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DOCTORAL DISSERTATION

Validation and characterization of novel autoantibody biomarkers for rheumatoid arthritis

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“One doesn't discover new lands without consenting
to lose sight of the shore for a very long time.”
-André Gide

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List of Abbreviations

3D	three-dimensional
aa	amino acid
Ab	antibody
ACPA	antibodies against citrullinated proteins
ACR	American College of Rheumatology
ANOVA	analysis of variance
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
AU	arbitrary units
BAFF	B cell activating factor
BCR	B cell receptor
BiP	binding immunoglobulin protein
BLyS	B-lymphocyte stimulator
BRAF	g v raf murine sarcoma viral oncogene homologue
C	control peptide
CII	collagen type II
CAIA	collagen antibody-induced arthritis
CarP	carbamylated protein
CCP2	cyclic citrullinated peptides second generation
CCP3	cyclic citrullinated peptides third generation
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund’s adjuvant
CIA	collagen-induced arthritis
COMP	cartilage oligomeric matrix protein
CRP	C-reactive protein
CT	computed tomography
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DAS(28)	disease activity score (based on 28 joints)
DKK-1	dickkopf-1 wnt signaling pathway inhibitor 1
DMARD	disease-modifying anti-rheumatic drug
DNA	deoxyribonucleic acid

EAC	Early Arthritis Clinic
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
Fc	fragment crystalline
FcR	fragment crystalline receptor
FcRn	neonatal fragment crystalline receptor
G6PI	glucose-6-phosphate isomerase
GC	germinal center
h	hour(s)
HAQ	health assessment questionnaire
HC	healthy controls
HECTOR	high-energy computed tomography system optimized for research
HLA	human leukocyte antigen
HP	high performance
HRP	horseradish peroxidase
ICAM-1	intracellular adhesion molecule 1
IFA	incomplete Freund’s adjuvant
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
JAK-STAT	Janus kinase / signal transducers and activators of transcription
KLH	keyhole limpet hemocyanin
KW	Kruskal Wallis
L	linker
LFA-1	lymphocyte function-associated antigen 1
LMM	linear mixed model
LPS	lipopolysaccharide
MAB	monoclonal antibody
MBS	3-maleimidobenzoic acid N-hydroxysuccinimide
MCM2	minichromosome maintenance complex component 2
MCTD	mixed connective tissue disease
MHC	major histocompatibility complex

MPBS	phosphate-buffered saline containing skimmed milk powder
min	minute(s)
MWU	Mann-Whitney U
N	number
NA	not available
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	N-hydroxysuccinimide
NS	not stated
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OD	optical density
OPG	osteoprotegerin
p	probability
P	specific peptide
PAD	peptidyl arginine deiminase
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PGE_2	prostaglandin E2
PM	paramalignant
PsA	psoriatic arthritis
PTPN22	protein tyrosine phosphatase non-receptor 22
RA	rheumatoid arthritis
RANK	receptor activator for nuclear factor kappa-B
RANKL	receptor activator for nuclear factor kappa-B ligand
RC	rheumatic controls
RF	rheumatoid factor
RNA	ribonucleic acid
RS3PE	remitting seronegative symmetrical synovitis with pitting edema
RT	room temperature
SAS	serological antigen selection
SD	standard deviation
SEM	standard error of the mean
SJC	swollen joint count
SNP	single nucleotide polymorphism

SpA	spondyloarthropathy
SS	Sjögren syndrome
STAT4	activator of transcription 4
TCR	T cell receptor
TJC	tender joint count
TGF	transforming growth factor
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
Treg	regulatory T cell
U	units
UA	undifferentiated arthritis
UGCT	centre for x-ray tomography of Ghent University
UH	Hasselt University
ULN	upper limit of normal
UTR	untranslated region
VAS	visual analogue scale

1

INTRODUCTION AND AIMS

1.1 Rheumatoid arthritis

The pathology of rheumatoid arthritis (RA) was first described in 1800 by Landré-Beauvais, a French surgeon and in 1859 the term "rheumatoid arthritis" was used for the first time by the English physician Garrod [1, 2]. RA is now known as the world's most common autoimmune disease mainly characterized by a chronic inflammation of multiple synovial joints [3, 4]. Up to 0.5-1% of the Western population is affected and each year 5-50 new cases per 100,000 people develop RA [4-6]. The prevalence increases with age and women are three times more at risk to develop RA. Prevalence also varies between geographical regions indicating the importance of genetic background and environmental triggers [6]. Despite intensive investigation worldwide, the underlying pathogenesis is still not fully understood. There is no cure but new drugs are increasingly available to manage the disease and prevent joint destruction.

1.1.1 Clinical presentation and diagnosis

Clinical presentation

The primary symptoms of RA are joint tenderness, swelling, pain and morning stiffness. RA presents as a symmetric arthritis, thus affecting synovial joints at both sides of the body. Many joints are targeted in RA, mainly the metacarpophalangeal and proximal interphalangeal joints of hands, small joints of feet, but also wrists, elbows, shoulders, ankles and knees [7-9]. Uncontrolled active RA will lead to progressive destruction and deformity of joint cartilage and bone, accompanied with disability and reduction of quality of life. Most patients suffer fatigue, malaise, weakness or weight loss, and many patients are affected by vasculitis, cardiovascular disease, anemia, lymphadenopathy and osteoporosis [10-12]. These systemic features are responsible for an increased mortality associated with RA [13]. The wide variety in clinical manifestation is linked to differences in genetic susceptibility and environmental triggers, autoantibody profiles and therapy response, pointing towards multiple pathways in RA all leading to chronic joint inflammation and bone erosion.

Classification criteria and disease monitoring

The first classification criteria for RA were developed in 1956 and amendments have been made regularly since then [14-16]. Currently, RA is diagnosed clinically by fulfillment of criteria revised in 2010 by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR), based on the 1987 criteria [8 17]. The classification criteria consist of the presence of synovitis in at least one joint with absence of a better, alternative diagnosis, and of the achievement of an individual score greater than or equal to six out of ten from individual scores in four domains: number and site of involved joints (0-5), serological abnormality (0-3), elevated acute-phase reactants (0-1) and symptom duration of six weeks or more (0-1) (Figure 1.1) [8].

ACR 1987 criteria

Four of the seven criteria must be present. Criteria 1-4 must have been present for at least 6 weeks.

1. Morning stiffness (at least 1 hour)
2. Arthritis of three or more joint areas
3. Arthritis of hand joints (at least one or more swollen joint)
4. Symmetric arthritis
5. Rheumatoid nodules
6. Serum rheumatoid factor
7. Radiographic changes (erosions or unequivocal decalcification)

ACR/EULAR 2010 criteria

A score of six or higher out of ten is needed to classify patient as having definite RA.

		Score
1.	Joint involvement	
	1 large joint	0
	2-10 large joints	1
	1-3 small joints	2
	4-10 small joints	4
	>10 joints (at least one small)	5
2.	Serology	
	RF- and ACPA-	0
	Low-pos RF or low-pos ACPA	2
	High-pos RF or high-pos ACPA	3
3.	Acute-phase reactants	
	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
4.	Duration of symptoms	
	<6 weeks	0
	≥6 weeks	1

Figure 1.1. Revised classification criteria for rheumatoid arthritis in 2010 based on the 1987 criteria in order to improve diagnosis of early rheumatoid arthritis patients. ACPA, anti-citrullinated protein antibodies; ACR, American College of Rheumatology; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; RA, rheumatoid arthritis; RF, rheumatoid factor.

Latest revision of the criteria was primarily aimed at shifting the focus to early diagnosis as the importance of early therapeutic intervention became more evident [8, 18]. Firstly, early intervention is highly desirable since joint damage starts early in disease course [19-22]. Secondly, several studies have implied the existence of a 'window of opportunity' during early disease marked by better treatment response and outcome [23-29]. The 1987 ACR classification criteria for RA were criticized by their lack of sensitivity in early disease. Indeed, the criteria highly relied on clinical symptoms, persistent erosion and extra-articular manifestations which are often not easily detected in recent-onset RA. Moreover, new treatments were more effective in decelerating these disease complications, further delaying the definitive diagnosis of RA patients. Inclusion of antibodies against citrullinated proteins (ACPA) as RA-specific disease markers further enhanced diagnosis in an early stage as they are present before joint destruction occurs and they can even be detected years before symptom onset [30]. As will become more evident further in this chapter, the breakthrough of ACPA has divided RA patients in two distinct disease subsets based on the presence or absence of ACPA, with different clinical manifestations, pathogenic mechanisms, and genetic and environmental associations [31-33].

After diagnosis, assessment of RA patients is mainly based on thorough examination of inflamed joints. Disease activity is mostly monitored by the disease activity score based on 28 joints (DAS28), integrating measures of physical examination (TJC, tender joint count and SJC, swollen joint count), acute phase response (erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP)), and patient self-assessment on a visual analogue scale (VAS) [34]. Additional patient assessment is evaluated by a health assessment questionnaire (HAQ), an important indicator of disability [35].

