+$EC may be indicative of high angiogenic activity in the inflamed ST, which is in line with previous work from our group and others demonstrating that NIK is a key regulator of pathological angiogenesis and is requisite for pathology in animal models of arthritis [16,30]. In light of the importance of angiogenesis in the progression from acute to chronic inflammation, and the fact that NIK also regulates endothelial expression of CXCL12, an important chemokine in the attraction of immune cells, NIK$^+$EC may contribute to the persistence of synovial inflammation [7,31].

Of interest, we also showed that NIK$^+$EC are present in ST in 18.5% of autoantibody-positive individuals. However, NIK expression was very low compared to the expression in patients with early arthritis and did not correlate with cellular or other local and systemic markers for (subclinical) disease activity. Also, the presence of NIK$^+$EC at

baseline did not predict development of arthritis in this relatively small cohort. Previously, we have demonstrated that features of the synovium are similar between autoantibody-positive individuals and healthy controls, all showing very low scores for phenotypic cellular markers, adhesion molecules and vascularity [27]. This may explain the lack of an association between synovial NIK$^+$EC and (subclinical) inflammation or the development of arthritis. Nonetheless, NIK$^+$EC are sometimes present before the onset of clinical arthritis, and therefore targeting these cells in the earliest phases of the disease may be beneficial.

With respect to arthritis outcome (*self-limiting, persistent nonerosive,* or *erosive disease*), we did not find a correlation with synovial NIK expression. This may seem surprising given the important role of NIK and the downstream NF-κB subunit RelB in osteoclast biology and the bone-destructive components of inflammatory arthritis [30,32]. In a human study, genetic association analysis showed that single nucleotide polymorphisms in *MAP3K14,* the gene encoding for NIK, affect bone mineral density and bone turnover [33]. However, we did not study the synovial tissue-bone interphase (pannus), but regular ST biopsies in which NIK was predominantly expressed in EC. Therefore, we cannot exclude the possibility that any potential expression of NIK in osteoclasts in pannus tissue may be predictive of development of erosive disease, but this was outside the scope of the present study.

Our study had some limitations; there was a limited number of patients with UA who progressed to RA (n=18), and multiple subsets were used for various analysis who was largely based on the availability of ST sections. Nevertheless, we provide clear evidence that NIK$^+$ EC correlate positively with markers of local inflammation, such as ST B and T cells and MRI scores. Given the important role of angiogenesis in the perpetuation of arthritis, targeting this process has been proven to be beneficial in (pre-)clinical disease models [34,35]. Importantly, targeting angiogenesis will probably not result in the severe immune suppression that is induced by treatment with biologics that target cytokines or specific immune cells, and may cause infectious complications. We have recently established that NIK is only involved in pathological angiogenesis in a preclinical model of arthritis and not in normal developmental angiogenesis [16]. Therefore, selective targeting of NIK in EC may have several advantages over targeting other well-known proangiogenic pathways, such as VEGF, which also blocks physiological angiogenesis and can have adverse effects suchn as hypertension and thrombo-embolic events [36].

Our combined data identify NIK as a potential therapeutic target in arthritis [16]. To target NIK selectively in EC, a multi-modular recombinant protein that specifically binds to cytokine-activated endothelium under inflammatory conditions, including arthritis, could be used [37]. Because we demonstrate that NIK$^+$EC can already be observed in the earliest phase of the disease, targeting NIK in patients with early arthritis may block

pathological angiogenesis and prevent the switch from acute to chronic inflammation. This could be done via local intraarticular treatment (e.g., gene therapy) or by using small molecule inhibitors [38]. The crystal structure of the catalytic domain NIK has been identified [39,40], which may facilitate the development of new potent NIK inhibitors. This may lead to new treatment strategies that could be beneficial in RA, and in other types of arthritis and other inflammatory diseases.

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SUPPLEMENTARY METHODS

Synovial tissue biopsy sampling, stainings for immunohistochemistry and immunofluorescence, and quantification

At baseline, all study subjects underwent arthroscopic synovial tissue (ST) biopsy sampling as described [1-3]. In patients with early arthritis ST biopsy sampling was performed in inflamed wrist, ankle, knee, or other (metacarpophalangeal or metatarsophalangeal) joints. Autoantibody-positive individuals underwent ST biopsy sampling from a knee joint[3]. No major complications of the arthroscopy were reported. At least six specimens were collected for immunohistochemistry, as described before [4], to correct for sampling error. The ST biopsy samples were snap-frozen en bloc in Tissue-Tek OCT (Sakura Finetek Europe B.V., Alphen aan de Rijn, the Netherlands) immediately after collection. Cryostat sections were cut (5 µm each) and mounted on Star Frost adhesive glass slides (Knittelglass, Braunschweig, Germany). Sealed slides were stored at -80 °C until further use.

ST sections were stained in two sessions for the patients with early arthritis and in one session for the autoantibody-positive individuals. The sections were fixed with acetone, and endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide and 0.1% sodium azide in phosphate-buffered saline (PBS) for 20 minutes. Slides were incubated overnight at 4°C with primary antibody diluted in 1% bovine serum albumin/PBS. The primary antibody used in this study was monoclonal mouse antibody specific for NIK (sc-8417, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed with PBS and incubated with goat anti-mouse antibodies (p0447, DAKO, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP. Biotinylated tyramide was used for amplification, as previously described [5], and development with the AEC peroxidase substrate kit (SK-4200, Vector Laboratories, Burlingame, CA). In a subset of the patients with early arthritis (n=52), depending on the availability of the tissue, ST sections were stained in one session using a monoclonal anti-von Willebrand factor (vWF; F8/86; DAKO) antibody for blood vessels [6]. A three-step immunoperoxidase protocol was used to detect specific staining for vWF, as described previously [7]. Slides were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaisers glycerol gelatin (Merck). As a negative control, isotype-matched immunoglobulins were applied to the sections instead of the primary antibody.

ST was only further used for analysis if the quality of the tissue sections were sufficient according to the strict quality control system based on the presence of an intimal lining layer. In the early arthritis cohort the expression of synovial NIK and vWF was quantified by digital image analysis within one week after staining, as previously described

[8]. For each slide 18 representative high power fields (2.2 mm^2) were analyzed. To correct for between-session variation, the factor correction program was used [9]. In the autoantibody-positive individuals the expression of synovial NIK was much lower and therefore analyzed by semi-quantitative analysis (SQA) by two independent observers (KIM and KvZ), as described [10]. Minor differences in assessment between the two observers were resolved by mutual agreement. The expression of synovial NIK was scored as either positive or negative.

In a random subset of the patients with early arthritis, sections were stained for CD68 to detect macrophages (n=51), CD3 to detect T cells (n=51), and CD22 to detect B cells (n=61), and analyzed by SQA, as described before [11].

In 10 randomly selected patients with early arthritis from the previously mentioned subset we performed double-immunofluorecence stainings on NIK and vWF using the same mouse monoclonal anti-NIK antibody (sc-8417, Santa Cruz Biotechnology) and a polyclonal rabbit anti-vWF antibody (0082, DAKO). After incubation with goat anti-mouse-HRP (p0447, DAKO), the slides were incubated with streptavidine-Alexa-594 (S-32356, Molecular Probes Europe, Leiden, the Netherlands) and Alexa-488-conjugated goat anti-rabbit (A-11008, Molecular Probes Europe). The slides were mounted with Vectashield containing DAPI (Brunschwig VC-H-1500, Amsterdam, the Netherlands). As a negative control, sections were incubated with isotype controls. The slides were analyzed using a Leica DMRA fluorescence microscope (Leica, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Dutch Vision Components, Breda, the Netherlands).

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Supplementary table 1. Expression of NIK and cellular markers of inflammation in the ST of the various biopsied joints

Joint	NIK expression median (IQR)	Swelling biopsied joint median (IQR)	CD68 lining median (IQR)	CD68 sublining median (IQR)	CD3+ T cells median (IQR)	CD22+ B cells median (IQR)
Knee (n=100)	154.9 (24.6-444.9)	2 (1-2)	464.1 (174.5-652.2)	1595.5 (526.7-1789.3)	392.9 (188.9-989.8)	305.5 (118.6-812.4)
Ankle (n=33)	4.7 (0.0-56.0)	1 (1-2)	56.8 (12.2-226.7)	126.9 (38.0-396.4)	88.5 (33.7-273.2)	252.9 (25.6-607.9)
Wrist (n=19)	0.0 (0.0-18.7)	1 (1-1)	50.0 (13.7-132.2)	30.1 (8.7-135.0)	41.1 (6.7-109.4)	190.1 (87.0-301.8)
Other (n=2)	32.4 (30.2-32.4)	3 (2-3)	179.0 (108.4-179.0)	432.9 (295.1-432.9)	165.8 (79.0-165.8)	374.8 (34.9-374.8)

Supplementary table 2. Development of arthritis in autoantibody individuals in relation to baseline NIK expression.

	Autoantibody-positive individuals		
Characteristic	No arthritis developed N= 41	Arthritis developed N=13	Total
NIK negative individuals (n(%))	33 (80,5)	11 (84,6)	44 (81,5)
NIK positive individuals (n(%))	8 (19,5)	2 (15,4)	10 (18,5)

Parameters are described as number (n (%)). For the frequencies of NIK negative individuals and NIK positive individuals in the two outcome groups, Pearson χ^2 test was used (P=0,739).

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DYNAMIC CONTRAST-ENHANCED MAGNETIC RESONANCE IMAGING USING PHARMACOKINETIC MODELING: INITIAL EXPERIENCE IN PATIENTS WITH EARLY ARTHRITIS

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ABSTRACT

Objective

Analysis of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) using pharmacokinetic modeling (PKM) provides quantitative measures that mirror microvessel integrity and can be used as an objective marker of the level of synovial inflammation. The aim of this study was to investigate the PKM parameters K^{trans}, k_{ep} and v_e in a prospective cohort of disease-modifying antirheumatic drug (DMARD)-naïve patients with early arthritis, and to validate the results by assessing their correlation with the number of synovial endothelial cells (ECs).

Methods

Forty-seven patients with early arthritis (arthritis duration <1 year, DMARD naïve; comprising 14 patients with rheumatoid arthritis, 22 with unclassified arthritis, 6 with spondyloarthritis (SpA), and 5 with other arthritides) were included. At baseline, DCE-MRI was performed on an inflamed knee joint of each patient. These images were used to calculate the K^{trans} (volume transfer constant between the plasma and extracellular extravascular space [EES]), the k_{ep} (transfer constant between the EES and plasma) and the v_e (fractional volume of the EES). Second, markers of disease activity were collected. Finally, vascularity was evaluated by immunohistochemical analysis of synovial tissue samples obtained from the inflamed knee joints, using antibodies to detect von Willebrand factor (vWF), a marker of ECs.

Results

The 3 PKM parameters differed significantly between diagnostic groups at baseline, with the highest K^{trans} value being observed in patients with SpA (median 0.050/minute, interquartile range (IQR) 0.041-0.069). Furthermore, the K^{trans}, k_{ep} and v_e values correlated significantly with markers of disease activity. Finally, the PKM parameters K^{trans} and k_{ep}, but not v_e, correlated significantly with synovial expression of vWF ($r=0.647$; $p=0.004$ for K^{trans}, $r=0.614$; $p=0.007$ for k_{ep}: $r=0.398$; $p=0.102$ for v_e).

Conclusion

These results suggest that the K^{trans}, k_{ep} and v_e, can be used to detect synovial inflammation in patients with early arthritis, and these PKM parameters may be helpful in differential diagnosis. This approach may also be useful in translational research analyzing tissue microcirculation and angiogenesis.

INTRODUCTION

Inflammatory joint diseases mainly affect the synovium and are characterized by increased tissue perfusion, capillary permeability, and angiogenesis, hypertrophy, and influx of inflammatory cells [1-4]. In the management of inflammatory joint diseases, there is a need for reliable noninvasive methods to closely monitor synovial inflammation, to predict the diagnoses, and, finally, to predict a patient's prognosis with respect to the future development of bone destruction [5]. However, conventional methods, such as clinical examination and radiography, do not allow detailed evaluation of synovial inflammation [6, 7].

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is an MRI technique consisting of the time-dependent registration of changes in the MR signal intensity during and after an intravenous bolus injection of a gadolinium (Gd)-based contrast agent. The resulting time-intensity curve (TIC) can be postprocessed to produce descriptive parameters such as the rate of (early) enhancement and maximum enhancement (ME), or to produce heuristic parameters such as the shape of the TIC-shape [8]. With a quantitative approach, it is also possible to obtain physiology-related parameters by converting the TICs to Concentration-Time Curves, and subsequently fitting these data with pharmacokinetic models (PKMs) such as the Tofts model [9]. In patients with rheumatoid arthritis (RA), descriptive DCE-MRI parameters are correlated with clinical disease activity parameters such as the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level [10-12], as well as with histologic measures of inflammation such as vascularity and perivascular edema [11, 13-16]. Furthermore, these descriptive parameters have been shown to be predictive of the progression of erosive disease [17], and have been found to decrease after successful treatment [18-22]. Therefore, DCE-MRI parameters have been suggested to represent potential objective markers of synovial inflammation.

Analysis of DCE-MR images using descriptive parameters has, however, some significant disadvantages. Parameters such as the ME or slope reflect signal changes that occur when monitoring contrast uptake, but these are dependent on the scanner parameters and field strength. These parameters are therefore unsuitable to compare studies that are based on data acquired with different scanners or with different MRI settings. For this reason, the use of physiology-related parameters that are obtained by means of PKM is preferred. These models should provide absolute measures of the microvessel integrity, such as the K^{trans} (the volume transfer constant between the plasma and the extracellular extravascular space (EES)), the v_e (the fractional volume of the EES), the k_{ep} (the transfer constant between the EES and plasma), and the v_p (the plasma volume) [9]. Besides being used as objective markers of synovial inflammation, pharmacokinetic DCE-MRI

parameters can also be used for research into certain aspects of tissue microcirculation [23, 24] and for assessment of treatment by monitoring microvascular changes [25, 26].

In this study, we investigated the PKM parameters K^{trans}, k_{ep}, and v_e in a prospective cohort of disease-modifying antirheumatic drug (DMARD)-naïve patients with early arthritis. Since the K^{trans} and v_e provide absolute measures of the microvessel integrity, we validated our results by assessing their correlation with synovial expression of the endothelial cell (EC) marker von Willebrand Factor (vWF).