1.1.2 Immunopathogenesis

The primary target of RA are synovial joints, which are characterized by a joint cavity between bones filled with synovial fluid (Figure 1.2A). Synovium is a thin highly-organized structure between the joint capsule and joint cavity [36]. It is responsible for providing structural support to the joints and delivering nutrients to the cartilage, and for the production of synovial fluid. Synovial tissue consists of two separate layers: an intimal lining layer (or synovial lining layer) and a synovial sublining layer (or subsynovium). The latter is a relatively acellullar structure composed of extracellular matrix, blood vessels and lymphatics, nerves, and some scattered fibroblasts, macrophages and mast cells. The intimal lining layer consists of 2-3 cell layers of type A (macrophage-like) and type B (fibroblast-like) synoviocytes.

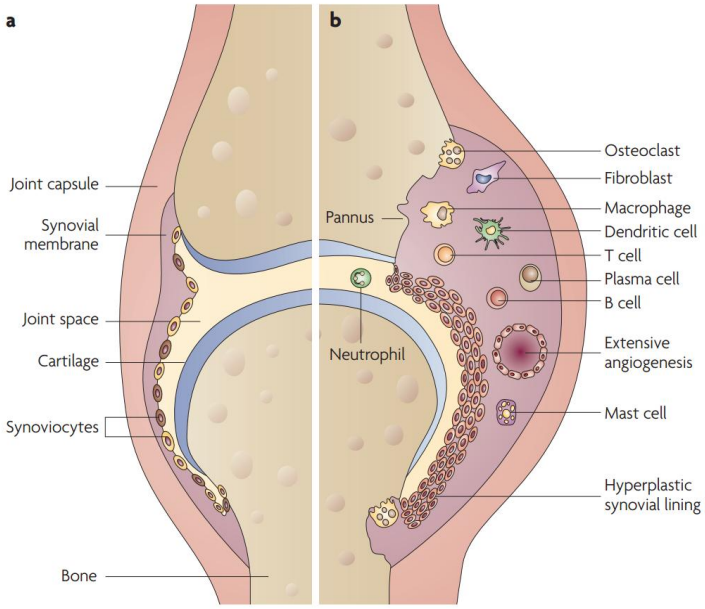

Figure 1.2. Illustration of a normal synovial joint (a) and a rheumatoid arthritis joint (b). In rheumatoid arthritis, synovial joints are infiltrated by a broad range of immune cells. The synovial or intimal lining layer becomes hyperplastic (ten or more cell layers) and starts forming erosive pannus tissue which migrates into the joint cavity and overgrows subchrondal bone and articular cartilage, leading to bone erosion and cartilage destruction. Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Drug Discov. 6:75-92 copyright 2007 [37]

In RA, the synovium undergoes striking changes (Figure 1.2B) [3, 37, 38]. The subsynovium gets massively infiltrated by mononuclear cells such as macrophages, and T and B lymphocytes. The synovial lining becomes hyperplastic (ten or more cell layers) probably due to apoptosis-resistance rather than increased proliferation [39]. Increased angiogenesis (new blood-vessel formation) is present already early in the disease enhancing the transport of nutrients to the hyperplastic synovial lining and recruiting inflammatory cells towards the joint [40, 41]. The hyperplastic cells start forming pannus, a structure containing macrophages, osteoclasts, fibroblast-like synoviocytes and few lymphocytes. While the synovium loses some of its protective functions such as the production of lubricant, the pannus tissue migrates into the joint cavity and starts to overgrow subchrondal bone and articular cartilage [42]. Bone is eroded following osteoclast stimulation by macrophage-like synoviocytes while proteases produced by fibroblast-like synoviocytes destroy cartilage [43]. The spreading of arthritis between different joints is considered at least partly mediated by fibroblast-like synoviocytes as they have been shown to migrate to unaffected joints in severe combined immune-deficient mice [44, 45]. Clonal T cell expansions are also widely distributed throughout the body [46, 47]. Next to T cells, B cells are abundantly represented, they are not only enriched in the inflamed synovial tissue, but also in bone marrow of affected joints and lungs of RA patients [48, 49]. Of note, the aggregation of B and T cells in bone marrow of RA patients is one of the features raising the hypothesis that RA starts in the bone marrow subsequently involving synovial tissue [39, 50, 51]. This hypothesis is supported by recent studies claiming changes in bone metabolism prior to inflammatory processes and onset of clinical symptoms [52, 53].

Changes in bone metabolism primarily manifest as bone loss, essentially due to the actions of osteoclasts, key players in bone erosion during RA disease course [54]. Differentiation of osteoclasts is stimulated via receptor activator of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (RANK) after binding by RANK ligand (RANKL). RANKL is primarily expressed by synovial fibroblasts but also by T cells [3, 55]. The interaction between RANK and RANKL can be blocked by osteoprotegerin (OPG), and thus bone metabolism is controlled by the RANKL/OPG balance [56, 57]. This balance is influenced by a complex network of cytokines, small soluble proteins mediating the communication between cells

involved in immune responses [58]. One of the most important cytokines in RA pathology – and other autoimmune disorders – is tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine which is produced by CD4+ T cells and macrophages. High levels of TNF-α are found in synovial fluid and tissue of affected joints, in the synovial lining and sublining [59]. TNF-α is a stimulator of a wide spectrum of effector functions in the synovium, and often acts synergistically with interleukin (IL)-1. In RA, osteoclasts can be stimulated directly by TNF-α and IL-1, and also by RANKL or prostaglandin E2 (PGE_2). Osteoclasts are also modulated indirectly via actions on other cells leading to an overweight of RANKL (IL-11, IL-17, IL-22) or an overweight of OPG (transforming growth factor (TGF-)β, IL-12, IL-18) [7, 39, 60, 61]. Even a direct link between autoantibody formation and bone loss was described with the observation that ACPA induce osteoclastogenesis and bone resorption in a TNF-α-mediated way [62]. Not only is bone resorption enhanced in RA, also mechanisms of bone repair are debilitated. One of the major pathways in bone remodeling is the Wnt pathway, which can be inhibited by dickkopf-1 (DKK-1), which is upregulated by TNF-α [63]. Cartilage repair by collagen synthesis would be restored by a Th2 response, if the T cell response would not be shifted towards Th1 and Th17 cells [64]. Therefore, mechanisms of bone or cartilage repair are seriously impaired in RA. Still, in about one out of ten RA patients repair has been observed [65-67].

While the pathogenesis of RA is mediated by a disturbed immuneregulation, numerous autoantigens have been described, either joint-related (e.g. collagen type II) or not joint-related [68-71]. This latter group can contain foreign antigens strongly resembling human proteins (e.g. heat shock proteins), post-translationally modified proteins (e.g. citrullinated vimentin) or the proteins mediating the modifications (e.g. peptidyl arginine deiminase) [68, 70, 72-76]. Also antibody reactivities have been identified towards ubiquitous proteins such as glucose 6 phospho isomerase, calpastatin, heterogenous nuclear ribonucleoprotein A2 (RA33) and many others [68-70, 77]. Autoantibodies and their targets form immune complexes which can activate different immune cells or complement and cause depositions thereby contributing to tissue damage [78, 79]. As addressed further in this chapter, these autoantibodies can be useful as biomarkers for diagnosis and prognosis.

1.1.3 Genetic susceptibility and environmental risk factors

RA is a multifactorial disease, resulting from a complex interplay of genetic, hormonal, immunologic and environmental factors. The risk for RA is for over 50% attributable to genetic risk factors [4, 80, 81]. Although no single genetic cause is responsible for RA, several genotypes have been shown to increase susceptibility for RA, most of them affecting T cell activation. The strongest associations have been found for major histocompatibility complex (MHC) class II genes such as human leukocyte antigen (HLA)-DRB1. The HLA-DRB1 alleles share a common motif in the third hypervariable region, known as the shared epitope, consisting of five amino acids in residues 70-74 of the HLA-DRβ chain [82]. The shared epitope is associated with ACPA-positive and more severe RA, probably due to specific formation of the antigen-binding groove, allowing citrullinated antigens to bind [83, 84]. Other genetic contributors are polymorphisms in the PTPN22 gene, encoding the protein tyrosine phosphatase non-receptor 22, which is involved in the negative regulation of lymphocyte activation. One single nucleotide polymorphism (SNP) in the PTPN22 gene reduces the activity of regulatory T cells and inhibits the negative selection of autoreactive T and B cell precursors [85]. A specific PTPN22 allele has also been demonstrated to increase the risk for ACPA-positive RA [86, 87]. In addition to the shared epitope and SNPs in PTPN22, a multitude of other genes have been linked to the risk for developing ACPA-positive RA [88-93], such as genes encoding for cytotoxic T-lymphocyte-associated protein 4 (CTLA4) which is expressed on helper T cells and provides inhibitory signals to T cells [90], and for peptidylarginine deiminase (PAD), responsible for the post-translational modification from arginine to citrulline, although the latter is restricted to certain populations [94]. Only few susceptibility loci seem to predispose for ACPA-negative RA, such as signal transducer and activator of transcription 4 (STAT4) involved in T cell regulation [95], and HLA-DR3 [96].

Besides genetic loci, also environmental factors influence the onset and severity of RA with different effects in ACPA-positive and ACPA-negative disease. The most dominant environmental determinant for the development and progression of RA is smoking [4, 97, 98]. This factor is particularly present for ACPA-positive patients and lead to the concept that smoking activates PAD enzymes in the

lungs initiating citrullination of novel antigens [99]. Interaction with genetic predisposition (HLA-DR1 and PTPN22) strongly enhances this pathway [87, 100, 101]. *Porphyromonas gingivalis* causing periodontitis, appears another important source of PAD in RA [102]. Additional environmental risk factors for RA are vitamin D status, alcohol and coffee intake, oral contraceptive use and female hormones, and low socioeconomic status [4, 103, 104].

1.1.4 Treatment

So far, there is no cure for RA but the treatment options for RA patients have improved enormously the last decades. Treatment of RA currently aims for reducing inflammation as soon as possible and as deep as possible to avoid damage and functional decline, and this together with additional focus on co-morbidities and all the aspects that matter for patients. Non-steroidal anti-inflammatory drugs (NSAIDs) are administered to manage symptoms and daily function is maintained by occupational therapy [4]. Glucocorticoids are effective in rapidly suppressing synovitis after local administration [105]. But the main therapeutic strategy is treat-to-target [106-108] and this is attempted by the first-line therapy for RA: disease-modifying anti-rheumatic drugs (DMARDs) [28, 109, 110], key therapeutics of which the mechanisms of action are not completely understood. Many theories have been proposed and probably multiple pathways are targeted, explaining the effectiveness in different phenotypes of RA. Initially, patients were first prescribed NSAIDs and glucocorticoids, while now they are treated more aggressively immediate from disease onset onwards, during the "window of opportunity". Most commonly prescribed DMARDs include methotrexate, sulfasalazine, hydroxychloroquine and leflunomide. DMARD monotherapy may not fully suppress disease progression and often combination therapies are applied, although the higher efficacy is often accompanied by an increase in toxicity and adverse effects [111-114]. Inadequate response and toxic effects are the major reasons to switch from conventional DMARDs to biologicals, genetically engineered drugs copying the effects of natural *in vivo* substances. The first biologicals were aimed at TNF-α, the major cytokine involved in RA pathology and for which targeting in both mice and humans has been shown to ameliorate disease [4, 115-120]. TNF-α inhibitors approved for the treatment of RA are infliximab, etanercept, adalimumab, golimumab and

certoluzimab – all proven to be highly effective, especially in combination with methotrexate [114, 121-123]. Despite high effectiveness in many patients, anti-TNF-α therapy responses are variable and still a considerable number of patients do not respond [114, 124, 125]. Moreover, TNF-α inhibition holds risks for serious adverse effects such as infection or cancer [28, 126, 127]. Finally, biologicals are very expensive [127]. Therefore, the need for improved treatments beyond TNF-α inhibitors remains. New biologicals target cytokine pathways (anakinra, anti-IL-1RA and tocilizumab, anti-IL-6 receptor), B cells (rituximab, anti-CD20) and T cell activation (abatacept, CTLA4-antibody fusion protein targeting CD80/CD86 on antigen-presenting cells (APC)) [123, 128-134]. Additional drugs in the pipeline target the Janus kinase / signal transducers and activators of transcription or JAK-STAT pathway and the IL-17/Th17 pathway [135]. In general, the development of biologicals has greatly improved disease management of RA. In addition to the treatment of the rheumatic processes, the management of comorbidities has progressed, further promoting better prognostics for RA patients. Regarding the highly variable responses of different therapeutic regimens and the differences in phenotypic presentations of RA, treatment is heading towards a personalized medicine approach [136, 137].

1.1.5 Animal models

The animal model most resembling human RA, is collagen-induced arthritis (CIA), first described in rats [138] and later expanded to mice [139]. Mice are immunized with heterologous collagen type II (CII), a known target for autoantibodies in RA [140], in complete Freund's adjuvant (CFA) [141]. The subsequent strong anti-CII antibody response causes a chronic and progressive form of arthritis [142]. Clinical arthritis symptoms are visible 21-25 days after the first immunization [141]. Similar to RA, the inflammatory arthritis affects the synovial joints leading to cartilage damage and bone erosions, appearing 10 days after disease onset [142-144]. As RA susceptibility is linked to specific MHC II molecules, susceptibility to CIA is attributed to the q haplotype of the mouse MHC ($H\text{-}2^q$) [145, 146]. Based on their $H\text{-}2^q$ background, DBA/1 mice are generally chosen for CIA. The link with MHC II implies an important role for T cell-specific responses, but also B cells are highly involved as B cell-deficient mice do not develop CIA [147] and B cell depletion delayed disease onset in CIA [142, 148]. In

contrast to human RA, male mice are more prone to CIA and the arthritis is self-limiting [141]. Nevertheless, because of the common features between CIA and RA, CIA has emerged as the most widely studied model for RA.

A second animal model for RA relies on arthritis elicited by antibodies against CII: collagen antibody-induced arthritis (CAIA) [149-151]. In this model, anti-CII antibodies are passively transferred to mice. The pathophysiological properties of these anti-CII antibodies are enhanced by lipopolysaccharide (LPS), causing an acute, rapid-onset arthritis (48 hours after antibody-transfer) [152]. For optimal immune reactivity, mice are administered anti-CII antibodies directed against different epitopes spread over the entire CII protein [153]. Clinically, CAIA resembles RA and CIA, but the role of the adaptive immune system is far less applicable while players of the innate immune system like macrophages are highly involved. Since antibodies are transferred, the CAIA model is not dependent on its own humoral response, and thus unrelated to MHC II background [153]. This model allows to study the inflammatory processes, without the complex interference of the immune system.

In addition to (anti-)CII (antibody)-induced arthritis, animal models are available in which arthritis is induced using adjuvant (adjuvant-induced arthritis or AIA), antigens (antigen-induced arthritis), proteoglycans such as pristane [154], or bacterial cell wall structures such as zymosan [144, 153]. Furthermore, some genetically manipulated mice strains appeared to develop a spontaneous arthritis. For instance, an over-expression of human TNF-α leads to chronic inflammatory arthritis marked by bone erosion. This mouse model has provided major insights into the role of TNF-α and its downstream effector functions in RA. Furthermore, it is an acknowledged model to study treatments targeting this pivotal cytokine. Spontaneous arthritis also develops in K/BxN mice which are the result from crossing T cell receptor (TCR) transgenic mice with mice expressing MHC II-A^{g7}. Typical to this model is the pathological role played by anti-glucose-6-phosphate isomerase (G6PI) antibodies, also reported in RA patients [155]. The pathological properties of these antibodies are further illustrated by the development of arthritis in serum recipients (serum transfer model). Both models have been helpful in studying autoimmunity and pathogenicity of autoantibodies in joint-related inflammation [144, 153, 156].

1.2 B cells in RA

1.2.1 The adaptive immune system in RA

The adaptive immune system is highly involved in the pathogenesis of RA. This is illustrated by the genetic susceptibility loci linked to T cell activation and recognition [82, 85, 87], and the presence of autoantibodies [157]. For a long time, research has focused on the role of T cells in RA pathogenesis. At disease initiation, CD4+ helper T cells responding to (self)arthritogenic substances might start producing cytokines, which in turn activate synovial cells and B lymphocytes [158]. Within synovium, T cell- and macrophage-derived cytokines are found, as well as high expression levels of molecules involved in antigen presentation and T cell activation [59, 159]. Additionally, macrophages and fibroblast-like synoviocytes are activated by T cells via an antigen-independent T cell-contact-dependent pathway through expression molecules such as ICAM-1 (intracellular adhesion molecule 1) and LFA-1 (lymphocyte function-associated antigen 1) [160-165]. The notion that most genetic susceptibility genes – described later – are linked to T cell pathways further supports the importance of T cells in RA pathogenesis. Whereas RA has long been considered a Th1 disease, the alternative Th17 type (IL-17-producing CD4+ T cells) has come forward [166-170]. Th17 cells require TGF-β, IL-1β and IL-6, provided by macrophages, dendritic cells and B cells [60, 171]. Th17-derived cytokines (IL-17A, IL-17F, IL-21, IL-22 and TNF-α) activate fibroblast-like synoviocytes and chondrocytes and block the activation of regulatory CD4+CD25+ T cells (Tregs), which function is also directly suppressed by TGF-β and IL-1β [172-175]. Not only the suppressive effects of Tregs on CD4+ T cells is impaired in RA, also reduced counteracting effects of the Th2 cell population are associated with worse prognosis [165].