PATIENTS AND METHODS

Study subjects

The study included patients enrolled between March 2004 and April 2009 in a prospective early arthritis cohort, the Synoviomics cohort of the Academic Medical Center (AMC) at the University of Amsterdam (Amsterdam, the Netherlands), for whom DCE-MRI data from an inflamed knee joint were available (n= 54) [27]. All patients had the disease for under 1 year, as measured from the first clinical evidence of joint swelling. At the time of inclusion, all patients had active arthritis in at least 1 knee joint, and all patients were DMARD naïve. The study was approved by the Medical Ethics Committee of the AMC and was performed in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Study design

At baseline, demographic information as well as the following clinical and laboratory parameters were collected. The tender joint count (of 68 joints assessed), the swollen joint count (of 66 joints assessed), and Disease Activity Score in 28 joints (DAS(28)) were obtained. In addition, the amount of swelling of the knee joint was assessed by the operating physician in synovial tissue from the biopsied joint (score range 0-3, were 0 indicates no swelling and 3 indicates severe swelling), IgM rheumatoid factor (IgM-RF) levels were determined using a Sanquin IgM-RF enzyme-linked immunosorbent assay (ELISA) kit (with the upper limit of normal (ULN) defined as 12.5 IU/ml)) until December 2009, and thereafter using a Hycor Biomedical IgM-RF ELISA kit (with the ULN defined as 49 IU/ml)). Anti-citrullinated protein antibodies were measured using an anti-cyclic citrullinated peptide 2 ELISA (with the ULN defined as 25 kAU/liter) (CCPlus; Eurodiagnostica). Finally, the ESR (in mm/hour), CRP level (in mg/liter), and radiographs of the hands and feet were obtained.

A diagnosis was made at baseline in each patient. After followup, patients were re-

classified on the basis of the 2-year clinical diagnosis, determined according to the cumulative fulfillment of standard classification criteria for established rheumatic diseases, and patients who did not fulfill these criteria were designated as having unclassified arthritis (UA) (phase e, according to the European League Against Rheumatism (EULAR) Study Group for Risk Factors for Rheumatoid Arthritis) [28-34]. Furthermore, after 2 years of followup, patients with early arthritis fulfilling the American College of Rheumatology (ACR)/EULAR 2010 criteria for RA were classified according to arthritis outcome, as follows: 1) self-limiting disease, defined as no arthritis on examination and no treatment with DMARDs or steroids in the preceding 3 months; 2) persistent nonerosive disease, defined as the presence of arthritis in at least 1 joint and/or treatment with DMARDs or steroids in the preceding 3 months and no signs of erosions on conventional radiographs; or 3) erosive disease, defined as the presence of joint erosions on radiographs of the hands and/or feet [35].

MRI

At baseline, DCE-MRI of an inflamed knee of each patient was performed. Images were acquired on a 1.5T MRI scanner (Signa Horizon Echospeed, LX9.0; General Electric Medical Systems). Patients were placed in a supine position with the knee joint centered in the magnetic field in a dedicated extremity coil (quadrature detection). The DCE-MRI data were acquired using an axial T1-weighted 3-dimensional Fast Spoiled Gradient Echo (FSPGR) sequence (echo time 4.6 msec, repetition time 8.1msec, flip angle= 30 deg, field of view= 180x180x80 mm, voxel size 1.4 x 1.4 x 4 mm, acquisition time per volume= 21 sec, 20 dynamic scans). Before the DCE-MRI protocol, a series of FSPGR sequences (same parameters as described above, but using only 1 dynamic scan) were run with variable flip angles (θ= 5,10,20, 30 deg) in order to provide data to determine absolute pre-contrast T1 maps.

During the DCE-MRI examination, patients were given a gadopentetate dimeglumine contrast agent (Magnevist®; Bayer) at a dose of 0.1 mM/kg body weight, delivered by means of a pump at a speed of 3-5 mM/second. Delivery of the contrast agent was followed by a chase bolus of 12-20 ml of 0.9% NaCl. Because the knee joint was carefully fixed and padded within the coil, movement during the scan was minimal. Therefore, no image registration was performed on the Dynamic scan.

Pharmacokinetic modeling

Data were analyzed off-line using in-house software (Dynamo) [8]. First, the absolute pre-contrast T1 maps were calculated by fitting the data obtained with variable flip angle FSPGR sequences. The signal intensities were transformed into signal concentrations (in each voxel) using the pre-contrast T1 maps. The Gd concentration-time relationship,

or $C_{gd}(t)$, was then calculated according to the following equation (Equation 1)

$$C_{Gd}(t) = -\frac{1}{TR \cdot r_1}\left\{\ln\left(\frac{SE(t)\cdot\left(e^{-\frac{TR}{T_{10}}}-1\right)+e^{-\frac{TR}{T_{10}}}(1-\cos\vartheta)}{1+\cos\vartheta\left(SE(t)\left(e^{-\frac{TR}{T_{10}}}-1\right)-1\right)}\right)-\frac{TR}{T_{10}}\right\}$$

where r1 represents the tissue relaxivity constant of proportionality between the Gd concentration and increase in relaxation rate (we used an r_1 value of 4.52 s^{-1} mM^{-1} lt), SE(t) represents the signal enhancement at time t, calculated as ($SE(t) = \frac{S(t) - S(t_0)}{S(t_0)}$), with t_0 being the time before contrast enhancement, and T_{10} represents the native T_1 (the value before contrast enhancement) [36]. Thereafter, we applied Tofts model in its extended form, calculated with the following equation (Equation 2)

$$C_t(t) = v_p C_p(t) + K_{trans} \cdot C_p(t) \otimes e^{-\frac{K^{trans}}{v_e}\cdot t} \qquad (9).$$

where $C_t(t)$ is the tissue signal concentration at time t (as calculated with Equation 1), v_p is the fractional volume of the plasma, v_p is the fractional volume of the EES, and $C_p(t)$ is the arterial input function (AIF), i.e. the estimated plasma concentration of contrast agent in the capillaries feeding the tissue.

In order to measure the AIF, we manually selected a region of interest (ROI) in the popliteal artery in multiple sections of the scan. An arterial concentration-time curve, the $C_{blood}(t)$, was obtained using Equation 1, and using a T_{10}Blood value of 1,540 ms [37]. Plasma concentrations in the capillary (C_p) were estimated using the equation

$C_p = C_{blood}/(1\text{-Hct})$, where Hct represents the hematocrit constant (assigned a value of 0.45) [37].

To avoid partial volume effects, only the pixels with the highest enhancement were used to generate the arterial signal. Because of the low time resolution of the dynamic scan, the AIF needed adjustment before being used for the PKM analysis. The AIF was shifted and its peak amplitude corrected according to a previously published method [38].

The resulting plasma concentration-time curve, the $C_p(t)$, was fitted to the following formula (Equation 3)

$$C_p(t) = \beta\left(\chi \cdot t \cdot e^{-mb\cdot t} - e^{-mb\cdot t} + e^{-ma\cdot t}\right)$$

which provided the $C_p(t)$ curve a functional form that would allow Equation 2 to be solved in closed form [39].

The concentration in the tissue (C_{tiss}) then could be calculated with the following equation (Equation 4)

$$C_{tiss}(t) = \beta \left\{ v_p \left(\chi \cdot t \cdot e^{-mb \cdot t} - e^{-mb \cdot t} + e^{-ma \cdot t} \right) + \right.$$
$$\left. + v_e k_{ep} \left[\frac{\chi}{k_{ep} - mb} \left(t \cdot e^{-mb \cdot t} - \frac{\left(e^{-mb \cdot t} - e^{-k_{ep} \cdot t} \right)}{k_{ep} - mb} \right) + \frac{(e^{-ma \cdot t} - e^{-k_{ep} \cdot t})}{k_{ep} - ma} - \frac{\left(e^{-mb \cdot t} - e^{-k_{ep} \cdot t} \right)}{k_{ep} - mb} \right] \right.$$

The descriptors have been described previously in reference 39 (in which Equation 3 and Equation 7 are identical in form to Equation 3 and Equation 4 in the present study [39].

The PKM parameters K^{trans}, v_e, and k_{ep}= K^{trans}*v_e were determined by fitting Equation 4 to the calculated concentration-time curves in the tissue and using the patient-specific AIF, as described above. Nonlinear fitting was done on a voxel-by-voxel basis, using the Levenberg-Marquardt fitting algorithm using Matlab® (The Mathworks™, Natick, MA).

Selection of the ROIs and of the ROI-specific PKM parameters

An ROI excluding enhancing (muscle) tissue was manually drawn by a trained investigator (CvdL) on maps showing the maximum enhancement (ME). This was done in 12 adjacent levels, using the tibial plateau as distal landmark.

Bones were automatically segmented and excluded from the ROIs using the precontrast T1 signal intensity, leaving the synovium within the ROI (Figure 1). Within these 3-dimentional ROIs, the median values for the distribution of the PKM parameters (K^{trans} k_{ep} and v_e) were calculated.

PKM Exclusion criteria

The quality of the K^{trans} maps was not always consistent. As the analysis was done on a pixel-by-pixel basis, and because of the sensitivity of the fitting procedure to noise, the PKM parameters were not calculated on pixels that had a noise in the time-intensity curve that exceeded 30% of the average baseline (precontrast) values. If the amount of pixels excluded was > 30%, the patient was not included in the study. Furthermore, patients were excluded if the T1 map calculated from the variable flip angle method was unreliable, or if it was not possible to extract a good AIF in the field of view.

Collection of synovial biopsy tissue, immunohistochemical staining, and quantification of ECs

At baseline, all study subjects underwent arthroscopic synovial biopsy sampling, as previously described [40, 41]. The synovial tissue (ST) was harvested from the same inflamed knee joint on which the DCE-MRI had been performed at a maximum of 1

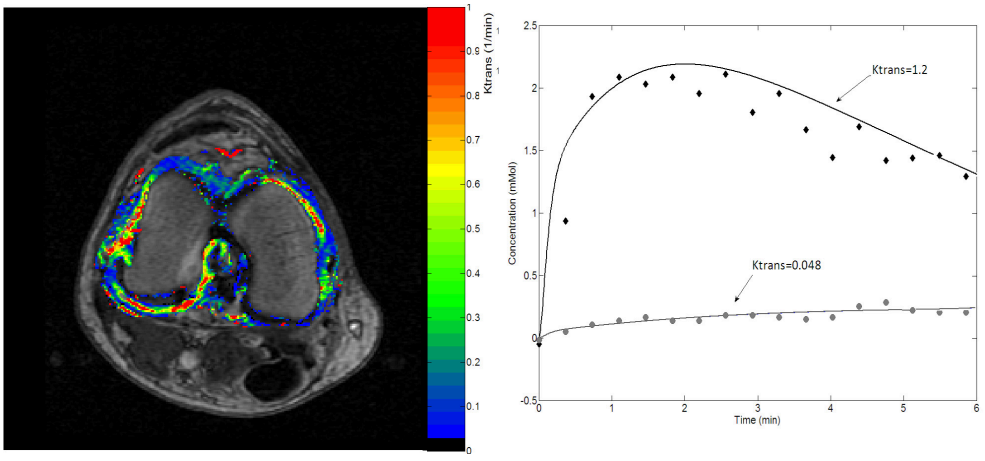

Figure 1. A representative image of the knee joint of a patient with early arthritis, as assessed by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI).

The map shows the K^{trans} values in the segmented region of interest (the synovium), superimposed on a T1-weighted image of the DCE-MRI scan. The concentration-time curves as well as the fitted model are shown for 2 pixels with low and high K^{trans} values

week before the biopsy [42]. To correct for sampling error, at least 6 biopsy specimens were collected for immunohistochemical analysis, as has been described previously [43]. The ST was snap-frozen en bloc in Tissue-Tek OCT medium (Sakura Finetek Europe) immediately after collection. Cryostat sections were cut (5 µm each) and mounted on Star Frost adhesive glass slides (Knittelglass). Sealed slides were stored at -80 until further used.

For detection of ECs, ST sections were stained using monoclonal antibodies to vWF (F8/86; Dako) [44].

Statistical analysis

Categorical data were depicted as the number (%), while non-normally distributed variables were depicted as the median (interquartile range (IQR)) or as the median (range) when 3 or fewer patients were analyzed. In comparing the PKM parameters between the different diagnostic groups at baseline or between the different arthritis outcome groups (self-limiting disease, persistent nonerosive disease, or erosive disease), the Kruskal-Wallis test was used when more than 2 groups were compared. Bivariate correlations of non-normally distributed variables were analyzed using Spearman's rank correlation test. All statistical analyses were performed using SPSS software (version 20.0; IBM). P values less than or equal to 0.05 were considered statistically significant.

RESULTS

Characteristics of the patients

Baseline characteristics of the patients with early arthritis are shown in Table 1. Of the 54 patients , 1 was excluded because the data could not be fitted to the PKM models (met the PKM exclusion criterion), 2 were excluded based on the AIF, and 4 were excluded based on the T1 map. At baseline, of the remaining 47 patients with early arthritis, 14 were classified as having RA, 22 as having UA, 6 as having spondyloarthritis (SpA), and 5 as having other arthritides. The other arthritides group consisted of 2 patients with gout, 2 patients with inflammatory osteoarthritis, and 1 patient with systemic lupus erythematosus. The overall disease duration at baseline was a median of 2 months (IQR 1-6 months). Of the 22 patients who were initially classified as having UA, 4 fulfilled the ACR/EULAR 2010 criteria for RA after 2 years of followup (UA-RA), 16 remained unclassified (UA-UA), and 2 were classified as having SpA after 2 years of followup (UA-SpA).

Of the total of 18 patients who fulfilled the ACR/EULAR 2010 criteria for RA after 2 years of followup, 3 patients had self-limiting disease, 5 patients had persistent nonerosive disease, 3 patients had erosive disease, while for 7 patients, the arthritis outcome data were not available, and these patients were therefore excluded from the arthritis outcome analysis.

DCE-MRI PKM parameters in different diagnostic and arthritis outcome groups

To evaluate potential differences in microvessel integrity between the different baseline diagnoses and between the different arthritis outcome groups (self-limiting disease, persistent nonerosive disease, or erosive disease), we compared the PKM parameters in those groups. We observed a significant difference in the PKM parameters K^{trans} and k_{ep} between the different groups classified according to diagnosis at baseline (K^{trans}: p=0.026, k_{ep}: p=0.024 and v_e: p=0.094) (Figure 2A-C). The K^{trans} value was highest in patients with SpA (median 0.050/minute, IQR 0.041-0.069) and lowest in those with other arthritides (median 0.003/minute, IQR 0.000-0.036). Intermediate K^{trans} values were obtained in the DCE-MR images from patients with UA (median 0.028/minute, IQR 0.015-0.042) and from those with RA (median 0.026/minute, IQR 0.008-0.044) (see supplementary data, table 1).

This analysis was followed by the investigation of PKM parameters in relation to arthritis outcome (self-limiting, persistent nonerosive, or erosive disease) in patients fulfilling the ACR/EULAR 2010 criteria for RA after 2 years of followup. The K^{trans} value was highest in

the persistent nonerosive disease group (median 0.041/minute, IQR 0.024-0.045)

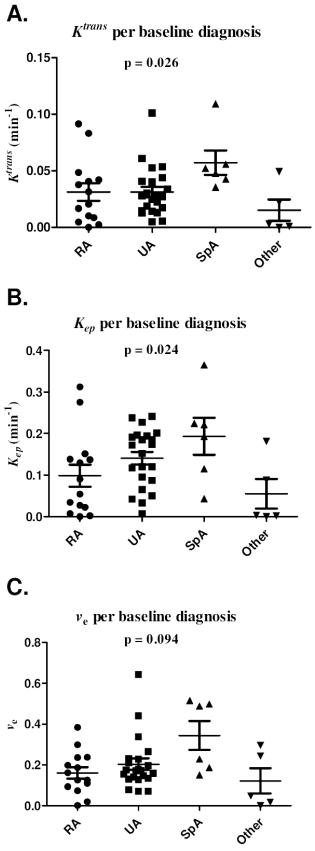

Figure 2. Baseline pharmacokinetic modeling (PKM) parameters from dynamic contrast-enhanced magnetic resonance imaging in relation to different diagnoses in patients with early arthritis.