In most autoimmune disorders, T cells cooperate closely with B cells. T cells are important for B cell activation and B cells play a pivotal role in activation of CD4+ T cells [176]. There are additional indications that T cells are not the only players in RA pathogenesis such as the relatively small increase of T cell cytokines in synovial tissue [177]. The awareness of the contribution of B cells has increased although the pathogenic mechanism behind B cell autoimmunity and its role in RA is still not clear. Current evidence and knowledge on B cell tolerance and function is discussed in the next paragraphs.

1.2.2 Break in B cell tolerance

In order for the adaptive immune system to discriminate between invading antigens and self- (autologous) antigens, lymphocytes are taught to be unresponsive to self-antigens. This *immunological tolerance* is established by central (thymus and bone marrow) and peripheral (lymph nodes and other tissues) mechanisms [178]. Within central tolerance mechanisms, immature B cells that express self-reactive receptors, undergo receptor editing in the bone marrow [179]. In this process, the light chain of the immunoglobulin (Ig) receptor is changed in a way that the B cell is no longer responsive to the self-antigen. If this process fails, self-reactive B cells become anergic (functionally inactive) or are triggered into apoptosis (deletion or negative selection). Autoreactive B cells escaping this central tolerance checkpoint, encounter additional tolerance checkpoints in the periphery, where reactivity to self-antigens in the absence of T cell help will induce anergy or cell death [180, 181]. In RA patients, defects were found in both central and peripheral tolerance checkpoints [181-184]. A higher frequency of polyreactive B cells was found among the transitional B cells leaving the bone marrow in RA patients [183]. Furthermore, peripheral checkpoints seem not capable to remove these autoreactive B cells that escape central tolerance [183]. Although the exact mechanisms behind these defects are not fully elucidated yet, RA patients have been found to display unusual Ig light chain repertoires showing impaired regulation of secondary recombination, presumably resulting from defects in B cell receptor (BCR) signaling [182, 183, 185-187]. One of the possible causes disrupting BCR signaling, is a missense polymorphism in the PTPN22 gene, strongly associated with RA [85, 188]. The loss of tolerance in RA probably occurs as early as in the pre-clinical stages of the disease, since autoreactive antibodies appear years before the onset of clinical symptoms [30, 182, 184].

1.2.3 Roles of B cells in RA

The humoral response can exert different functions (Figure 1.3) and one of its key components are antibodies, normally produced to provide protection to invading pathogens. Upon binding their antigens, they neutralize foreign substances by forming immune complexes, mark them for phagocytosis by macrophages or stimulate other immune mechanisms such as the complement

pathway [77-79]. Unfortunately, binding to (self)antigens, can also drive persistent and harmful inflammation. For instance, antibodies aggregating in immune complexes, cause further activation of B cells and follicular dendritic cells [78, 79]. Antibodies interact with other players of the immune system via the Fragment crystalline (Fc) constant region in their heavy chain. Variations in this region divide them into different classes and subclasses each with different ability to activate complement and bind Fc receptors. The first Ig on B cell surfaces are IgD and IgM and the latter is the first secreted antibody in response to (self)antigenic stimulation. Upon further stimulation, a switch is made to isotypes or classes other than IgM, such as IgG, IgA and IgE. The most studied autoantibody systems in RA are RF and ACPA (more extensively described further in this chapter) and the presence of IgM isotypes of these antibodies proves a continuous reactivation of B cells [189].

The pathogenic action of the humoral immune system in RA is illustrated by the efficacy of treatment with an anti-CD20 antibody (rituximab). A reduction in autoantibody titers has been reported following rituximab treatment [128, 190]. However, the antibody-producing plasma cells (CD20-) are not directly affected by rituximab, thus this treatment's effectiveness points towards additional effector functions of B cells [191] (Figure 1.3). B cells can also act as APC, presenting antigens to T cells in the synovium [60, 176, 192-195]. Upon interaction between an antigen and the Ig receptor on the B cell surface, the antigen is processed into antigenic peptides which are then presented to T cells via MHC class II molecules. B cells also express co-stimulatory molecules necessary for T cell activation [79, 192]. Activated T cells then further enhance the inflammatory cascade.

B cells do not only act as APCs themselves, they also activate other APC and modulate autoimmunity by their cytokine production. Although these cytokines can act pro- and anti-inflammatory [196, 197], they predominantly sustain the inflammatory immune response by increasing the expression of B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) thereby promoting B cell survival [198] and germinal center (GC) formation [199], encouraging synovitis by interacting with fibroblast-like synoviocytes [200], affecting several types of T lymphocytes [197, 201, 202], even stimulating osteoclastogenesis as source of IL-6 and RANKL [60, 201] and many other effects (bystander activation) [203].

The involvement of B cells in RA pathogenesis is marked by lymphocyte accumulation and the formation of ectopic lymphoid structures resembling GCs [78, 204, 205]. The development of these GCs is not only dependent on the presence of B cells [176], it is also the place where B cells encounter their specific antigen, are selected and start proliferating and differentiating towards antibody-producing plasma cells or memory B cells. Critical factors for B cell survival and T cell-independent B cell activation, such as APRIL and B-lymphocyte stimulator (BLyS) are upregulated within RA synovial GCs [198, 206-208]. How GCs contribute to loss of tolerance is not clearly defined yet. However, somatic hypermutation in the GC, involved in affinity maturation, might change the specificity of a B cell clone for a foreign epitope into specificity for a self-antigen [180, 209]. In addition, it is likely that B cells with a weak self-reactivity – who have failed to induce control mechanisms in tolerance checkpoints – are activated within the GC by self-antigen aggregated to foreign antigens [209]. Autoreactive B cells thereby clonally expand within the GC. The ectopic GC-like structures thus promote ongoing local autoimmunity and inflammatory injury. And as the GC is also an essential site for class-switching, ectopic lymphoid structures in the synovium of RA patients support ongoing production of class-switched autoantibodies [205, 210]. These autoantibodies reflect the strong autoimmune humoral aspect typifying RA.

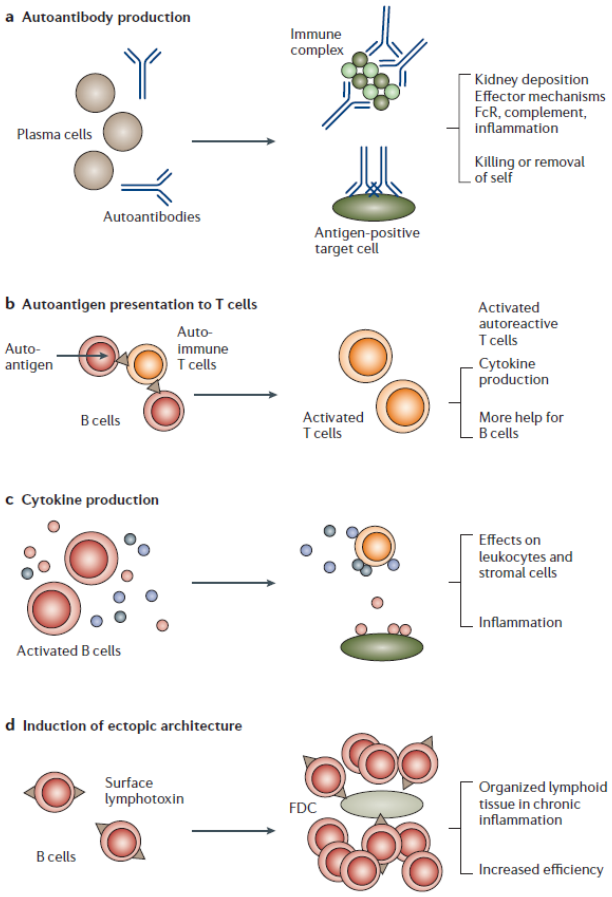

Figure 1.3. Pathogenic roles of B cells

B cells can exert different pathological functions in rheumatoid arthritis (RA). (a) One of the key features of the humoral response and RA, is the production of autoantibodies by plasma cells. These antibodies can form immune complexes with antigens or antigen-expressing cells, thereby activating downstream effector mechanisms. (b) Activated B cells act as antigen-presenting cells. Antigens bound by the immunoglobulin receptor on the B cell surface, are processed and antigenic peptides are presented to T cells. Activated T cells on their turn sustain the inflammatory cascade by the production of cytokines and the activation of B cells. (c) B cell-derived cytokines modulate autoimmunity via different pathways, driving persistent inflammatory arthritis. (d) The expression of lymphotoxin on the surface of activated B cells contributes to the formation of ectopic germinal centers in RA-affected joints. Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Drug Discov. 5(7):564-576 copyright 2006 [79]

1.3 Biomarkers

A biomarker, or biological marker, is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [211]. In other words, the presence or features of a biomarker can aid in distinguishing health from disease, making the right diagnosis, or predicting how the disease will progress in particular patients. Moreover, it can tell whether – and to what extent – a patient responds to a certain treatment, or even predict the most efficient therapy before the intervention is initiated. Biomarkers can thus play a pivotal role in optimizing clinical management of RA patients. The performance of a biomarker is generally assessed by its sensitivity and specificity. The sensitivity of a diagnostic marker is defined as the proportion of patients testing positive for the biomarker, while specificity refers to the proportion of non-patients testing negative. An increase in sensitivity is generally accompanied by a loss of specificity and vice versa.

Several factors have been investigated as biomarker in RA disease course [197], such as serum markers of B cell activation (e.g. β2-microglobulin which was found at higher levels in early RA compared to UA [212]) and cytokines (e.g. IL-6 and IL-21 which were associated with higher proportions of autoantibodies and higher levels of B cell activation markers [213]). However, as RA is mediated by a strong humoral response, the next paragraphs focus on the role of autoantibodies in particular, as biomarkers for RA.

1.3.1 Clinical biomarkers for RA

Currently, two serological biomarkers are part of the RA diagnosis: RF and ACPA [8]. RF is the classical biomarker for RA, first described by Waaler in 1939 and rediscovered by Rose in 1948 – nowadays RF is still often referred to as Waaler-Rose [214, 215]. RF was identified as a factor able to agglutinate sheep red blood cells sensitized with rabbit Ig. Later it was found that RF is an antibody directed against the Fc portion of IgG, so in fact an anti-antibody antibody [216]. Binding of RF to Ig-Fc is mediated at an unconventional part of the B cell receptor leaving space for other antigens to be bound and presented to T cells [217]. Immune complexes formed by RF activate B cells and complement thereby triggering the

release of chemotactic factors and recruiting other inflammatory cells [218]. By this pro-inflammatory mechanism of action, RF-positive patients have worse outcome [3, 219]. RF is found in 69-80% of RA patients [3, 220]. Unfortunately, RF is also detected in elderly and in other chronic inflammatory conditions limiting RA-specificity to no more than 85% [220].

Most but not all patients positive for RF, are also positive for ACPA. ACPA made it into the classification criteria in 2010, fifteen years after their actual discovery [8, 221]. In contrast to RF, ACPA are highly specific (>95%) for RA with a sensitivity of approximately 70% [220, 222]. ACPA are antibodies recognizing citrullinated peptides which are formed by the post-translational modification of arginine into citrulline, mediated by PAD enzymes that are probably released in the inflammatory environment [99, 223-225]. Citrullination takes place in a variety of biological processes, but rather than the citrullinated antigens, the development of ACPA has a high RA-specificity [226]. ACPA have also been demonstrated in unaffected relatives of patients with RA, with a different antigen recognition profile [220, 227-231]. ACPA can recognize several citrullinated antigens including citrullinated filaggrin [221, 232, 233], vimentin [72, 73], fibrin [234], fibrinogen [235], type II collagen [236-238], α-enolase [239], Epstein-Barr virus nuclear antigen 1 [240] and many others. Initial tests detected ACPA using citrullinated filaggrin epitopes. Few years later the first enzyme-linked immunosorbent assay (ELISA) was developed using synthetic citrullinated peptides that were cyclized to allow better antibody binding and thus provide a more sensitive measurement of ACPA (CCP). This CCP test was swiftly followed by an upgraded second-generation CCP test (CCP2) commercialized in 2003 [223, 241-243]. Recently, also a third-generation assay (CCP3) was developed and reported to be even more sensitive than the previous assays [244-246]. ACPA are detected years before symptom onset and have even proven to be useful in the prediction of progression from arthralgia and undifferentiated arthritis (UA) to RA [30, 230, 243, 247-249]. Testing for ACPA provides important prognostic information, as ACPA-positive patients are more likely to develop extra-articular manifestations [250, 251] and have more radiological progression [22, 252, 253]. These observations from clinical assessment were supported by the finding that ACPA can directly induce osteoclastogenesis as has been demonstrated for antibodies against citrullinated vimentin [62]. The bad

prognostic outcome for ACPA-positive patients requires aggressive intervention as soon as possible, and clinical studies have demonstrated that ACPA-positive and ACPA–negative patients respond differently to treatment with methotrexate [28, 254] or rituximab [255]. The discovery and characterization of ACPA highlights the heterogeneous character of RA and the need to subclassify RA patients in studying biological pathways and treatment options [31-33].

1.3.2 The serological gap

RF is widely used as diagnostic marker for RA – despite its relatively low specificity – and the more recent discovery of ACPA has greatly improved RA diagnostics. However, still up to one third of RA patients test negative on these currently used serological RA biomarkers [220, 256]. The lack of biomarkers for these autoantibody-negative or "seronegative" patients is referred to as the "serological gap". In early disease stages, this serological gap is even larger than in established RA [222, 257] while at these stages irreversible joint damage already occurs and treatment initiation yields best results. The importance of early diagnosis and intervention has thus become increasingly evident. Obviously, there is a need for additional biomarkers for RA, preferably detectable during or even prior to disease onset. Besides, the characterization of ACPA responses in RA patients has pointed towards different genetic and environmental interactions, pathways and severity of disease. These observations propose that ACPA-positive and ACPA-negative RA are different disease entities [31-33, 229, 258, 259]. This further increases the need for novel biomarkers especially for the ACPA-negative subpopulation, not only to aid in its diagnosis but also to reveal new disease mechanisms. Seronegative patients generally have a milder disease course which initially shifted the research priority towards seropositive disease. The presence of radiological damage in seronegative patients and the lack of insight into seronegative disease, now urges for knowledge and biomarkers for this subgroup [33, 260].

1.3.3 Candidate biomarkers for RA

Over the past decades researchers have intensively looked for additional biomarkers for RA, to complement the sensitivity of RF and ACPA, but also to

assess prognosis and progression of the disease, monitor disease activity, and predict the response to certain therapies.
As a wide variety of antibodies are typically present in RA patients, they have been primarily of interest in the search for biomarkers. The spectrum of antibody specificity differs between patients, pointing towards different disease entities with varying clinical manifestations and severity, and different susceptibility for genetic and environmental factors. As such, ACPA were the first to subclassify RA patients [31-33].

Recently, a novel antibody system has been discovered, anti-carbamylated protein (anti-CarP) antibodies, directed towards another post-translational modification [75, 261]. Carbamylation is the conversion from lysine to homocitrulline by cyanate. The production of cyanate is induced by myeloperoxidase, released by neutrophils in inflammatory conditions. Higher cyanate concentrations have been detected after smoking, the most prominent environmental risk factor for the development of RA [262]. Although structurally homocitrulline resembles citrulline, and some overlap was found for the two antibody systems, ACPA and anti-CarP recognize their own specific peptides. Carbamylated fibrinogen and vimentin have already been described and probably there are many others to be yet identified [73, 263]. Anti-CarP antibodies are found in 45% of RA patients and 16-30% of the ACPA-negative RA patients, and they are associated with more severe joint damage, also within these ACPA-negative RA patients [75].
Besides citrullination and carbamylation, also other enzymatic and chemical modifications create new antigens, further described by Burska *et al* [264]. Autoantibodies are not only formed against modified proteins, but also against the catalyzing enzymes. For instance, antibodies have been described against citrullinating PAD4, specific for RA and associated with disease severity [74]. Interestingly, these antibodies enhance the activation of PAD4 providing a feed-forward loop for their own production [76].
Other antigenic targets in RA that have been described, include antibodies directed against calpastatin, phospholipids, α-enolase, stress proteins (binding immunoglobulin protein (BiP or p68)), G6PI, ribosomal proteins (anti-RA33), cartilage proteins (collagen, fibronectin, cartilage oligomeric matrix protein (COMP)), g v raf murine sarcoma viral oncogene homologue (BRAF) and many others, summarized in [68-71].

Extensive characterization and validation of these candidate biomarkers is required to determine which are likely to become available for clinical use. The simultaneous measurement of diagnostically relevant biomarkers into a multiplex biomarker approach, may further improve the biomarkers' performance in diagnosing RA patients within the heterogeneous disease population [71].

1.3.4 Antibodies to novel UH-RA peptides

Previously, at Hasselt University novel candidate autoantibody biomarkers for RA (UH-RA) were identified by means of serological antigen selection (SAS), a powerful high-throughput screening technology based on phage display (Table 1.1) [265-267]. A cDNA phage display library was constructed from RA synovial tissue. This library was screened for reactivity with antibodies in pooled RA patient sera to select those antigens recognized by the humoral immune system of RA patients. Two pools were created from either early and seronegative RA patients. This approach led to the identification of 22 clones which were screened for immunoreactivity in individual patients with RA or other (non-)inflammatory rheumatic diseases (RC) and healthy controls (HC). Fourteen clones were not detected in HC, from these clones 11 were only present in RA patients (RA-specific) while 3 clones were not only found in RA patients but also some RC (RA-associated) (Table 1.1). Combining either these 11 RA-specific or these 14 RA-associated clones into one diagnostic panel, resulted in a sensitivity of 37% and 54% for RA, with associated specificities of 100% and 90%, respectively.