The PKM parameters K^{trans} (volume transfer constant between the plasma and extracellular extravascular space [EES]) (**A**), k_{ep} (transfer constant between the EES and plasma) (**B**), and v_e (fractional volume of the EES) (**C**) were assessed in patients who were classified as having rheumatoid arthritis (RA), unclassified arthritis (UA), spondyloarthritis (SpA), or other arthritides at baseline. The Kruskal-Wallis test was used for group comparisons. Symbols represent individual patients; bars show the median and interquartile range.

and lowest in the self-limiting disease group (median 0.006/minute, range 0.005-0.022). Intermediate K^{trans} values were obtained in images from patients with erosive disease (median 0.027/minute, range 0.000-0.083) (see supplementary data, table 1). However, none of the PKM parameters showed a statistically significant difference between the different arthritis outcome groups (K^{trans}: p=0.212, k_{ep}: p=0.262 and v_e: p=0.357).

Taken together, these data indicate that the PKM parameters K^{trans}, k_{ep} and v_e may be useful in classifying patients according to diagnosis in the early phase of inflammatory joint diseases.

Table 1. Baseline demographic and clinical characteristics of the study patients with early arthritis (n=47)

	Patients with early arthritis
Characteristic	N = 47
Sex, female (n (%))	23 (49)
Age, years (median (IQR))	47 (36-56)
IgM-RF positive (n (%))	11 (23)
ACPA positive (n (%))	9 (19)
IgM-RF and ACPA positive (n (%))	6 (13)
ESR, mm/hr (median (IQR))	17 (7-36)
CRP, mg/L (median (IQR))	5.6 (1.7-21.3)
Swelling of biopsied joint, score 0-3 (median (IQR))	1 (1-2)
DAS28 (median (IQR))	3.91 (2.80-5.18)
68 TJC (median (IQR))	3 (1-14)
66 SJC (median (IQR))	2 (1-5)

Parameters are described as number (n (%)) or median (interquartile range, IQR) as appropiate. IgM-RF = immunoglobulin M rheumatoid factor; ACPA = anti-citrullinated protein antibody; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score in 28 joints; 68 TJC = tender joint count of 68 joints; 66 SJC = swollen joint count of 66 joints

Correlations of DCE-MRI PKM parameters with local and systemic disease activity markers in patients with early arthritis

To evaluate the PKM parameters as markers of disease activity in inflammatory joint diseases, we correlated them with local and systemic makers of disease activity. We found a statistically significant correlation between the PKM parameters and the severity of swelling of the biopsied joint as assessed by the operating physician (K^{trans}: r=0.505; p<0.001, k_{ep}: r=0.456; p=0.001 and v_e: r=0.500; p<0.001).

Furthermore, all PKM parameters correlated significantly with the CRP levels as a systemic marker of disease activity (K^{trans}: r=0.412; p=0.004, k_{ep}: r=0.295; p=0.046 and v_e: r=0.316; p=0.032). There was no significant correlation of any of the PKM parameters with the DAS28 (K^{trans}: r=0.375; p=0.126, k_{ep}: r=0.377; p=0.123 and v_e: r=0.318; p=0.198), and only the v_e was significantly correlated with the ESR (K^{trans}: r=0.286; p=0.054, k_{ep}: r=0.417; p=0.085 and v_e: r=0.525; p=0.025) (Figure 3A-C).

Correlation of the K^{trans} with synovial tissue expression of vWF

In order to validate the use of PKM parameters reflecting the microvessel integrity as objective markers of synovial inflammation, we studied the correlation of these parameters with the number of synovial blood vessels. Based on the availability of ST of good quality, 18 individuals could be included in this analysis. The PKM parameters K^{trans} and k_{ep} each correlated significantly with synovial expression of vWF (K^{trans}: r=0.647; p=0.004, and k_{ep}: r=0.614; p=0.007) (Figure 4), whereas the parameter v_e was

not significantly correlated with the expression of vWF (v_e: r=0.398; p=0.102). These data demonstrate that the PKM parameters mirror the number of synovial ECs.

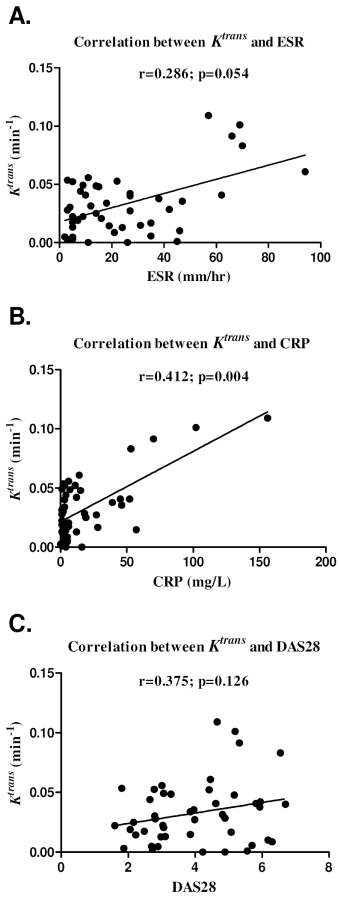

Figure 3. Correlations of the dynamic contrast-enhanced magnetic resonance imaging pharmacokinetic parameter K^{trans} and systemic markers of disease activity at baseline in patients with early arthritis.

Correlations between the K^{trans} and erythrocyte sedimentation rate (ESR) (**A**), K^{trans} and C-reactive protein (CRP) level (**B**), and K^{trans} and Disease Activity Score in 28 joints (DAS28) (**C**) were analyzed using Spearman's rank correlation test.

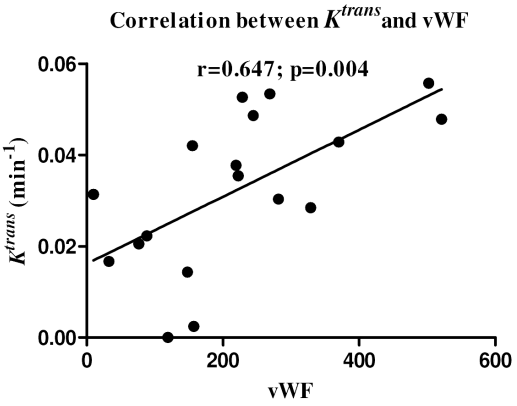

Figure 4. Correlations of the dynamic contrast-enhanced magnetic resonance imaging pharmacokinetic parameter K^{trans} and the expression of the endothelial cell marker von Willebrand factor (vWF) in the synovial tissue of patients with early arthritis.

Correlations were analyzed using Spearman's rank correlation test.

DISCUSSION

This is the first study investigating the value of DCE-MRI PKM parameters (K^{trans}, k_{ep} and v_e) in the Tofts model, each of which represents an absolute measure of microvessel integrity, in a prospective cohort of DMARD-naïve patients with early arthritis. Our results show that K^{trans}, k_{ep} and v_e differ between diagnostic groups and correlate with local and systemic markers of disease activity. Importantly, we validated our results by demonstrating that these parameters are correlated with the expression of the EC marker vWF in synovial biopsy tissue from the same joint. Taken together, these data suggest that PKM parameters have value in differentiating patients with a different inflammatory joint disease diagnosis. In addition, this approach can be used to detect synovial inflammation, and may be helpful in investigating certain aspects of tissue microcirculation and angiogenesis.

Research on the relationship between DCE-MRI PKM parameters and aspects of the disease is relatively new in the arthritis field. Qualitative DCE-MRI analysis has been applied in early arthritis [45], while its value has been proven in distinguishing between malignant and benign musculoskeletal lesions and other tumors [46-51]. Extensive evidence has been presented to support the value of PKM analysis in patients with cancer [52]. Since the inflamed synovium exhibits the same features as those found in malignant tumors, such as increased tissue perfusion, increased capillary permeability, and invasive

growth of the ST into bone and cartilage, we hypothesized that PKM parameters may also be of use in the diagnostic and/or prognostic classification of arthritis patients. Indeed, we found that the K^{trans}, k_{ep} and v_e were significantly different between diagnostic groups, with the highest values in patients with SpA. This is consistent with the findings in earlier studies showing a significantly higher microvessel density in the ST from SpA patients with active inflammation compared to controls or patients with other forms of arthritis, such as RA [53, 54]. The data presented herein support the rationale for future studies to confirm the value of PKM parameters for differential diagnosis.

Since the K^{trans}, k_{ep} and v_e correlated significantly with the severity of swelling of the joint, with the highest values for the severity of swelling observed in patients with SpA (see supplementary data, table 2), the K^{trans}, k_{ep} and v_e may be more representative of local inflammation than of a specific diagnosis. Another potential caveat is the fact that the K^{trans}, k_{ep} and v_e were analyzed using the whole 3-dimensional image of the synovium (and not only in regions of high K^{trans}), resulting in the median values being shifted towards lower values. It is possible that a better depiction of the diagnosis could be represented by averaged values in the most affected area of the synovium. This hypothesis needs further testing.

The K^{trans}, k_{ep} and v_e significantly correlated with local markers of disease activity and with CRP levels. However, these PKM parameters did not correlate with other systemic markers of inflammation, such as the DAS28 and ESR. Perhaps this is not surprising, as we only examined a single knee joint of patients with mostly systemic polyarticular diseases.

Earlier studies showed that the synovial vasculature is related to disease progression [47, 49]. In our study, we did not find a correlation between PKM parameters and arthritis outcome (self-limiting, persistent nonerosive or erosive disease) in patients fulfilling the ACR/EULAR 2010 criteria for RA after 2 years of followup. These results need to be interpreted with caution, as we could only evaluate the outcome in a small number of patients (3 with self-limiting disease, 5 with persistent nonerosive disease, and 3 with erosive disease).

The PKM parameter K^{trans} represents the transfer constant of the contrast agent between the plasma space and the extracellular space, and the value increases in an environment of high capillary permeability (although K^{trans} is not a direct measure of permeability). The results of our study show a clear correlation with the expression of vWF in the ST from the same joint, which is consistent with findings of increased permeability in newly formed vessels due to neoangiogenesis in tumor tissue [55]. It has been suggested that the number of blood vessels is significantly increased in RA patients whose disease is long-standing, and this is correlated not only with higher disease activity and severity, but also with increased inflammatory cell infiltration [1]. Taken together, the findings indicate

that increased angiogenesis is considered an important factor in the pathogenesis of RA [56] and also in other inflammatory joint diseases such as SpA. Based on our results, K^{trans} is a non-invasive marker of synovial angiogenesis in patients with early arthritis.

Despite the advantages of PKM compared to descriptive parameters in DCE-MRI, one significant limitation is that its implementation is far from straightforward. The absolute contrast agent concentrations must be calculated from the MR signal, a process that requires additional scans, and the AIF needs to be either measured or approximated. In both cases, potential errors are introduced. In our study, a low temporal resolution (21 seconds) was used to favor spatial resolution and coverage of the whole knee, and a correction method was therefore needed to estimate the AIF. The calculation of the contrast agent concentration is hampered by the fact that some constants, such as the hematocrit, relaxivity, and blood T1 constants, have not been measured, and therefore values from the literature have been used. All this contributes to the inaccuracy of the PKM parameters [57].

Moreover, noise and inadequate temporal resolution severely limit the robustness of the (nonlinear) fitting procedure: it is thus important to exclude pixels that are not suitable for fitting. A thorough optimization of the protocol is essential to ensure sufficient signal-to-noise ratio. The standardization of the infusion technique is also important. To control for variability as much as possible, we standardized several parameters in our study, such as the diameter of the needle and duration of the infusion. Although routine use of this model in clinical practice may be challenging due to these limitations, we think it is feasible to implement DCE-MRI PKM in clinical practice in specific cases.

Taken together, our results indicate that the DCE-MRI PKM parameters K^{trans}, k_{ep} and v_e are thought to provide absolute measures of microvessel integrity, allowing comparison between patients and between studies, and therefore offering a great advantage over the use of DCE-MRI descriptive parameters. We demonstrated that these PKM parameters correlated with the expression of the EC marker vWF in ST from patients with early arthritis, which can be of use in the context of translational research bridging aspects of tissue microcirculation and angiogenesis with advanced imaging techniques. Our results also suggest that K^{trans}, k_{ep} and v_e can be used as diagnostic biomarkers in early inflammatory joint disease. Finally, these PKM parameters may be used to detect synovial inflammation in patients with early arthritis, which could facilitate the evaluation of joints inaccessible to proper clinical examination or joints with suspected, but not definite, synovitis or the detection of subclinical synovitis in patients at risk of developing RA.

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SUPPLEMENTARY INFORMATION

Supplementary table 1. Dynamic contrast-enhanced magnetic resonance imaging pharmacokinetic parameters in different diagnostic and arthritis outcome groups

A

	RA (n=14)	UA (n=22)	SpA (n=6)	Other (n=5)	p-Value
K^{trans}, min^{-1} (median (IQR))	0.026 (0.008-0.044)	0.028 (0.015-0.042)	0.050 (0.041-0.069)	0.003 (0.000-0.036)	0.026
K_{ep}, min^{-1} (median (IQR))	0.072 (0.018-0.141)	0.162 (0.082-0.195)	0.207 (0.097-0.259)	0.003 (0.001-0.135)	0.024
v_e (no units) (median (IQR))	0.143 (0.088-0.236)	0.172 (0.133-0.230)	0.359 (0.177-0.503)	0.048 (0.009-0.270)	0.094

B

	self-limiting (n=3)	persistent nonerosive (n=5)	erosive disease (n=3)	p-Value
K^{trans}, min^{-1} (median (IQR or range))	0.006 (0.005-0.022)	0.041 (0.024-0.045)	0.027 (0.000-0.083)	0.212
K_{ep}, min^{-1} (median (IQR or range))	0.033 (0.007-0.117)	0.137 (0.044-0.213)	0.120 (0.000-0.138)	0.262
v_e (no units) (median (IQR or range))	0.078 (0.073-0.176)	0.186 (0.136-0.237)	0.232 (0.001-0.384)	0.357

Parameters are described as median (interquartile range) as appropriate.

In **A**, the median (IQR) K^{tran}, $k_{ep,}$ and v_e of patients with early arthritis who were classified as rheumatoid arthritis (RA), unclassified arthritis (UA), spondyloarthritis (SpA), or other arthritides at baseline. In **B,** the median (IQR) K^{tran}, $k_{ep,}$ and v_e per arthritis outcome group (*self-limiting disease*, *persistent nonerosive disease*, or *erosive disease)* in patients fulfilling the ACR/EULAR 2010 criteria for RA after 2 years of followup.

Supplementary table 2. Severity of swelling of the biopsied joint per diagnosis.

Diagnosis	**Severity of swelling biopsied joint (n=47; 0-3 median (IQR))**
RA (median (IQR)) (n=14)	1 (0-1)
UA (median (IQR)) (n=22)	2 (1-2)
SpA (median (IQR)) (n=6)	2 (1-2)
Other (median (IQR)) (n=5)	1 (1-1)

Parameters are described as median (interquartile range) as appropriate.
RA = rheumatoid arthritis, UA = unclassified arthritis, SpA = spondyloarthritis.