Table 1.1. The novel candidate UH biomarkers for rheumatoid arthritis

Clone	Sensitivity	Specificity	Size [a]	Homology on amino acid level (Swissprot accession number) [b]
UH-RA.1	10 %	97 %	9	7/9 (77%) T cell specific transcription factor 1 (P36402)
UH-RA.2	2 %	100 %	5	5/5 (100%) DnaJ homolog subfamily B member 6 (O75190)
UH-RA.7	3 %	100 %	5	5/5 (100%) histone acetyltransferase MYST3 (Q92794)
UH-RA.9	4 %	100 %	23	6/9 (66%) T cell surface glycoprotein CD1d precursor (Q9UBG0)
UH-RA.10	7 %	100 %	34	15/16 (93%) Plakophilin-2 (Q99959)
UH-RA.11	13 %	100 %	65	65/65 (100%) MHC class I A-2 (P01892)/A-68 (P01891)/A-69 (P10316)
UH-RA.13	2 %	98 %	7	6/7 (85%) U3 small nucleolar RNA-associated protein 18 homolog (Q9Y5J1)
UH-RA.14	12 %	100 %	7	6/6 (100%) trichohyalin (Q07283)
UH-RA.15	5 %	100 %	49	49/49 (100%) minichromosome maintenance complex component 2 (MCM2) (P49736)
UH-RA.16	5 %	100 %	176	176/176 (100%) 40S ribosomal protein S6 (P62753)
UH-RA.17	3 %	100 %	76	58/58 (100%) selenoprotein P, precursor, SEPP1 (P49908)
UH-RA.20	2 %	100 %	22	6/7 (85%) collagen alpha-2 (VIII) chain precursor (P25067)
UH-RA.21	29 %	95 %	28	7/7 (100%) B cell scaffold protein with ankyrin repeats (Q8NDB2)
UH-RA.22	2 %	100 %	32	10/14 (71%) Transcription factor 15 (Q12870)
11 RA-specific antibodies	37 %	100 %		
14 RA-associated antibodies	54 %	90 %		

[a] size in amino acids. [b] For most peptides only partial protein homologies were found, these probably comprise mimotopes. For short sequences, multiple hits were obtained but only one is reported in this table. UH, Hasselt University. Reprinted with permission from Somers K et al. J Autoimmun. 2011;36:33-46. [267]

Among the candidate biomarkers, the one with the highest sensitivity was the antibody response against UH-RA.21 (Table 1.1), a 28-amino acid sequence (PGGFRGEFMLGKPDPKPEGKGLGSPYIE) resulting from the translation of a normally untranslated region (3'UTR) of messenger RNA. UH-RA.21 probably constitutes a mimotope, an epitope mimicking an *in vivo* antigen, and the identity of the corresponding antigen is unknown [265, 268]. A mimotope and its corresponding antigen are not necessarily identical or similar at amino acid level, although sequence similarity is not excluded. For UH-RA.21, homology on protein level was found for, for instance, B cell scaffold protein with ankyrin repeats (7/7 amino acids, 100%), Epstein-Barr virus induced 3 protein (7/8 amino acids, 87%), La-related protein 1 (8/10 amino acids, 80%) and Islet cell autoantigen 1 (7/9 amino acids, 77%). Antibodies against UH-RA.21 were found in 29% of the RA patients with a 95% RA-specificity [267]. Importantly, UH-RA.21 resulted from screenings on both early and seronegative plasma pools and anti-UH-RA.21 antibodies were detected in seronegative RA patients and in early disease, suggesting a promising potential for the improvement of RA diagnostics [267]. The antibodies were present in both plasma and synovial fluid samples from RA patients. Moreover, immunohistochemical staining with human affinity-purified polyclonal anti-UH-RA.21 antibodies showed expression of the antigenic target at sites of inflammatory cell infiltration and in the hyperproliferating synovial lining [267]. For the remaining candidate biomarkers, individual sensitivities ranged from 2% to 13% with RA-specificities from 97% to 100% (Table 1.1). The combination of individual markers into one panel resulted in a sum of individual sensitivities, preserving a high specificity. These results support the biomarkers' potential in a multiplex biomarker approach. Further validation screenings are warranted to accurately define diagnostic and prognostic potential of the antibody responses against the novel UH-RA peptides. Characterization of the antibody responses might reveal a putative role in rheumatic disease mechanisms.

1.4 Aims of the study

In the search for biomarkers for RA, novel candidate antibody markers to UH-RA peptides as presented above, were identified using SAS based on phage display technology [266, 267]. The antibodies were also detected in some RA patients in early disease and negative for the current diagnostic markers RF and ACPA. The goal of this study is to further investigate the diagnostic and prognostic potential of the anti-UH-RA autoantibodies in RA, and to characterize their biological properties. Candidate biomarkers were selected based on their prevalence in early and seronegative RA, and constituting small phage-displayed peptides: UH-RA.1, UH-RA.9, UH-RA.10, UH-RA.14 and UH-RA.21.

Table 1.2. Peptide clones selected for further validation

Clone	Peptide sequence	Size (aa)
UH-RA.1	EKRQEITTE	9
UH-RA.9	RSCHHGCTFTEDQHWECGEDDAV	23
UH-RA.10	SNALENFVYNKFQQNNCVWPGAVAHACNPSTLRG	34
UH-RA.14	KEELWRQ	7
UH-RA.21	PGGFRGEFMLGKPDPKPEGKGLGSPYIE	28

aa, amino acids

AIM 1: Translate the phage-mediated research test into a clinically applicable format

To study the prevalence of the anti-UH-RA autoantibodies in larger cohorts and evaluate their diagnostic and prognostic potential, it is important to have reliable and robust test assays. Explorative screenings after initial discovery were performed by phage ELISA, a lab technique which is unfortunately not suitable for clinical laboratories. The use of phages requires specific safety measures and is very labor intensive. Moreover, phage ELISA are characterized by relatively high background reactivities, high inter- and intra-assay variability and therefore low reproducibility. The first step in this project is to develop sensitive assays to test for the presence of the autoantibodies (**Chapter 2**). The phage ELISA will be replaced by peptide ELISA in which only the antibody-binding part expressed on the phages will be retained, the corresponding sequences will be produced as synthetic peptides and applied in ELISA.

AIM 2: Validate the diagnostic and prognostic potential of the novel autoantibodies

Using the improved assays, we will investigate the prevalence of the novel antibodies in RA patients and controls. In **Chapter 3**, two large cohorts will be implemented in the validation of the candidate biomarkers. A first cohort of RA patients, rheumatic controls and healthy controls, is assembled in collaboration with Belgian rheumatology clinics. The second cohort is the Dutch Early Arthritis Clinic (EAC) cohort, a large cohort provided by the Leiden University Medical Center which allows us to study the autoantibodies in the early stages of RA. The diagnostic value of the autoantibodies will be evaluated and putative links with prognostic information will be addressed. The collection of UA patients in the EAC cohort will demonstrate whether the antibodies can aid in the prediction of progression from UA to RA. Whether fluctuations in levels of the UH-RA antibodies, together with RF and ACPA, can be detected and potentially linked to disease activity, will be investigated during a longitudinal study in **Chapter 4**.

AIM 3: Characterize the biological role of antibodies towards the UH-RA peptides

Antibody biomarkers are not only useful in clinical testing for diagnosis or treatment decisions. The production of these antibodies can be related to a secondary effect of specific disease mechanisms, or the antibodies can be actively involved in the pathogenesis. Characterizing antibodies found in RA patients will provide insight into the underlying etiology of RA. As pathogenic properties of antibodies are partially determined by their corresponding Ig class, the isotype profile of the novel antibodies will be studied in **Chapter 5**. And in **Chapter 6** the biological role of the antibodies will be addressed by investigating the *in vivo* effects in animal models for RA.

2

DEVELOPMENT AND OPTIMIZATION OF A SENSITIVE PEPTIDE ELISA FOR AUTOANTIBODY TESTING IN RHEUMATOID ARTHRITIS

Abstract

Using serological antigen selection (SAS), 14 novel candidate autoantibody markers were discovered that can be detected in early and seronegative RA patients [267]. So far, screening for plasma antibodies were performed by means of phage enzyme-linked immunosorbent assays (ELISA). The goal of this study, was to translate the phage ELISA into sensitive peptide ELISA, which are easy reproducible and fit for large-scale screening. Synthetic peptides corresponding to phage-displayed peptides, were applied in competition ELISA with phage clones, in order to confirm specific target recognition. After successful competition between phage displaying the specific peptide of interest and the corresponding synthetic peptide, the synthetic peptide was implemented in a solid-phase ELISA. Clones were selected based on presence in early and seronegative RA and constituting small phage-displayed peptides: UH-RA.1, UH-RA.9, UH-RA.10, UH-RA.14 and UH-RA.21 [267]. To illustrate our approach, results reported in this chapter focus on the most sensitive candidate marker, antibodies against UH-RA.21. For this clone, synthetic peptides covering overlapping fragments or the full sequence were implemented. Although one fragment located in the middle of the sequence was successful in competition assays, the full length sequence performed best in a solid-phase peptide ELISA, not markedly improved by cyclization, citrullination or biotinylation of the peptide. Following similar strategies, peptide ELISA were also successfully developed and optimized for UH-RA.1, UH-RA.9 and UH-RA.14. Interestingly, one harmonized protocol was developed for the different markers facilitating simultaneous testing and favoring the feasibility to combine them in a multiplex biomarker panel. Although the peptide ELISA need further fine-tuning on their way into clinic, the current assays are found highly suitable as robust test assays in order to measure antibodies in a research setting. They will serve as valuable tests in further large-scale screenings of validation cohorts.

2.1 Introduction

For the diagnosis of rheumatoid arthritis (RA) two serological markers are currently used, namely rheumatoid factor (RF) and antibodies directed against citrullinated proteins (ACPA). However, one third of established RA patients are seronegative for these two markers, and the sensitivity of the markers is lower in early stages of the disease [220, 222, 256, 257]. Previously, 14 novel candidate autoantibody markers were discovered by serological antigen selection (SAS), a phage display technology [266, 267]. These novel autoantibodies could be detected in early and seronegative RA by use of enzyme-linked immunosorbent assays (ELISA) [267]. ELISA is a powerful method to detect antibodies or other specific proteins in a complex mixture, even when they are present in very small quantities [269]. For the detection of antibodies in serum, the antigen is immobilized directly onto a surface or by a capturing antibody (sandwich ELISA), after which target-specific antibodies are allowed to bind. These antibodies themselves are then detected using an enzyme-labeled secondary antibody. Addition of a substrate causes a visible color change or fluorescence, read by a spectrophotometer and translated into a quantitative output. ELISA is a valuable tool in both basic and clinical research, and in clinical diagnostics [269]. Within the rheumatology field, it plays a major role in the detection of ACPA [222]. Also in the discovery of novel candidate biomarkers for RA, autoantibody reactivity was detected using phage ELISA [265, 267], in which the phages displaying the antibody targets are immobilized by an anti-phage capture antibody (Figure 2.1A). Unfortunately, phage ELISA are very labor intensive and the use of phages requires specific safety measures, making this format not suitable for clinical laboratories. Furthermore, we observed a relatively high background reactivity, high inter- and intra-assay variability and therefore low reproducibility. To this end, we aimed at translating the original phage ELISA into a more robust peptide ELISA. Within a peptide ELISA, only the antibody-binding part expressed on the phage will be retained (Figure 2.1B).

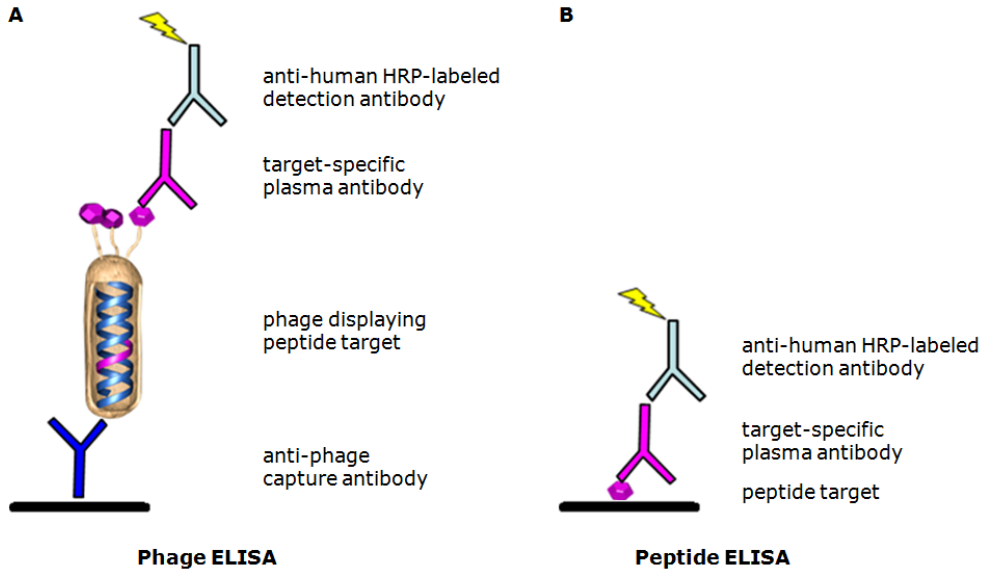

Figure 2.1. Schematic representation of two different enzyme-linked immunosorbent assays (ELISA). (A) Using a phage ELISA, the phage is captured by an anti-phage antibody coated on a solid surface. Target-specific antibodies present in patients' plasma bind the phage-displayed antigen. Bound antibodies are detected using an enzyme-labeled detection antibody. (B) To improve antibody detection, the phage ELISA can be translated into a more reproducible and robust peptide ELISA in which a synthetic peptide corresponding to the expressed cDNA products on the phage, are directly immobilized on an assay plate. HRP, horseradish peroxidase

To confirm specific recognition of phage-displayed peptides, synthetic peptides are used in competition ELISA with phage clones. After successful competition between phage displaying the specific peptide of interest and the corresponding synthetic peptide, peptide ELISA are developed. The development of peptide ELISA requires optimization of the peptide immobilization and of the antigen-antibody interaction. Peptides can be immobilized by passive adsorption through hydrophobic and ionic interactions between the peptide and the surface, or by a more direct approach using biotin-labeled antigens that interact with streptavidin-coated plates and orientate the antigen for optimal antibody-binding. Further optimization concerns different materials (ranging from assay plates to antibodies), the composition and pH of buffers used for coupling,

washing, blocking and binding, the duration and conditions of the incubation steps, and the detection method applied (enzyme, substrate and signal detection). To evaluate the performance of the newly developed assays, results will be compared to phage ELISA outcomes from the original screening [267]. Among the clones constituting small phage-displayed peptides, were UH-RA.1, UH-RA.9, UH-RA.10, UH-RA.14 and UH-RA.21, selected based on their prevalence in (early and seronegative) RA in exploratory screenings, for the development of peptide ELISA. To illustrate our approach in the development and optimization of the assays, we here mainly describe the results obtained for UH-RA.21. UH-RA.21 is a 28-amino acid sequence constituting a mimotope, an epitope mimicking an *in vivo* antigen of which the identity is yet unknown [267]. The autoantibody response against UH-RA.21, yielded the highest RA-sensitivity (29%) with an associated specificity of 95%, and was detected in early and seronegative RA patients [267]. For the other candidate biomarkers a similar approach was implemented to translate phage ELISA into peptide ELISA.

2.2 Materials and methods

2.2.1 Peptide materials

Synthetic peptides corresponding to the phage-displayed peptides were commercially obtained (GL Biochem, Shanghai, China) (Table 2.1). For the shorter peptides UH-RA.1 and UH-RA.14, and for UH-RA.10, eight additional N-terminal amino acids (aa) from the phage vector backbone were included (italic sequence).

Table 2.1. Peptide sequences corresponding to the phage displayed clones

Clone	Peptide sequence	Size (aa)
UH-RA.1L*	*GLQEFGTR*EKRQEITTE	17
UH-RA.9	RSCHHGCTFTEDQHWECGEDDAV	23
UH-RA.10	SNALENFVYNKFQQNNCVWPGAVAHACNPSTLRG	34
UH-RA.10L*	*PSRPDLLE*SNALENFVYNKFQQNNCVWPGAVAHACNPSTLRG	42
UH-RA.14L*	*PSRPDLLE*KEELWRQ	15
UH-RA.21	PGGFRGEFMLGKPDPKPEGKGLGSPYIE	28
Control	WTKTPDGNFQLGGTEP	16

* L, linker. Eight additional N-terminal amino acids from the phage vector backbone were included (italic sequence). aa, amino acids

For UH-RA.21, overlapping peptide sequences were tested next to the full sequence (Table 2.2), together with a citrullinated peptide in which the arginine residue at the fifth position was replaced by a citrulline residue: PGGF**R**GEFMLGKPDPKPEGKGLGSPYIE. In order to improve the antigen-antibody interaction, the full length sequence was also biotinylated at the N-terminal end (with or without a spacer) or cyclized (N- and C-termini joined together) (GL Biochem). These modified peptides were included in the competition assays.

Table 2.2. Peptide variants corresponding to UH-RA.21

Peptide variant	Peptide sequence	Size (aa)
UH-RA.21-P1	PGGFRGEFMLGKP	13
UH-RA.21-P2	EFMLGKPDPKPEG	13
UH-RA.21-P3	PDPKPEGKGLGSP	13
UH-RA.21-P4	GKGLGSPYIE	10
UH-RA.21 unmodified	PGGFRGEFMLGKPDPKPEGKGLGSPYIE	28
UH-RA.21 cyclized*†	PGGFRGEFMLGKPDPKPEGKGLGSPYIE	28
UH-RA.21 citrullinated°	PGGF**R**GEFMLGKPDPKPEGKGLGSPYIE	28
UH-RA.21 biotinylated*	Biotin-PGGFRGEFMLGKPDPKPEGKGLGSPYIE	28
UH-RA.21 biotinylated with spacer*‡	Biotin-$[PEG]_6$-PGGFRGEFMLGKPDPKPEGKGLGSPYIE	28

* Identical modifications were made to the control peptide (WTKTPDGNFQLGGTEP, 16 amino acids)

† N- and C-termini joined together

° The arginine residue at the fifth position was replaced by a citrulline residue

‡ A polyethylene glycol (PEG) spacer was placed between the biotin molecule and the peptide in order to increase the accessibility of the peptide sequence for the interacting antibodies

2.2.2 Competition assays

The specificity of the measured antibodies against phage-displayed peptides was confirmed in a competition assay between synthetic peptide and phage displaying the corresponding peptide. The phage ELISA was performed as described previously [267, 270, 271] with minor modifications and an additional pre-incubation step of plasma samples with the synthetic peptides (Figure 2.2).