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DOES THE MULTI-BIOMARKER DISEASE ACTIVITY SCORE HAVE DIAGNOSTIC VALUE IN EARLY RHEUMATOID ARTHRITIS AND UNCLASSIFIED ARTHRITIS?

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The 2010 American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) updated classification criteria for rheumatoid arthritis (RA) focus on identifying patients at early disease stage[1]. They facilitate early implementation of RA disease-modifying therapy, which has been associated with improved clinical and structural outcomes[2, 3]. Some patients with inflammatory oligoarthritis or polyarthritis initially not meeting RA classification criteria and classified with undifferentiated arthritis (UA) based on clinical and laboratory assessments, might later fulfill those criteria.[4] Diagnosing these patients earlier would enable better therapeutic intervention and suppression of RA disease activity[5-7].

The Multi-biomarker Disease Activity (MBDA) score, calculated from the concentrations of 12 serum biomarkers, is an objective validated disease activity measure for patients with RA. It has been shown to track disease activity in patients with early and established RA, treatment-naïve or not, and to associate with risk of radiographic progression[8]. Here we investigated whether the MBDA score might inform RA diagnosis in patients with UA.

We evaluated 126 patients from the prospective Synoviomics cohort [9]; 81 fulfilled ACR/EULAR 2010 criteria for RA and 45 for UA[1, 4]. At study entry, all patients had ≥1 swollen joint, <1 year of clinical symptoms, and were naïve to disease-modifying anti-rheumatic drugs and corticosteroids. Following baseline clinical assessment and serum collection, patients were treated according to EULAR guidelines. Patients were grouped as UA-UA (n=29), UA-RA (n=16), or RA-RA (n=81) based on their fulfillment of ACR/EULAR 2010 classification criteria for RA at baseline and after 2-years.

Baseline MBDA score, DAS28, joint counts, acute phase protein concentrations, and autoantibody status differed significantly between UA and RA patients ($p<0.005$) (Table 1). Significant correlations ($p<0.001$) were observed between baseline MBDA score and DAS28 ($r=0.62$), erythrocyte sedimentation rate (ESR) ($r=0.67$), and C reactive protein (CRP) ($r=0.84$) in the overall population and separately in the RA ($r=0.57$; 0.59; 0.83) and UA ($r=0.63$; 0.70; 0.82) groups.

To test whether baseline MBDA score or other disease activity measures were associated with fulfilment of RA classification criteria after 2 years, trends across the three groups (RA–RA, UA–RA and UA–UA) were evaluated using Jonckheere-Terpstra test. Statistically significant ($p<0.05$) decreasing trends were observed for all measurements tested (Figure 1). Pairwise comparisons by Wilcoxon's rank-sum test showed that baseline disease activity scores based on 28 joints (DAS28), ESR, CRP, swollen joint count based on 66 joints, tender joint count based on 68 joints (TJC68), and MBDA score were significantly greater in patients with RA–RA versus UA–RA ($p<0.01$). MBDA score was not significantly different between UA–RA versus UA–UA ($p=0.132$). Only baseline TJC68 was significantly greater in UA–RA versus UA–UA (median [IQR]: 5 [1–7.25] versus 1 [1–3], $p=0.010$);

Table 1. Baseline demographic and clinical characteristics by diagnosis group

Variables	Overall (N=126)	RA (N=81)	UA (N=45)	p value
Female, n (%)	88 (70%)	61 (75%)	27 (60%)	0.07
Age (years), median (IQR)	51 (40–58)	51 (39–57)	51 (43–59)	0.457
Disease duration (months), median (IQR)†	4 (2–7.5)	4 (2–8)	3 (2–6)	0.28
IgM-RF positive, n (%)	56 (44%)	49 (60%)	7 (16%)	<0.001
Anti-CCP positive, n (%)	51 (40%)	49 (60%)	2 (4%)	<0.001
IgM-RF and anti-CCP positive, n (%)	43 (34%)	41 (51%)	2 (4%)	<0.001
IgM-RF and anti-CCP negative, n (%)	62 (49%)	24 (30%)	38 (84%)	<0.001
ESR (mm/h), median (IQR)†	20 (9–35)	25 (11–37)	12 (5–25)	0.003
CRP (mg/L), median (IQR)‡	6.2 (3–22.7)	8 (3.7-28.3)	3 (1.9–12.5)	0.003
TJC68, median (IQR)	7.5 (2–17)	14 (5–23)	2 (1–4)	<0.001
SJC66, median (IQR)	5 (1–9)	7 (4–12)	2 (1–3)	<0.001
DAS28, median (IQR)§	4.5 (3.1–5.7)	5.1 (4.3–6.1)	3.3 (2.7–3.9)	<0.001
MBDA score, median (IQR)	42 (32–59)	46 (34–61)	35 (18–44)	0.001
HAQ Score, median (IQR)¶	1.1 (0.5–1.6)	1.3 (0.8–1.8)	0.6 (0.3–1.3)	0.001

Parameters were summarised as number (n [%]) or median (interquartile range [IQR]) as appropriate. P values were calculated using the chi-square test for categorical variables and Wilcoxon's rank-sum test for continuous variables. †-¶Values missing for †1 RA and 1 UA patients; ‡1 RA patient; §2 RA and 1 UA patients; ¶4 RA patients. Anti-CCP, anti-cyclic citrullinated peptide; CRP, C-reactive protein; DAS28, disease activity score based on 28 joints (based on ESR); ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; IgM-RF, immunoglobulin M rheumatoid factor; IQR, interquartile range; MBDA, multi-biomarker disease activity; RA, rheumatoid arthritis; SJC66, swollen joint count based on 66 joints; TJC68, tender joint count based on 68 joints; UA, unclassified arthritis.

this difference remained statistically significant after adjustment for multiple testing (p=0.019)[10]. Female gender was also associated with UA–RA (14/16) versus UA–UA (13/29) (p=0.005).

In summary, baseline MBDA score did not inform fulfilment of RA classification criteria in patients with UA. However, consistent with MBDA score measuring active disease in patients with RA, baseline MBDA scores were higher in patients with an initial RA diagnosis compared with UA. Limitations to be considered include the relatively small number of patients with UA–RA and possible treatment effect on disease course in patients with UA.

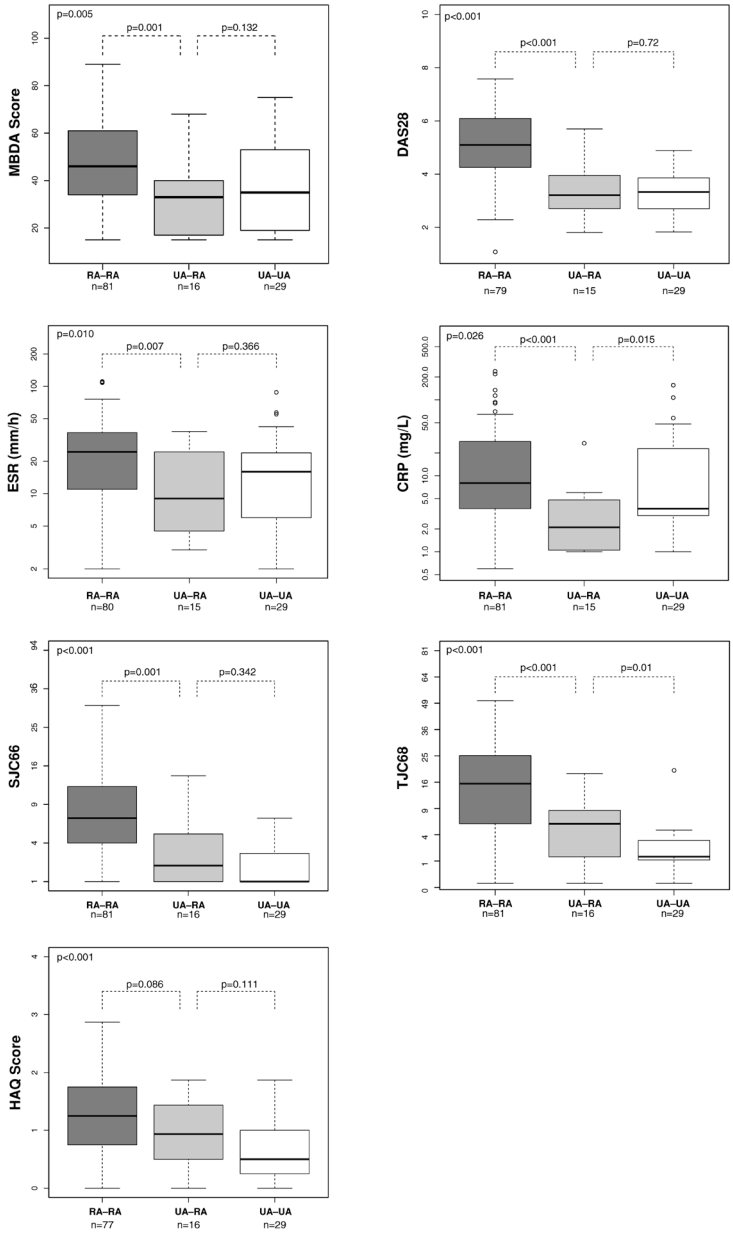

Figure 1. Comparison of baseline disease activity measures across the diagnosis groups.

Disease activity measures are shown for patients grouped according to their diagnosis at baseline and at 2 years. Thick horizontal line: median; box: interquartile range (IQR); whiskers: most extreme points within 1.5x IQR. P-values were derived by Wilcoxon's test. P-values in the upper left corners represent the significance of the trends across the 3 groups by Jonckheere-Terpstra test.

CRP, C-reactive protein; DAS28, disease activity score based on 28 joints (based on ESR); ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; MBDA, multi-biomarker disease activity; RA, rheumatoid arthritis; SJC66, 66 swollen joint count; TJC68, 68 tender joint count; UA, unclassified arthritis.

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NEO-EPITOPES – FRAGMENTS OF CARTILAGE AND CONNECTIVE TISSUE DEGRADATION IN EARLY RHEUMATOID ARTHRITIS AND UNCLASSIFIED ARTHRITIS

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ABSTRACT

Objectives

Tissue destruction in rheumatoid arthritis (RA) is predominantly mediated by matrix metalloproteinases (MMPs), thereby generating protein fragments. Previous studies have revealed that these fragments include MMP-mediated collagen type I, II, and III degradation, citrullinated and MMP-degraded vimentin and MMP degraded C-reactive protein. We evaluated if biomarkers measuring serum levels of specific sequences of the mentioned fragments would provide further information of diagnostic and/or prognostic processes in early arthritis.

Methods

Ninety-two early arthritis patients (arthritis duration<1 year, DMARD naïve) were enrolled. Patients either fulfilled the ACR/EULAR2010 criteria for RA (n=60) or had unclassified arthritis (UA) (n=32). Patients fulfilling the RA criteria after 2 years follow-up were classified into *non-erosive* (n=25), or *erosive disease* (n=13). Concentrations of the biomarkers: C1M, C2M, C3M, VICM and CRPM were measured in baseline serum.

Results

C1M, C3M and CRPM were able to discriminate between the UA and RA baseline diagnosis in 92 patients with an AUROC of 0.64 (95%CI 0.517 to 0.762), 0.73 (95%CI 0.622 to 0.838) and 0.68 (95%CI 0.570 to 0.795). C2M showed a potential for discrimination between *non-erosive* and *erosive disease in 38 patients* with an AUROC of 0.75 (95%CI 0.597 to 0.910). All of the applied biomarkers correlated with one or more of the disease activity parameters: DAS28, ESR, CRP, SJC66, TJC68 and/or HAQ.

Conclusions

This is the first study evaluating the applied biomarkers at this early stage of arthritis. C1M, C3M, CRPM might be the best diagnostic marker, whereas high levels of C2M indicated progression of disease at follow-up in early RA patients.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology, characterized by synovial inflammation in multiple joints [1]. Moreover, RA is associated with excessive turnover of connective tissues of the joints, specifically the extracellular matrix (ECM) in cartilage, bone and synovium. Consequently joints become damaged and disabled [2,3].

Throughout the last 25 years several treatment regimens have been developed, but none of them are effective in all patients [4,5]. It is therefore of interest to subclassify patients for further characterization of the pathogenesis of the disease, which may lead to a better understanding of the disease [6,7]. Early detection of joint damage may be identified and characterized by biochemical markers that predict which patients have severe ongoing joint damage and hence are in need of most aggressive treatment [8,9].

The ECM of the cartilage consists mainly of type II collagen, while type I and III collagens are the main proteins of soft tissue surrounding the joint, as the synovium and entheses [10]. Examining the turnover of these and other collagens may aid the understanding of RA pathogenesis. In RA, inflammation leads to excessive remodelling and tissue turnover. Tissue destruction of the ECM in RA is mediated by enzymatic cleavage predominantly by matrix metalloproteinases (MMPs). MMPs have been shown to be highly up-regulated in RA [11,12]. Consequently, a range of protein-degradation products are generated, which results in the exposure of *de novo* sites of these fragmented proteins, referred to as neo-epitopes [13]. Moreover, these protein-degradation products may be specific for the tissue of origin and for the involved enzymes, and may therefore be used as diagnostic and prognostic biomarkers [14].

Such biomarkers include C1M, which is a product of MMP-cleavage of type I collagen and a biomarker of soft tissue destruction [15]. This biomarker has proved its value in RA as it is able to depict fast progressors from slow progressing disease in the phase III tocilizumab trial LITHE [15]. C1M in combination with MMP3 and CRPM were able to predict, which patients had an increased chance of responding to treatment in the LITHE study [16]. CRPM is the MMP-depended degradation product of C-reactive protein (CRP) [17]. Other soluble biomarkers of interest include C2M, C3M, and VICM. C2M is a serum biomarker that measures a MMP-generated neo-epitope of type II collagen, thereby reflecting cartilage degradation [18]. C3M is a biomarker of soft tissue turnover associated with inflammation [19,20], and VICM evaluates citrullinated and MMP-degraded vimentin [21].

Since all of the mentioned biomarkers have proved useful in evaluation and characterization of established RA [15,16] the objective of this study was to evaluate and characterize the tissue turnover of the joints as reflected by C1M, C2M, C3M, VICM,

and CRPM in early arthritis patients. Furthermore, we studied whether these biomarkers could provide additional information for the diagnostic and/or prognostic process in the very early phase of inflammatory arthritis, when peripheral blood samples are collected during the patient's first visit to the rheumatology department.

PATIENTS AND METHODS

Patients

Ninety-two early arthritis patients were enrolled in the prospective early arthritis 'Synoviomics' cohort at the Academic Medical Center (AMC) in Amsterdam between April 2004 and January 2013 in this study [22]. At baseline the selected patients either fulfilled the ACR/EULAR 2010 criteria for RA classification (n=60) [23] or had unclassified arthritis (UA) that did not fulfill classification criteria of established rheumatic disease (n=32) (phase e according to the EULAR Study Group for Risk Factors for Rheumatoid Arthritis) [24]. All patients enrolled in the study had less than 1 year disease duration, as measured from the first clinical evidence of joint swelling. Patients had active arthritis in at least one joint and were disease-modifying antirheumatic drug (DMARD) naïve. All patients provided written informed consent. The study was performed according to the Declaration of Helsinki and approved by the Medical Ethics Committee of the Academic Medical Center (AMC).

Study design

At baseline, demographic data were collected and the following clinical and laboratory parameters were obtained: serum levels of C-reactive protein (CRP); erythrocyte sedimentation rate (ESR); 68 tender and 66 swollen joint count (TJC68 and SJC66); Disease Activity Score in 28 joints (DAS28); IgM-RF levels using IgM-RF ELISA (Sanquin, Amsterdam, the Netherlands (upper limit of normal (ULN) 12.5 IU/ml)) until December 2009 and thereafter using IgM-RF ELISA (Hycor Biomedical, Indianapolis, IN (ULN 49 IU/ml)); anti-citrullinated protein antibodies (ACPA) using anti-citrullinated cyclic peptide (CCP)2 ELISA CCPlus (Eurodiagnostica, Nijmegen, the Netherlands (ULN 25 kAU/l)); and radiographs of hands and feet.

Patients were followed for 2 years and those with UA were categorized for diagnostic outcome as having either converted to RA (UA-RA; n=6) or remained unclassified (UA-UA; n=23). Three patients were not available for follow-up, and were therefore excluded from the diagnostic outcome analysis. Patients fulfilling the ACR/EULAR 2010 criteria for RA after 2 years follow-up were further classified for prognostic outcome into: (1) non-erosive disease (n=25), or (2)erosive disease (n=13), defined as presence of joint

erosions on radiographs of the hands and/or feet [25]. The group of *non-erosive disease* consisted of patients with self-limiting disease (n=3), and persistent *non-erosive disease* (n=22). Self-limiting disease was defined as no arthritis on examination and no use of DMARDs or steroids in the preceding three months. Persistent disease was defined as the presence of arthritis in at least 1 joint and/or DMARDs or steroids use in the preceding three months but who had no evidence of joint erosion. The prognostic outcome data were not available for 28 of the patients and were therefore excluded from the prognostic outcome analysis. Finally, patients were classified as being in remission (DAS < 2.6) or not (DAS ≥2.6) [26].

Biomarker measurements

Levels of five protein biomarkers (MMP degraded type I collagen [C1M], cartilage degradation [C2M], MMP degraded type III collagen [C3M], citrullinated and degraded vimentin [VICM], and MMP-degraded CRP [CRPM]) were measured in baseline patient serum samples. Measurements were performed manually on blinded samples using competitive enzyme-linked immune sorbent assays (ELISAs) developed and produced by Nordic Bioscience (Herlev, Denmark).

Briefly, for C1M; 96-well streptavidin plates (Roche Diagnostics, Mannheim, Germany) were coated with biotinylated synthetic peptide Biotin-K-GSPGKDGVRG dissolved in assay buffer (50 mM Tris, 1% BSA, 0.1% Tween-20, pH 7.4) adjusted and incubated 30 min at 20°C. 20 µL of peptide calibrator or sample were added to appropriate wells, followed by 100 µL of conjugated monoclonal antibody 4D3-HRP and incubated 1 hour at 20°C. Finally, 100 µL/well tetramethylbenzinidine (TMB) (Kem-En-Tec cat. no. 438OH) was added and the plates were incubated 15 min at 20°C in the dark. When C2M was measured; 4 ng/mL of biotin-KPPGRDGAAG (American peptide, Sunnyvale, CA) was coated onto the streptavidin pre-coated 96-well plates (Roche Diagnostics, Mannheim, Germany) and left for 30 min at 20°C. The calibrators, controls, and undiluted serum samples were added followed by peroxidase-conjugated monoclonal antibody NB44-3C1, and incubated at 4°C for 20 hours. The peroxidase reaction was visualized by 15 min incubation with 3,3′,5,5′-tetramethylbenzidine (TMB, Kem-En-tec, Taestrup, Denmark) at 20°C. For C3M, 96-well streptavidin-coated plates (Roche Diagnostics, Mannheim, Germany) were coated with 0.4 ng/mL of KNGETGPQGP-biotin and left for 30 min at 20°C. Calibrators, controls, and serum samples (diluted 1:1 in incubation buffer) were added, followed by peroxidase-conjugated antibody NB51-G12. The sample–antibody mix was incubated at 20°C for 60 min. TMB was added afterwards and incubated at 20°C and stopped after 15 min. CRPM measurement followed the same procedure as C3M; however, applying a different peroxidase-conjugated antibody (NB94-1A7) and coater (KAFVFPKESDK-biotin). For measurement of serum, VICM samples were prediluted 4 times in incubation buffer. Streptavidin-coated 96-well plates were coated with 100 µL

biotin–RLRSSVPGV–citrulline and left for 30 min at 20°C. The calibrators, controls, and prediluted serum samples were added followed by 100 µL of peroxidase-conjugated monoclonal antibody NB212-1C5 and incubated at 4°C for 20 hours. Afterwards sample/calibrator incubation 100 µL of TMB was added and plates were incubated at 20°C for 15 min. All of the mentioned incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 µL of stopping solution (0.1M sulfuric acid) and measured at 450 nm with 650 nm as the reference. Calibration curves were plotted using a 4-parametric mathematical fit model.

Statistical analysis

Categorical data were depicted as number (%) and differences between study groups analyzed using Chi-square test. Not normally distributed variables were depicted as median (interquartile range, IQR). To compare baseline characteristics and biomarker concentration between the different classification groups, the Kruskal-Wallis test was used when more than 2 groups were compared: subsequently the Mann-Whitney U test was used to compare differences between two subgroups. Bivariate correlations of not normally distributed variables were analyzed using Spearman's rank correlation test. In order to assess the discriminating power of the biomarkers studied we generated ROC curves by using baseline diagnosis (UA or RA), by using diagnostic outcome of the UA patients only (UA-RA or UA-UA), by mean of prognostic outcome (non-erosive or erosive disease) and by classifying patients into remission or not as outcomes. To examine the relationship between the biomarkers and baseline diagnosis, diagnostic outcome, and prognostic outcome, we performed binary logistic regression. All statistical analyses were performed by using SPSS v20.0 software (IBM Corp., Armonk, NY) and MedCalc version 14.8.1 (MedCalc Software bvba, Ostend, Belgium). A p-value of <0.05 was considered statistically significant. Bonferroni correction was applied to correct for multiple comparisons.

RESULTS

Early arthritis patients

Baseline characteristics of the early arthritis patients are shown in Table 1.

Age and gender were comparable between the RA and UA patients. IgM-RF positivity and ACPA positivity were lower in the UA group compared to the RA group. Baseline ESR and CRP were comparable between the groups, whereas DAS28 and ACPA positivity were higher in the RA group. A schematic overview of the study is presented in figure 1.

Table 1. Baseline characteristics of patients with early arthritis

	Rheumatoid arthritis	Unclassified arthritis	p Value
Characteristic	N = 60	N=32	
Sex, female (n (%))	42 (70)	17 (53)	0.110
Age, years (mean (SD))	53 (40-61)	46 (34-59)	0.097
IgM-RF positive (n (%))*	35 (58)	1 (3)	<0.001
ACPA positive (n (%)) **	44 (73)	3 (9)	<0.001
IgM-RF and ACPA both positive (n (%))	30 (50)	0	<0.001
ESR, mm/hr (median (IQR))	17 (7-37)	9 (5-22)	0.079
CRP, mg/L (median (IQR))	6.4 (1.6-16.1)	3.0 (1.2-7.9)	0.117
DAS28 (median (IQR))	4.7 (3.3-6.1)	3.4 (2.6-4.5)	<0.001

Parameters are described as number (n (%)), mean (standard deviation) or median (interquartile range) as appropriate. IgM-RF = immunoglobulin M rheumatoid factor; ACPA = anti-citrullinated protein antibodies; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score in 28 joints Significance levels were set to p<0.0056 when corrected for multiplicity.

Baseline biomarker concentrations are higher in RA patients compared to UA patients

We observed a significantly higher concentration of C3M and CRPM in RA compared to UA based on the diagnosis at baseline (p<0.001 and p=0.004, respectively). In patients diagnosed with RA, the median (IQR) baseline concentrations of C3M, and CRPM were 34.1 ng/mL (24.5-41.2) and 12.8 ng/mL (9.2-16.0), respectively. For UA diagnosis at baseline, the median (IQR) concentration of C3M and CRPM were, 23.7 ng/mL (21.5-29.4) and 9.6 ng/mL (7.6-12.4.), respectively. C1M had a tendency to be higher in patients diagnosed with RA with a median (IQR) level of 37.7ng/ml (26-64.1) compared to patients with UA with the median level of 27.7ng/ml (20.4-51.3) (p=0.028). There was no difference in concentrations of C2M or VICM between the RA and UA diagnosis at baseline (p=0.84 and p=0.13, respectively). The analysis was followed by the investigation of the biomarkers in relationship to diagnostic outcome. After 2 years

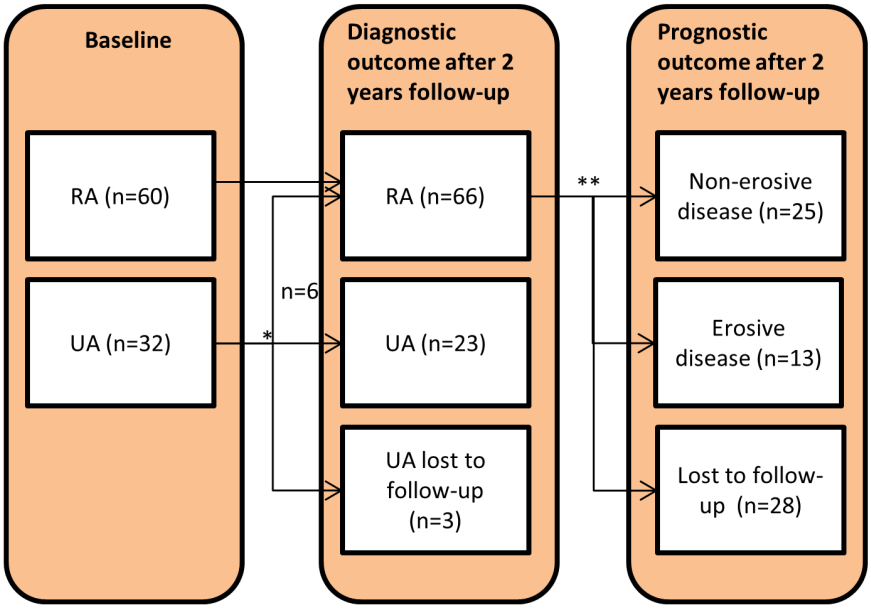

Figure 1. Schematic overview of the study.

At baseline the selected patients either fulfilled the ACR/EULAR 2010 criteria for the classification of rheumatoid arthritis (RA; n=60) or had unclassified arthritis (UA; n=32). Patients were followed for 2 years and those with UA were categorized as having either converted to RA (UA-RA; n=6) or remained unclassified (UA-UA; n=23). Patients fulfilling the ACR/EULAR 2010 criteria for RA after the 2 years follow-up were further classified for arthritis outcome into non-erosive disease (n=25) or erosive disease (n=13).

** Three patients were not available for follow-up and were therefore excluded from the diagnostic outcome analysis.*

*** Of 28 patients the arthritis outcome data were not available and were therefore excluded from the prognostic outcome analysis.*

follow-up period, 6 (18%) of the patients initially classified as UA fulfilled the classification criteria for RA. The patients were stratified in one of following three groups considering their baseline and 2 years follow-up diagnosis: UA-UA (n=23), UA-RA (n=6) (three UA patients were lost to follow-up) and RA-RA (n=60). C3M levels were significantly different between the groups (p=0.005). C3M concentrations (median (IQR)) were higher in RA-RA (34.1 ng/mL (24.6-41.2)) compared to UA-RA (23.9 ng/mL (20.40-31.9)) and UA-UA (24.1 ng/mL (21.6-29.6)). CRPM concentrations were also higher RA-RA (12.8 ng/mL (9.2-16.0)) than in UA-UA (9.6 ng/mL (7.6-12.0)) and UA-RA (10.8 ng/mL (7.2-14.8)). There was no statistically significant difference in concentrations of C1M, C2M and VICM between the different diagnostic outcome groups (p=0.16, p=0.90 and p=0.23, respectively).

Diagnostic power of biomarkers to discriminate between RA and UA diagnosis

To evaluate the diagnostic power of the biomarkers of joint destruction and inflammation and of other disease activity markers to discriminate between the UA and RA groups regarding baseline diagnosis, we calculated the area under the receiver operating characteristic (AUROC). The diagnostic power of C1M, C3M and CRPM was highly significant with an AUROC of 0.64 (95% CI 0.52 to 0.76), 0.73 (95% CI 0.62 to 0.84) and 0.68 (95% CI 0.57 to 0.80), respectively (Table 2A). The same evaluation was performed with CCP and the disease activity parameter DAS28, which resulted in AUROC values of 0.85 (95% CI 0.762 to 0.914) and 0.74 (95% CI 0.64 to 0.84) respectively. C2M, VICM, CRP and ESR were not sufficient to reach statistical significance.

Table 2. Area under the receiver operating characteristic (AUROC

A

Biomarker	AUROC	Std. Error	95% confidence interval	P value
C1M	0.64	0.062	0.52 to 0.77	0.028
C2M	0.51	0.065	0.39 to 0.64	0.838
C3M	0.73	0.055	0.62 to 0.84	<0.001
VICM	0.60	0.061	0.48 to 0.72	0.130
CRPM	0.68	0.057	0.57 to 0.80	0.004
ESR	0.61	0.060	0.49 to 0.73	0.080
CRP	0.60	0.062	0.48 to 0.72	0.117
DAS28	0.74	0.052	0.64 to 0.84	<0.001
CCP	0.85	0.039	0.76 to 0.91	<0.001

B

Biomarker	AUROC	Std. Error	95% confidence interval	P value
C1M	0.49	0.124	0.24 to 0.73	0.91
C2M	0.52	0.134	0.26 to 0.79	0.87
C3M	0.49	0.134	0.23 to 0.76	0.96
VICM	0.32	0.119	0.09 to 0.55	0.18
CRPM	0.45	0.142	0.17 to 0.73	0.71
ESR	0.68	0.135	0.41 to 0.94	0.19
CRP	0.61	0.147	0.32 to 0.90	0.42
DAS 28	0.37	0.171	0.04 to 0.70	0.33
CCP	0.53	0.14	0.34 to 0.72	0.82

In **A**, for discriminating between RA and UA baseline diagnosis and in **B**, for discriminating between UA patients that progress to RA after 2 years of follow-up and those that remain UA after 2 years of follow-up. Significance levels were set to p<0.0056 when corrected for multiplicity.

An univariate logistic analysis was used to assess the relationship between C1M, C2M, C3M, VICM, CRPM, ESR, CRP, DAS28, RF positivity and ACPA positivity, on one hand, and baseline diagnosis on the other. C3M was related to baseline diagnosis (OR

1.07, 95% CI 1.02 to 1.13, p=0.006), and there was an association between CRPM and baseline diagnosis (OR 1.78, 95% CI 1.14 to 2.77, p=0.012). Evaluated clinical parameters were significantly associated with baseline diagnosis: DAS28 (OR 1.94, 95% CI 1.34 to 2.80, p<0.001), RF positivity (OR 43.4, 95% CI 5.6 to 340, p<0.001), and ACPA positivity (OR 26.6, 95% CI 7.1 to 99.4, p<0.001).

Next, we performed multivariate logistic regression analysis with C3M and CRPM in combination with RF positivity, ACPA positivity and/or DAS28. C3M and CRPM did not reach statistical significance in this analysis.

We also calculated the AUROC to assess the ability of the biomarkers to discriminate between patients who subsequently progressed from UA to RA after 2 years and patients who remained UA after 2 years. None of the soluble biomarkers could statistically significantly predict whether patients would be in the UA-RA group compared to the UA-UA group (Table 2B).

Diagnostic power of biomarkers to discriminate between non-erosive and erosive disease after 2 years follow-up

Prognostic outcome after 2 years follow-up was assessed in 66 patients with RA. 28 (42%) patients could not be classified due to missing data. There were no significant differences in baseline characteristics (including age, DAS28, sex or CRP) between the remaining patients and those that were lost to follow-up. Among the remaining RA patients 2 years after initiation of the study, 25 (38%) had *non-erosive disease* and 13 (20%) had *erosive disease*. Baseline C2M concentrations were slightly higher in patients with *erosive* disease (0.23 ng/mL (0.19-0.26)) compared to patients with non-erosive disease (0.18 ng/mL (0.15-0.22) as a prognostic outcome (p=0.011); this difference was borderline significant when adjusted for multiple testing, with the highest (median (IQR)) concentration in the *erosive* group and the lowest concentration in the *non-erosive* group. Baseline C1M, C3M, VICM and CRPM were comparable between the prognostic outcome groups.

This analysis was followed by the investigation of the power of the biomarkers to discriminate between groups with different prognostic outcome. The prognostic power of C2M had an AUROC of 0.75 (95% CI 0.60 to 0.91) (Table 3). Baseline C1M, C3M, VICM, CRPM or any of the standard clinical parameters ESR, CRP, CCP, and DAS28 were not statistically significant predictors of outcome in this study.

Next, univariate logistic analysis was applied to assess the relationship between C1M, C2M, C3M, VICM, CRPM, ESR, CRP, DAS28, RF positivity and ACPA positivity on the one hand and prognostic outcome on the other. None of the markers reached statistical significance using this approach.

Table 3. Area under the receiver operating characteristic (AUROC)

Biomarker	AUROC	Std. Error	95% confidence interval	P value
C1M	0.51	0.11	0.3 to 0.71	0.96
C2M	0.75	0.08	0.60 to 0.91	0.01
C3M	0.51	0.11	0.30 to 0.73	0.89
VICM	0.57	0.10	0.38 to 0.76	0.50
CRPM	0.57	0.11	0.36 to 0.79	0.47
ESR	0.51	0.10	0.31 to 0.71	0.93
CRP	0.59	0.10	0.40 to 0.78	0.38
DAS28	0.46	0.10	0.27 to 0.66	0.70
CCP	0.62	0.09	0.49-073	0.18

AUROCs discriminating between non-erosive and erosive disease for patients fulfilling the RA criteria after 2 years of follow-up. Significance levels were set to p<0.0056 when corrected for multiplicity.

Biomarker concentrations correlate significantly with clinical disease activity measurements

The correlations between the biomarkers of joint destruction and inflammation and measures of clinical disease activity were assessed in the overall population and separately in the patients diagnosed with RA at baseline (Table 4). All soluble biomarkers tested showed statistically significant correlations with measures of disease activity.

Table 4. Correlations between baseline biomarker concentration and other parameters for disease activity. The correlations between the biomarkers of joint destruction and inflammation and measures of clinical disease activity were assessed in A, the overall population (UA+RA) and B, separately in the patients diagnosed with RA at baseline.

A

	DAS28	ESR	CRP	SJC66	TJC68	HAQ
C1M (p; r)	**<0.001; 0.53**	**<0.001; 0.66**	**<0.001; 0.87**	**0.003; 0.31**	0.040; 0.21	**0.004; 0.30**
C2M (p; r)	0.15; 0.15	**0.029;** 0.23	0.067; 0.19	0.21; 0.13	0.93; -0.010	0.34; -0.10
C3M (p; r)	**<0.001; 0.63**	**<0.001; 0.73**	**<0.001; 0.65**	**0.001; 0.34**	**0.009; 0.27**	0.14; 0.16
VICM (p; r)	0.019; 0.25	0.13; 0.16	0.015; 0.25	0.039; 0.22	0.047; 0.21	0.11; 0.17
CRPM (p; r)	**<0.001; 0.47**	**<0.001; 0.60**	**<0.001; 0.60**	**0.003; 0.31**	**0.10; 0.17**	0.12; 0.28

B

	DAS28	ESR	CRP	SJC66	TJC68	HAQ
C1M (p; r)	**<0.001; 0.57**	**<0.001; 0.69**	**<0.001; 0.89**	**0.007; 0.35**	0.16; 0.18	**0.003; 0.38**
C2M (p; r)	0.024; 0.29	0.026; 0.29	0.079; 0.23	0.19; 0.17	0.45; 0.10	0.88; 0.02
C3M (p; r)	**<0.001; 0.60**	**<0.001; 0.69**	**<0.001; 0.66**	**0.004; 0.37**	0.12; 0.20	0.15; 0.19
VICM (p; r)	0.15; 0.19	0.35; 0.12	0.27; 0.15	0.37; 0.12	0.59; 0.07	0.15; 0.19
CRPM (p; r)	**<0.001; 0.48**	**<0.001; 0.578**	**<0.001; 0.58**	0.010; 0.33	0.28; 0.14	0.13; 0.20

The correlations between the biomarkers of joint destruction and inflammation and measures of clinical disease activity were assessed in A, the overall population (UA+RA) and B, separately in the patients diagnosed with RA at baseline. UA = unclassified arthritis; RA = rheumatoid arthritis; DAS28 = disease activity score in 28 joints; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; SJC66 = 66 swollen joint score; TJC68 = 68 tender joint score; HAQ = health assessment questionnaire; C1M = matrix metalloproteinases (MMP) degraded type I collagen; C2M = MMP degraded type II collagen; C3M = MMP degraded type III collagen; VICM = citrullinated and degraded vimentin; CRPM = MMP degraded CRP. Significance levels were set to p<0.01 when corrected for multiplicity.

C1M and C3M can discriminate between patients in remission and those with persistent disease activity

Next, we investigated the power of the biomarkers to discriminate between patients in remission (DAS<2.6) versus those with active disease. First, we evaluated the power of C1M and C3M to discriminate between remission versus active disease in UA and RA patients (n=10 patients in remission; n=82 patients with active disease) and found that these were significant with an AUROC of 0.75 (95% CI 0.62 to 0.88) and 0.79 (95% CI 0.68 to 0.89), respectively. C2M, VICM, CRPM did not appear to provide any information in this regard. Secondly, we performed this analysis in RA patients only (n=3 patients in remission; n=57 patients with active disease) and found that C1M, C3M and CRPM were significant with an AUROC of 0.84 (95% CI 0.68 to 1.00), 0.94 (95% CI 0.87 to 1.00), and 0.91 (95% CI 0.80 to 1.00), respectively.

DISCUSSION

This early arthritis study investigated five neo-epitopes (C1M, C2M, C3M, VICM, CRPM), which are soluble biomarkers each representing different aspects of joint destruction and inflammation. We aimed to determine the ability of these biomarkers to improve the current diagnostic and/or prognostic process in early arthritis patients and to investigate the tissue turnover in the joints reflected by these biomarkers in early arthritis patients. The applied biomarkers evaluate neo-epitopes released during tissue turnover, and they

are therefore sensitive measures of alterations during pathological events in inflamed tissue. We investigated the biomarker profile in early RA as well as in early UA patients. We demonstrated that early RA is associated with significantly increased serum levels of C3M and CRPM hence increased connective tissue turnover compared to UA. Also, C1M levels appeared to be increased in RA compared to UA, although this tendency was not significant after Bonferroni correction for multiple testing. Furthermore, our results indicates that turnover of cartilage, as reflected by C2M levels, was higher in early RA patients who subsequently developed erosive disease after 2 years of follow-up.

The diagnostic utility of the biomarkers was explored in early RA patients and we found that the biomarkers C1M, C3M and CRPM all had a diagnostic power which was comparable to the standard disease parameter DAS28 when evaluated as AUROC. This is not unexpected since RA is characterized by massive changes of metabolic processes in the joints, which includes cartilage degradation and connective tissue turnover as a consequence of synovial inflammation. Thus C1M, C3M and CRPM add further information to the well-established clinical parameters. However, the diagnostic power of CCP was exceeding all of the evaluated biomarkers, as well as DAS28, with an AUROC value of 0.85. All of the applied biomarkers correlated with one or more of the established parameters for disease activity, such as DAS28, ESR, and/or CRP. These results presented here support the notion that connective tissue degradation relates to the inflammatory process in RA. However, the soluble biomarkers tested were unable to predict which of the UA patients at baseline would eventually fulfil classification criteria for RA since the levels of soluble biomarkers were not statistically significantly different between the UA-RA group and the UA-UA group. Single use of a biomarker may therefore not provide enough diagnostic information since RA is a very heterogeneous disease.

Type I, II and III collagen are the main collagens in the joint. Therefore, an increase in MMP fragments of these collagens may provide novel information about connective tissue balance. Indeed, earlier studies demonstrated an association between elevated levels of serum C2M and severity of osteoarthritis, suggesting that C2M could be applied as a biomarker for cartilage loss or degradation [27]. This is in line with our study, which indicated that C2M concentrations were different between the prognostic outcome groups, with a tendency of higher concentrations in *erosive* disease.

These findings suggest that the biomarkers may contribute with independent and additive information about the disease pathogenesis and may provide supplementary diagnostic tools for clinical diagnosis. These biomarkers should not compete with current diagnostic tools for clinical diagnosis or disease activity. Instead they provide additional information on tissue integrity.

A limitation of this study is the relatively small sample size; there was a limited number of UA-RA patients (n=6). Larger studies including higher numbers of early arthritis patients followed longitudinally are needed to confirm these initial findings. In addition it would be preferable to include healthy and age matched non-arthritic patients for the comparison.

This study shows for the first time that measurement of C1M, C3M, and CRPM may assist in differential diagnosis in early arthritis patients. In addition, C2M might be a prognostic biomarker predictive of the development of erosive disease. The results provide the rationale for larger studies in early arthritis patients to confirm and extend these findings.

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9

GENERAL DISCUSSION AND SUMMARY

RHEUMATOID ARTHRITIS:

FROM THE AT RISK PHASES ALL THE WAY UP TO THE DEVELOPMENT OF THE DISEASE

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by pain, swelling and stiffness of the joints due to synovial inflammation. Despite growing insights into the involvement of specific molecules and pathways in synovial tissue inflammation in established RA, the local processes leading to initiation of the disease have not yet been elucidated. Although the synovium is the most important site of pathology in the established phase of RA, it is most probably not the site where the disease is initiated [1]. The analysis of serum and tissues from articular as well as from extra-articular sites obtained from individuals at risk of RA and/or from early RA patients, may aid understanding of the processes leading to synovial tissue inflammation and detrimental autonomous disease progression. Furthermore, accurately defining individuals at high risk of developing RA may facilitate communication between researchers and comparisons between studies in order to further improve the understanding of the disease process and may lead to the discovery of targets for preventive therapy. An example of the latter is the recently completed PRAIRI study.

Preclinical phase

As mentioned above, studies of tissues potentially involved in the aetiology of rheumatoid arthritis (RA), such as synovium, lymph node, gingiva and lung, are critical to understand the development from a healthy to a diseased state. In **chapter 2** we systematically review data from studies on various tissues collected during at risk phases leading up to the development of RA. For future studies on tissues we recommend the use of standardised definitions of the different stages of the disease together with an accurate description of the duration of symptoms to facilitate communication between researchers and comparisons between studies in order to start to understand the processes underlying the development of RA.

During the last couple of years, RA research has focused more on the earliest stages of the disease, leading to the discovery that circulating autoantibodies and elevated acute phase reactants, cytokines and chemokines can be present years before the clinical signs of the disease appear. Individuals with arthralgias who are positive for ACPA have a chance of ~30% of developing RA within 1 year. Moreover, in the presence of ACPA the risk for arthritis development is enhanced by IgM-RF: about 40% of ACPA-positive and IgM-RF-positive individuals with arthralgia develop arthritis after 2 years . Furthermore, multiple genes determine RA disease susceptibility , as well as lifestyle and environmental factors, such as overweight, smoking and an altered function of the autonomic nervous system. Unaffected first degree relatives (FDRs) of RA patients share at least some genetic and environmental risk factors with RA patients and may therefore provide an opportunity to enrich the population at risk of developing RA. Therefore, we determined the prevalence of ACPA and IgM-RF in a large cohort of 577 FDRs of a European population of RA patients in **chapter 3**.

We found a prevalence of ACPA and IgM-RF in FDRs of 1.9% and 6.6%, respectively. These results are important for research programs aimed at the identification of individuals at risk of developing RA. Furthermore, there appeared to be more smokers in the ACPA positive tested individuals compared to those who were ACPA negative. This is in line with other studies reporting the presence of ACPA to be more likely in FDRs with a smoking history [5]. Indeed, smoking is the major known environmental risk factor for RA, although the exact mechanism remains to be elucidated. It has been suggested that smoking, in the context of the HLA-DR shared epitope (SE) genes, may trigger citrullination in the lungs and thereby provide a substrate for immune activation [6]. It can be expected that the prediction models to identify individuals at risk of developing RA will further improve by identification of novel molecular biomarkers as well as life style factors that contribute to the predictive model.

Obesity has also been suggested as an important risk factor in the development of RA and may increase the risk of developing RA in individuals who are already at increased risk for this disease [8]. As obesity is characterized by an increased volume of adipose tissue, producing adipokines, we hypothesized that serum levels as well as synovial expression of adipokines in autoantibody-positive individuals at risk of developing RA could be of importance in the development of RA. In **chapter 4** we show that serum vaspin levels were associated with the development of arthritis in these individuals, even after adjustment for being overweight. Also, serum adipokine levels correlated with body mass index and with systemic markers of inflammation, such as CRP levels and ESR, as well as with synovial expression of CD3+ cells. No association between the synovial expression of adipokines and the development of arthritis was found. Together, our results suggest that serum adipokines are associated with an increased inflammatory state in autoantibody-positive individuals at risk of developing RA. Furthermore, serum vaspin levels may assist in predicting the development of arthritis in these individuals.

Angiogenesis in early RA

Increased angiogenesis is considered an important factor in the pathogenesis of RA and might perhaps be a target for future therapies [9, 10]. The nuclear factor-κB (NF-κB) family of transcription factors is highly important in the development and perpetuation of (synovial) inflammation [11, 12]. We have recently demonstrated that non-canonical NF-κB signaling in endothelial cells (EC) regulates pathological angiogenesis [13]. The non-canonical NF-κB pathway is strictly dependent on NF-κB-inducing kinase (NIK) and IKKα homodimers . In **chapter 5** we investigated NIK expression in the earliest phases of RA compared to other forms of arthritis in a prospective cohort of disease-modifying antirheumatic drug (DMARD) naive patients with early arthritis, as well as in a cohort of autoantibody-positive individuals at risk of developing RA. We show that NIK is highly expressed in synovial blood vessels of patients with various forms of early arthritis.

Additionally, we demonstrate that synovial NIK expression is associated both with systemic (i.e. ESR and CRP levels) and local markers of disease activity such as joint swelling and MRI findings in early arthritis patients. Interestingly, NIK^+ blood vessels could also be found in some individuals at risk of developing RA. Collectively, our data identify NIK as a potential therapeutic target in arthritis since NIK inhibition can block pathological angiogenesis (Maracle et al, manuscript submitted for publication) and thereby may be able to prevent the switch from acute to chronic inflammation.

The analysis of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) using pharmacokinetic modeling (PKM) provides quantitative measures that mirror microvessel integrity and can be used as objective markers of the level of synovial inflammation and as a non-invasive marker of synovial angiogenesis. In **chapter 6** we investigated the value of the Tofts DCE-MRI PKM parameters (K^{trans}, K_{ep} and v_e), which represent absolute measures of microvessel integrity, in a prospective cohort of DMARD naive early arthritis patients. Our results show that K^{trans}, K_{ep} and v_e differ between diagnostic groups and thereby suggest that they may be used as a diagnostic biomarker in early inflammatory joint disease. However, whether these parameters can be used at the individual patient level remains to be determined. Furthermore, these PKM parameters may assist in detecting synovial inflammation in early arthritis patients, which could be of use for evaluation of joints that are relatively inaccessible for proper clinical examination (i.e. hip joints) or joints with suspected but not definite synovitis, or to detect subclinical synovitis in patients at risk for developing RA. Importantly, we validated our results by demonstrating that these parameters correlated with the EC marker vWF in synovial tissue biopsies of the same joint, which can be of use in the context of translational research bridging aspects of tissue microcirculation and angiogenesis with advanced imaging techniques. In addition, this technique may be used to evaluate anti-angiogenic treatment effects of (novel) targeted therapies.

Biomarkers in early RA

The 2010 American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) updated classification criteria for rheumatoid arthritis (RA) focus on establishing a diagnosis for research purposes during an early disease stage . They facilitate research on the effects of treatment of early RA, which has been associated with improved clinical and structural outcomes . Some patients with inflammatory oligoarthritis or polyarthritis initially not meeting RA classification criteria who are classified as undifferentiated arthritis (UA) based on clinical and laboratory assessments, might later fulfil classification criteria for RA. Classifying these patients earlier as RA would enable evaluation of therapeutic intervention and suppression of RA disease activity during an earlier stage of the disease .

The multi-biomarker disease activity (MBDA) score, calculated from the concentrations

of 12 serum biomarkers, is an objective validated disease activity measure for patients with RA. It has been shown to track disease activity in patients with early and established RA, independent of previous treatment, and is associated with the risk of radiographic progression . In **chapter 7** we investigated whether the MBDA score might inform RA diagnosis in patients with UA. This was not the case: baseline MBDA score did not have added value in establishing a diagnosis of RA in UA patients.

Tissue destruction of the extracellular matrix (ECM) in RA is mediated by enzymatic cleavage by proteinases, predominantly by matrix metalloproteinases (MMPs). MMPs have been shown to be highly up-regulated in RA [14, 15]. Consequently, a range of protein-degradation products are generated, which results in the exposure of *de novo* sites in these proteins, referred to as neo-epitopes [16]. These protein-degradation products may be specific for the tissue of origin and for the involved enzymes, and may therefore be used as diagnostic and prognostic biomarkers [17]. In **chapter 8** we investigated the concentration of 5 neo-epitopes (C1M, C2M, C3M, VICM, CRPM), markers of joint destruction and inflammation, in a cohort of early arthritis patients. We demonstrated that there was a significant difference in C1M, C3M, CRPM and connective tissue degradation levels between patients classified as RA and UA at baseline, with higher concentrations in the early RA patients. This was to be expected since RA is characterized by massive synovial inflammation and structural damage, which includes cartilage degradation and connective tissue turnover. Moreover, these markers correlated with other established markers of disease activity, such as DAS28, ESR, and/or CRP, supporting the concept that connective tissue degradation is related to the inflammatory component of RA. Finally, we showed that C2M concentrations are significantly higher in early RA patients that progress to erosive disease after 2 years of follow up. Taken together, these neo-epitopes may improve the current diagnostic and/or prognostic process in early arthritis patients.

Concluding remarks/Future directions

It is important to investigate the earliest phases of RA in order to better understand the processes underlying the development of RA from a phase in which individuals are at risk for developing RA, characterized by systemic autoimmunity associated with RA, to having clinically apparent disease.

Although the updated 2010 American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) classification criteria has been a great step forward in establishing an early diagnosis for research purposes, some patients remain unclassified. It should also be noted that these criteria were not developed to establish the prognosis of the individual patient. Taken together, there is still a need for better prediction models, to facilitate timely diagnosis and early therapeutic decisions.

The research presented in this thesis may help to develop better prediction models to identify subjects with an increased risk of developing RA, based on the identification of increased vaspin levels as an additional predictor in subjects who are positive for RF and/or ACPA. Other factors such as smoking, body mass index and autonomic dysfunction may also contribute to the prediction model. Studying the preclinical phase of RA is currently a hot topic in rheumatology research. The door for screening approaches that may help to identify individuals at risk of developing RA is opened, since it has become clear that ACPA and rheumatoid factors may be present years before the development of clinical signs and symptoms of RA appear. The ultimate objective is to get a deeper understanding of the aetiology of RA and to evaluate preventive strategies during the preclinical phase . An example of the latter is the recently completed PRAIRI study (Nederlands Trial Register, http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=1969, NTR1969), in which individuals at risk of developing RA with subclinical signs of inflammation (i.e. elevated CRP and/or ESR and/or subclinical synovitis as assessed by MRI or by ultrasound) were treated with a single course of rituximab (anti-CD20 B cell depleting therapy) in order to prevent or delay development of clinically manifest arthritis. Similar studies are currently underway with abatacept and simvastatin.

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NEDERLANDSE SAMENVATTING

RHEUMATOID ARTHRITIS:

FROM THE AT RISK PHASES ALL THE WAY UP TO THE DEVELOPMENT OF THE DISEASE

Reumatoïde artritis (RA) is een chronische ontstekingsziekte die gekenmerkt wordt door ontsteking van het weefsel dat de binnenzijde van het gewricht bekleedt, het synovium. Deze gewrichtsontsteking (artritis) leidt tot pijn, zwelling en stijfheid van de gewrichten en kan leiden tot permanente schade aan de gewrichten (aantasting van het kraakbeen en erosies van bot). Hierdoor kunnen de gewrichten minder goed functioneren, wat uiteindelijk kan leiden tot invaliditeit. Bij RA blijft de ontsteking niet altijd beperkt tot de gewrichten, maar kan ook voorkomen in andere organen, zoals de huid, longen en ogen. RA komt wereldwijd bij ongeveer 1% van de bevolking voor, waarbij vrouwen vaker zijn aangedaan dan mannen. Het risico op het krijgen van RA wordt door meerdere factoren bepaald. Naast erfelijke factoren spelen ook omgevingsfactoren een rol, maar de exacte oorzaken voor het ontstaan van RA zijn nog grotendeels onbekend.

Bij het merendeel van de RA-patiënten zijn bepaalde antistoffen aanwezig in het bloed, de zogenaamde reumafactoren (RF) en antistoffen tegen gecitrullineerde eiwitten (ACPA). Deze antistoffen kunnen tot 14 jaar voor het ontstaan van de eerste ziekteverschijnselen al aanwezig zijn in het bloed. Echter, een klein deel van de bevolking draagt deze antistoffen ook bij zich zonder dat zij ooit RA zullen ontwikkelen. Het is tot op heden niet bekend wie van deze personen met antistoffen uiteindelijk RA krijgt. Als het mogelijk is om te voorspellen wie in de toekomst RA zal ontwikkelen en wie niet, dan kunnen we wellicht personen selecteren voor preventieve behandeling en daarmee het ontstaan van ziekteverschijnselen uitstellen of zelfs voorkomen. Daarnaast kan meer inzicht in de factoren die betrokken zijn bij het ontstaan van RA leiden tot de ontwikkeling van nieuwe behandelingen. Omdat de genoemde RA-specifieke antistoffen vaak al voor het ontstaan van de ziekte aanwezig zijn in het bloed (soms al jaren), is het nu mogelijk om deze preklinische fase te onderzoeken. In deze fase zijn de antistoffen dus al aanwezig, maar is er nog geen sprake van artritis.

In dit proefschrift zijn zowel studies naar de preklinische fase als naar de vroege klinische fase van RA opgenomen. Hierbij zijn verschillende compartimenten bestudeerd, namelijk het synovium en het bloed, en is er gebruik gemaakt van verschillende technieken om deze verschillende fases van de ziekte te bestuderen, zoals een verscheidenheid aan laboratorium- en radiologische technieken.

Preklinische fase

Het bestuderen van weefsels die mogelijk betrokken zijn bij het ontstaan van RA, zoals het synovium, de lymfeklieren en longweefsel, is belangrijk om meer inzicht te krijgen in de mogelijke oorzaken voor het ontstaan van RA. In **hoofdstuk 2** voeren wij een systematische review uit, waarbij we de resultaten weergeven van studies die verschillende weefsels hebben onderzocht tijdens de preklinische fasen van RA. Voor toekomstige studies adviseren wij standaard definities te hanteren voor de verschillende fasen van de ziekte, evenals een precieze beschrijving van de duur van de symptomen.

Eerstegraads familieleden van RA patiënten delen een aantal genetische – en omgevingsfactoren met RA patiënten. Dit geeft ons een kans om bepaalde aspecten van de preklinische fase van RA te bestuderen door het bloed te onderzoeken van deze (nog) gezonde eerstegraads familieleden. In **hoofdstuk 3** laten we zien dat ACPA aanwezig zijn bij 1,9% eerstegraads familieleden die zelf geen RA hebben, terwijl RF zelfs aanwezig zijn bij 6.6% van de eerstegraads familieleden. Een andere interessante bevinding is dat er meer rokers bleken te zijn onder de ACPA positieve familieleden in vergelijking met de voor ACPA negatief geteste familieleden. Dit sluit goed aan bij het feit dat roken één van de grootste risicofactoren is voor het ontstaan van RA, al is nog niet bekend welk mechanisme hieraan ten grondslag ligt. De bevindingen van dit hoofdstuk kunnen daarom mogelijk bijdragen aan het identificeren van personen met een verhoogd risico op RA. Uiteindelijk verwachten we dat bestaande predictie-modellen (die de kans op het ontwikkelen van RA voorspellen) verbeterd kunnen worden door identificatie van (andere) omgevingsfactoren en specifieke biomarkers.

Een van de omgevingsfactoren die het risico op RA ook vergroot is de aanwezigheid van overgewicht (obesitas). Obesitas wordt gekenmerkt door een toename van vetweefsel, dat bepaalde ontstekingsbevorderende eiwitten kan produceren, de adipokinen. Wij postuleerden daarom dat de concentratie van bepaalde adipokinen in het bloed, danwel de expressie van deze adipokinen in het synovium, belangrijk zou kunnen zijn in de ontwikkeling van RA. In **hoofdstuk 4** laten we zien dat de concentratie van het adipokine ***vaspin*** geassocieerd is met de ontwikkeling van artritis in RF- en/of ACPA-positieve personen (zonder artritis), zelfs wanneer we corrigeren voor het overgewicht. De expressie van adipokinen in het synovium bij deze personen daarentegen, was niet geassocieerd met de ontwikkeling van artritis.

Vroege fase van RA

Angiogenese in vroege RA

De vorming van nieuwe bloedvaten (angiogenese), speelt een belangrijke rol in het ontstaansproces van RA en is daardoor mogelijk een goed aangrijpingspunt voor toekomstige therapieën. De nuclear factor-κB (NF-κB) familie van transciptiefactoren

is belangrijk in het ontwikkelen en in stand houden van (synoviale) ontsteking. Eerder onderzoek van onze afdeling heeft aangetoond dat de “non-canonical NF-κB activatieroute” in endotheelcellen van bloedvaten een belangrijke bijdrage levert aan pathologische angiogenese bij chronische ontstekingsziektes zoals langer bestaande RA en in tumoren. NF-κB-inducing kinase (NIK) is het centrale kinase dat deze activatieroute reguleert. In **hoofdstuk 5** vergelijken we de expressie van NIK in de vroegste fasen van RA met andere vormen van artritis bij patiënten met “early arthritis” die tot op heden geen antireumatische behandeling (DMARD) hebben gebruikt. Daarnaast bekijken we de expressie van NIK bij auto-antilichaam positieve personen die “at risk” zijn om RA te ontwikkelen. Hierbij tonen we aan dat NIK tot expressie komt in synoviale endotheelcellen van patiënten met verschillende vormen van artritis. Daarnaast tonen we aan dat deze expressie geassocieerd is met zowel systemische als lokale markers van ziekteactiviteit in “early arthritis” patiënten. NIK positieve bloedvaten werden af en toe zelfs al gevonden in het synovium van personen “at risk” op het ontwikkelen van RA. Samengevat kunnen we concluderen dat onze data laten zien dat NIK^+ bloedvaten al in de vroegste fase van de ziekte aanwezig zijn. Daarnaast identificeert dit NIK als een potentieel therapeutisch aangrijpingspunt in artritis, aangezien remming van NIK pathologische angiogenese kan blokkeren en daarbij mogelijk de switch van acute naar chronische ontsteking kan voorkomen.

Om synovium te kunnen onderzoeken, moeten biopten verkregen worden. Dit kan bijvoorbeeld echo-geleid of door middel van een mini-artroscopie. Een minder invasieve manier om het synovium te onderzoeken is door gebruik te maken van beeldvormende technieken, zoals echografie of magnetic resonance imaging (MRI). Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is een bepaalde MRI variant, waarmee het mogelijk is om de opname van contrast in het synovium in de loop van de tijd te bestuderen. Dit resulteert in een afspiegeling van verschillende factoren die betrokken zijn bij het ontstekingsproces, zoals de vorming van nieuwe bloedvaten. Door een specifiek rekenmodel toe te passen kan hierover relevante informatie verkregen worden. In **hoofdstuk 6** onderzoeken we de waarde van dit Tofts model bij DMARD-naïeve “early arthritis” patiënten. Onze resultaten tonen dat de hieruit voorkomende parameters K^{trans}, K_{ep} and v_e (welke maten zijn voor de integriteit van de kleine bloedvaten) verschillen tussen de diagnostische groepen, waardoor deze mogelijk gebruikt kunnen worden als diagnostische marker in vroege inflammatoire gewrichtsziekten. Verder zijn deze PKM parameters mogelijk behulpzaam bij het detecteren van synoviale ontsteking in “early arthritis” patiënten, wat nuttig kan zijn voor het beoordelen van gewrichten die minder goed toegankelijk zijn voor gedegen lichamelijk onderzoek (zoals bijvoorbeeld het heupgewricht), of voor gewrichten waarbij men twijfelt aan het al dan niet aanwezig zijn van synovitis. Deze resultaten zijn gevalideerd door te laten zien dat deze parameters correleren met de endotheelcel-marker von Willebrandt Factor (vWF) in synoviaal weefsel van hetzelfde gewricht.

Biomarkers in vroege RA

De American College of Rheumatology (ACR) en de European League Against Rheumatism (EULAR) hebben in 2010 de classificatiecriteria voor RA aangepast, waarbij meer aandacht wordt geschonken aan het vroegtijdig stellen van de diagnose RA voor onderzoeksdoeleinden dan voorheen. Het onderzoek naar de effecten van vroegtijdige behandeling van RA wordt hiermee vergemakkelijkt, hetgeen op termijn hopelijk leidt tot betere klinische en structurele uitkomsten. Sommige patiënten die initieel niet voldoen aan de RA classificatiecriteria en worden geclassificeerd als ongedifferentieerde artritis (UA), voldoen later alsnog aan de RA classificatiecriteria. Door deze patiënten eerder als RA te classificeren, zoals in de nieuwe criteria gebeurt, zou de juiste therapie eerder kunnen worden ingesteld waardoor ziekteactiviteit in een vroegere fase van de ziekte onderdrukt wordt, om uiteindelijk te resulteren in betere uitkomsten.

De multi-biomarker disease activity (MBDA) score, berekend vanuit de concentratie van 12 serum biomarkers, is een objectieve, gevalideerde maat voor ziekteactiviteit in RA patiënten. Daarnaast is de score geassocieerd met het risico op het ontstaan van structurele schade aan kraakbeen en bot, vastgesteld via radiologische progressie. In **hoofdstuk 7** onderzoeken we of de MBDA score mogelijk informeert over het uiteindelijk krijgen van de diagnose RA in UA patiënten. Dit bleek niet het geval te zijn.

Weefseldestructie in RA wordt gemedieerd door verscheidene enzymen (proteinases), voornamelijk door matrix metalloproteinasen (MMPs). MMPs komen in hoge mate tot expressie in de gewrichten van RA patiënten. Het gevolg hiervan is dat verschillende afbraakproducten worden gegenereerd, die resulteren in nieuwe epitopen in deze eiwitten, zgn. "***neo-epitopen***". Deze degradatieproducten zijn mogelijk specifiek voor het soort weefsel en voor de betrokken enzymen en zouden daardoor gebruikt kunnen worden als diagnostische en prognostische biomarkers. In **hoofdstuk 8** onderzoeken we de concentratie van 5 neo-epitopen (C1M, C2M, C3M, VICM, CRPM), gevalideerde markers voor gewrichtsdestructie en ontsteking, bij vroege artritis patiënten. We tonen aan dat er een significant verschil is in C1M, C3M, CRPM concentraties tussen vroege RA en UA patiënten (baseline), met hogere concentraties in de vroege RA patiënten. Dit is plausibel, aangezien RA gekarakteriseerd wordt door aantasting van het gewricht (destructie van kraakbeen, bot en bindweefsel) tijdens synoviale ontsteking. Daarnaast correleren deze markers met een of meer andere maten van ziekteactiviteit, zoals de DAS28, BSE en/of CRP. Dit ondersteunt de hypothese dat bindweefseldegradatie gerelateerd is aan de mate van ontsteking in RA. Tenslotte laten we zien dat de C2M concentratie significant hoger is in vroege RA patiënten die uiteindelijk erosieve ziekte ontwikkelen na 2 jaar follow up. Deze neo-epitopen kunnen daarom mogelijk gebruikt worden als diagnostische en prognostische biomarkers bij vroege artritis patiënten.

Conclusie en toekomstige plannen

Om de processen te begrijpen die aan de ontwikkeling van RA ten grondslag liggen, is het zeer belangrijk om de vroegste fasen van RA te onderzoeken; van een fase waarin personen een verhoogde kans hebben om RA te ontwikkelen ("at risk" fase) tot de aanwezigheid van klinisch evidente ziekte. Deze fasen kunnen onderzocht worden door verschillende compartimenten te bestuderen en door gebruik te maken van verschillende technieken, waaronder een verscheidenheid aan laboratorium- en radiologische technieken.

Hoewel de meest recente 2010 ACR/EULAR classificatiecriteria voor RA kunnen helpen bij het stellen van een vroege diagnose, blijven sommige patiënten ongeclassificeerd. Daarnaast zijn deze criteria niet ontwikkeld om de prognose van een individuele patiënt te voorspellen. Er bestaat dus nog steeds een noodzaak voor betere predictiemodellen, om vroegtijdige therapeutische beslissingen te ondersteunen.

Enerzijds helpen de onderzoeksresultaten beschreven in dit proefschrift mogelijk om betere predictiemodellen te ontwikkelen, om personen met een verhoogd risico op het ontwikkelen van RA vroegtijdig te identificeren, bijvoorbeeld gebaseerd op de aanwezigheid van een verhoogde serum vaspin concentratie als een additionele predictor bij personen die RF en/of ACPA positief zijn. Andere factoren kunnen mogelijk ook bijdragen aan het verbeteren van bestaande predictiemodellen , zoals roken, body mass index en activiteit van de nervus vagus.

Het bestuderen van de preklinische fase van RA is momenteel zeer actueel. Sinds het duidelijk werd dat ACPA en reumafactoren jaren voor het ontstaan van klachten aanwezig kunnen zijn, staat de deur naar het screenen van personen die "at risk" zijn om RA te ontwikkelen wagenwijd open. Het uiteindelijke doel is om de etiologie van RA beter te kunnen begrijpen en om preventieve therapieën die gebruikt kunnen worden tijdens de preklinische fase te ontwikkelen. Een voorbeeld hiervan is de door het AMC geïnitieerde PRAIRI studie, waarin personen die at risk zijn om RA te krijgen gerandomiseerd werden naar eenmalig rituximab of placebo (Nederlands Trial Register, http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=1969, NTR1969).

Daarnaast helpen deze onderzoekresultaten mogelijk in het stellen van een zo vroeg mogelijke diagnose van RA (bijv. door gebruik te maken van neo-epitopen danwel DCE-MRI parameters) en hebben we NIK geïdentificeerd als een potentieel therapeutisch aangrijpingspunt in artritis.

DANKWOORD

Dit proefschrift is het resultaat van een mooie, leerzame tijd en zou niet tot stand zijn gekomen zonder de hulp van vele mensen om mij heen. Ik wil daarom iedereen bedanken die (in)direct betrokken zijn geweest bij mijn onderzoek. Een aantal personen wil ik hier in het bijzonder bedanken.

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c'est la que mon coeur est

PHD PORTFOLIO

Name PhD student	K.I. Maijer
PhD period	June 2009 – December 2014

Courses		
The AMC World of Science	2009	0.7 ECTS
Basis cursus Regelgeving en Organisatie Klinisch Wetenschappelijk Onderzoek (BROK) AMC	2009	0.9 ECTS
Practical Biostatistics AMC	2010	1.1 ECTS
Cursus Snel lezen Tijdwinst.com	2010	0.2 ECTS
Postgraduate Course Advanced Immunology AMC/VUmc/Sanquin	2011	2.9 ECTS
Scientific Writing in English for Publication AMC	2012	1.5 ECTS
Systematic Reviews AMC	2013	0.3 ECTS
Clinical Epidemiology AMC	2013	0.6 ECTS
Oral presentation AMC	2014	0.8 ECTS
Presentations		
Smoking and overweight determine the likelihood for development of rheumatoid arthritis, 3rd International Pre-RA/RA-Risk Workshop, in Stockholm, Sweden (oral presentation)	2013	0.5 ECTS
Evaluation of a multi-biomarker disease activity (VECTRA ™ DA ALGORITHM) in early rheumatoid arthritis and unclassified arthritis patients , The Annual European Congress of Rheumatology EULAR 2013, in Madrid, Spain (poster presentation)	2013	0.5 ECTS
NF-κB-inducing kinase (NIK) expression in synovial blood vessels correlates with systemic markers of inflammation and local disease activity in early arthritis patients, but is not disease specific , The Annual European Congress of Rheumatology EULAR 2013, in Madrid, Spain (poster presentation)	2013	0.5 ECTS
NF-κB-inducing kinase (NIK) expression in synovial blood vessels correlates with systemic markers of inflammation and local disease activity in early arthritis patients, but is not disease specific , The ACR/ARHP Annual Scientific Meeting 2014, in Boston, USA (poster presentation)	2014	0.5 ECTS

Conferences		
NVR (Nederlandse Vereniging voor Reumatologie) Najaarsdagen in Arnhem, the Netherlands	2012	0.25 ECTS
EULAR Annual European Congress of Rheumatology in Madrid, Spain	2013	1 ECTS
ACR/ARHP Annual Scientific Meeting in Boston, USA	2014	1.25 ECTS
Seminars and workshops		
Weekly department research seminars	2009-2015	15 ECTS
Workshop on Specific immunity and immune memory in RA, Joint AutoCure/Masterswitch meeting, in Sigtuna, Sweden	2010	0.75 ECTS
Workshop ultrasound guided biopsies, Barts and the Royal Hospital, in London, England	2010	0.25 ECTS
Finnal annual meeting, Joint AutoCure/Masterswitch meeting, in Amsterdam, the Netherlands	2011	0.5 ECTS
International Workshop on “Pre-RA”, JBI, in Amsterdam, the Netherlands	2011	0.25 ECTS
EWRR Workshop in Amsterdam, the Netherlands	2011	0.25 ECTS
3rd International Pre-RA/RA-Risk Workshop, in Stockholm, Sweden	2013	0.75 ECTS
International Pre-RA/RA-Risk Workshop, in Madrid, Spain	2013	0.25 ECTS
Lecturing		
Rheumatoid arthritis: new developments, Patient meeting, in Harderwijk, the Netherlands (oral presentation)	2009	0.5 ECTS
Evaluation of a multi-biomarker disease activity (VECTRA ™ DA ALGORITHM) in early rheumatoid arthritis and unclassified arthritis patients for Projects in Knowledge (video presentation)	2013	0.5 ECTS
Other		
Member arthroscopy team	2009-2011	5 ECTS
Organising large-scale fairs and investigator meetings regarding the PRAIRI study	2009-2012	5 ECTS

CURRICULUM VITAE

Karen Inger Maijer werd op 28 mei 1983 geboren in Zeist en is opgegroeid in Huizen. Nadat zij in 2001 was geslaagd voor het VWO+ aan het Erfgooiers College in Huizen, begon zij aan de studie geneeskunde aan de Vrije Universiteit van Amsterdam. In januari 2008 behaalde zij haar artsendiploma. Vrijwel aansluitend werkte zij als ANIOS bij de Interne Geneeskunde in het Tergooiziekenhuis in Hilversum (opleider dr. S. Lobatto). In 2009 besloot zij promotieonderzoek te gaan doen bij de Klinische Immunologie en Reumatologie van het Academisch Medisch Centrum/Universiteit van Amsterdam onder supervisie van haar promotor prof. dr. P.P. Tak, waarvan dit proefschrift wat nu voor u ligt het resultaat is. Naast het verrichten van klinisch onderzoek, heeft zij ook laboratoriumonderzoek uitgevoerd, als mede onderzoek op de afdeling Radiologie. Verder verrichte zij mini-artroscopieën en organiseerde en coördineerde zij grootschalige wervingsprojecten betreffende trials. Daarnaast heeft zij geparticipeerd in de reguliere polikliniek en de diensten van de Reumatologie. In augustus 2015 is zij begonnen met de opleiding Dermatologie (opleider dr. M. Wintzen) aan het VU Medisch Centrum in Amsterdam.

LIST OF PUBLICATIONS

1. Ramwadhdoebe TH, Hähnlein J, **Maijer KI**, van Boven LJ, Gerlag DM, Tak PP, van Baarsen LG. Lymph node biopsy analysis reveals an altered immunoregulatory balance already during the at-risk phase of autoantibody positive rheumatoid arthritis. Eur J Immunol. 2016 Dec;46(12):2812-2821.

2. **Maijer KI**, Gudmann NS, Karsdal MA, Gerlag DM, Tak PP, Bay-Jensen AC. Neo-Epitopes-Fragments of Cartilage and Connective Tissue Degradation in Early Rheumatoid Arthritis and Unclassified Arthritis. PLoS One. 2016 Mar 28;11(3):e0149329.

3. **Maijer KI**, van der Leij C, de Hair MJ, Tas SW, Maas M, Gerlag DM, Tak PP, Lavini C. Dynamic Contrast-Enhanced Magnetic Resonance Imaging Using Pharmacokinetic Modeling: Initial Experience in Patients With Early Arthritis. Arthritis Rheumatol. 2016 Mar;68(3):587-96.

4. **Maijer KI**, Neumann E, Müller-Ladner U, Drop DA, Ramwadhdoebe TH, Choi IY, Gerlag DM, de Hair MJ, Tak PP. Serum Vaspin Levels Are Associated with the Development of Clinically Manifest Arthritis in Autoantibody-Positive Individuals. PLoS One. 2015 Dec 15;10(12):e0144932.

5. **Maijer KI**, Gerlag DM, Tak PP. Prevalence of Anti-Citrullinated Protein Antibodies and IgM Rheumatoid Factor in First-Degree Relatives of Dutch Rheumatoid Arthritis Patients. Arthritis Rheumatol. 2015 Dec;67(12):3324-6.

6. **Maijer KI**, Li W, Sasso EH, Gerlag DM, Defranoux NA, Tak PP. Does the multi-biomarker disease activity score have diagnostic value in early rheumatoid arthritis and unclassified arthritis? *Ann Rheum Dis*. 2015 Nov;74(11):2097-9.

7. **Maijer KI**, Noort AR, de Hair MJ, van der Leij C, van Zoest KP, Choi IY, Gerlag DM, Maas M, Tak PP, Tas SW. Nuclear Factor-κB-inducing Kinase Is Expressed in Synovial Endothelial Cells in Patients with Early Arthritis and Correlates with Markers of Inflammation: A Prospective Cohort Study. J Rheumatol. 2015 Sep;42(9):1573-81.

8. de Hair MJ, Leclerc P, Newsum EC, **Maijer KI**, van de Sande MG, Ramwadhdoebe TH, van Schaardenburg D, van Baarsen LG, Korotkova M, Gerlag DM, Tak PP, Jakobsson PJ. Expression of Prostaglandin E2 Enzymes in the Synovium of Arthralgia Patients at Risk of Developing Rheumatoid Arthritis and in Early Arthritis Patients. PLoS One. 2015 Jul 30;10(7):e0133669.

9. de Hair MJ, Lehmann KA, van de Sande MG, **Maijer KI**, Gerlag DM, Tak PP. The clinical picture of rheumatoid arthritis according to the 2010 American College of Rheumatology/European League Against Rheumatism criteria: is this still the same disease? Arthritis Rheum. 2012 Feb;64(2):389-93.