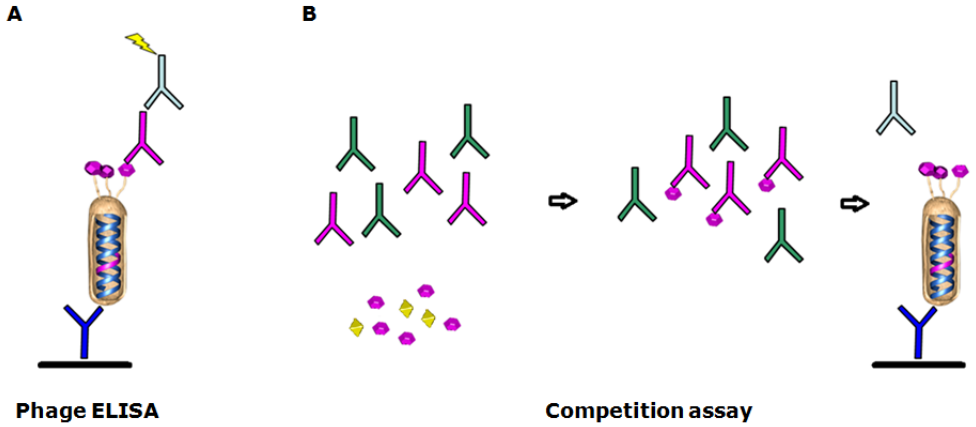

Figure 2.2. Schematic representation of competition enzyme-linked immunosorbent assays (ELISA). (A) The original phage ELISA detects antigen-specific antibodies which themselves are bound by an enzyme-linked detection antibody. (B) The phage ELISA is implemented in a competition assay in which the samples containing plasma antibodies are pre-incubated with synthetic peptides. These synthetic peptides compete with the phage-displayed peptides for binding the sample antibodies. Specific peptides will thus block the ELISA signal. This procedure is also conducted with control peptide to confirm the specificity of the antibody recognition.

Briefly, ninety-six-well flat-bottom microtiter plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 5 µg/mL anti-M13 antibody (GE Healthcare, Diegem, Belgium) in 0.1 M sodium hydrogen carbonate buffer, pH 9.6. Plates were washed twice with phosphate-buffered saline (PBS, 50 mM Tris, 150 mM sodium chloride, pH 7.5) and blocked with PBS containing 5% (w/v) skimmed milk powder (MPBS) for 2 hours (h) shaking at 37°C. Meanwhile, plasma samples were pre-incubated with increasing amounts of synthetic peptides in solution (PBS) for 2 h shaking at room temperature (RT). Phages precipitated overnight with 20% polyethylene glycol (PEG)-6,000 and 2.5 M sodium chloride at 4°C, were diluted to a concentration of $7x10^{11}$ colony-forming units per mL, applied onto the anti-M13 antibody-coated plates and incubated for 1 h at 37°C followed by 30 minutes (min) shaking at RT. The pre-incubated sample-peptide mix was applied onto the ELISA plates and incubated for 1 h at 37°C followed by 30 min shaking at RT. Antibody binding was detected using a

polyclonal goat anti-human IgG secondary antibody mouse-adsorbed and conjugated to horseradish peroxidase (HRP) (Invitrogen, Merelbeke, Belgium), 100 µL/well diluted 1:2,000 in 2% MPBS for 1 h shaking at RT. Staining was performed in the dark with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific, Erembodegem, Belgium). After 11 min, the reaction was stopped with 50 µL 2N H_2SO_4 and results were read at 450 nm (Microplate reader Infinite M1000 Pro, TECAN, Männedorf, Switzerland). Unless mentioned otherwise, plates were washed in between steps, for three consecutive times during 5 min with PBS containing 0.1% (v/v) Tween-20 (VWR, Leuven, Belgium), and once with PBS.

2.2.3 Solid-phase peptide ELISA formats

Peptides emerging as specific antibody targets based on competition assays, were applied in a solid-phase ELISA format. Unmodified and cyclized peptides were immobilized on polystyrene flat-bottom microplates (Greiner Bio-One) by passive adsorption. These peptides were coated in concentrations ranging from 0-8 µg/mL PBS overnight at RT. The biotinylated peptides were captured by streptavidin-coated flat-bottom microplates (Nunc Immobilizer Streptavidin, Thermo Scientific). Biotinylated peptides were coated at 0-1 µg/mL PBS for 1 h at RT. The subsequent procedure went according to a standard peptide ELISA protocol as described below. Samples were selected based on their reactivity on phage ELISA – either high-positive (>3x cutoff), low-positive (>1x cutoff) or negative according to a cutoff, as defined previously [267]. These samples were tested for reactivity on the different formats to determine which format performs best regarding sensitivity, background reactivity and usability. Of note, all samples were tested on both the specific peptide (P) and the irrelevant control peptide (C).

The peptide ELISA protocol was further optimized resulting in the following final protocol: Synthetic peptides were coated overnight at RT at 1 µg/mL in PBS (1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 130 mM NaCl) in ELISA plates (polystyrene flat-bottom microplates, Greiner Bio-One). Plates were blocked with 200 µL/well of 2% MPBS for 2 h shaking at 37°C. Samples (diluted 1:100 in 2% MPBS) were incubated for 2 h at RT (100 µL/well, shaking). Antibody binding was detected

using a polyclonal rabbit anti-human IgG secondary antibody conjugated to HRP (Dako, Heverlee, Belgium), 100 µL/well diluted 1:2,000 in 2% MPBS for 1 h shaking at RT. Staining was performed in the dark with 100 µL TMB (Thermo Scientific). After 6 min, the reaction was stopped with 50 µL 2N H_2SO_4 and results were read at 450 nm (Microplate reader Infinite M1000 Pro, TECAN). Washing steps were performed with PBS containing 0.05% (v/v) Tween-20 (VWR), during 5 min for three consecutive times. All samples were tested in duplicate within one experiment and experiments were performed independently twice at least.

2.2.4 Evaluation of the new test assays

In order to verify the performance of the new peptide ELISA, a small population originating from the explorative screenings by phage ELISA was tested. This population included 40 RA patients, 30 rheumatic controls (RC) and 30 healthy controls (HC) [267]. Results from both approaches were compared in order to determine whether the phage ELISA are successfully translated into peptide ELISA, and the peptide format is sensitive and accurate in measuring antibody reactivity.

2.3 Results

2.3.1 Competition assays

To translate phage into peptide ELISA, the specific target of the antibodies has to be determined. The specificity of the antibody interaction with phage-displayed peptides and corresponding synthetic peptides, was confirmed in competition ELISA, in which prior incubation with specific synthetic peptides was shown to decrease the signal in the phage ELISA, in contrast to prior incubation with irrelevant control peptide. Competition was absent when using an antibody-negative sample according to phage-ELISA testing.

The results for the UH-RA.21 clone are shown in Figure 2.3. Overlapping peptide sequences were tested in an effort to narrow down the epitope (Figure 2.3A-D), and pre-incubation steps were conducted with peptides ranging from 0 to 100 µg/mL. Fragments located at the beginning or end of the sequence were not competitive at all (P1 and P4, Figure 2.3A and 2.3D, respectively) while one other fragment caused limited competition at high concentrations (P3, Figure 2.3C): the specific signal of the antibody-positive sample was reduced with 28% after pre-incubation with 100 µg peptide per mL. This inhibition was not merely specific as a 17% competition was also observed when pre-incubating with a control peptide. A final fragment (P2), located in the middle of the original full sequence, was able to block up to 90% of the signal at peptide concentrations of 75 µg/mL or more (Figure 2.3B). Furthermore, successful competition was achieved by applying the full length sequence, but at considerably lower peptide concentrations compared to the short sequence from P2 (Figure 2.3E-H). For comparison, to reach a 50% competition, a peptide concentration of 0.16 µg/mL was sufficient for the full length sequence, while >2 µg/mL was required using P2. A cyclic version of the full length peptide was successful at even lower concentrations (<0.04 µg/mL for 50% competition) (Figure 2.3F), while citrullination (Figure 2.3G) and biotinylation (Figure 2.3H) of the full length sequence did not seem to influence the antibody interaction in solution – competition was similar to using the unmodified full length.

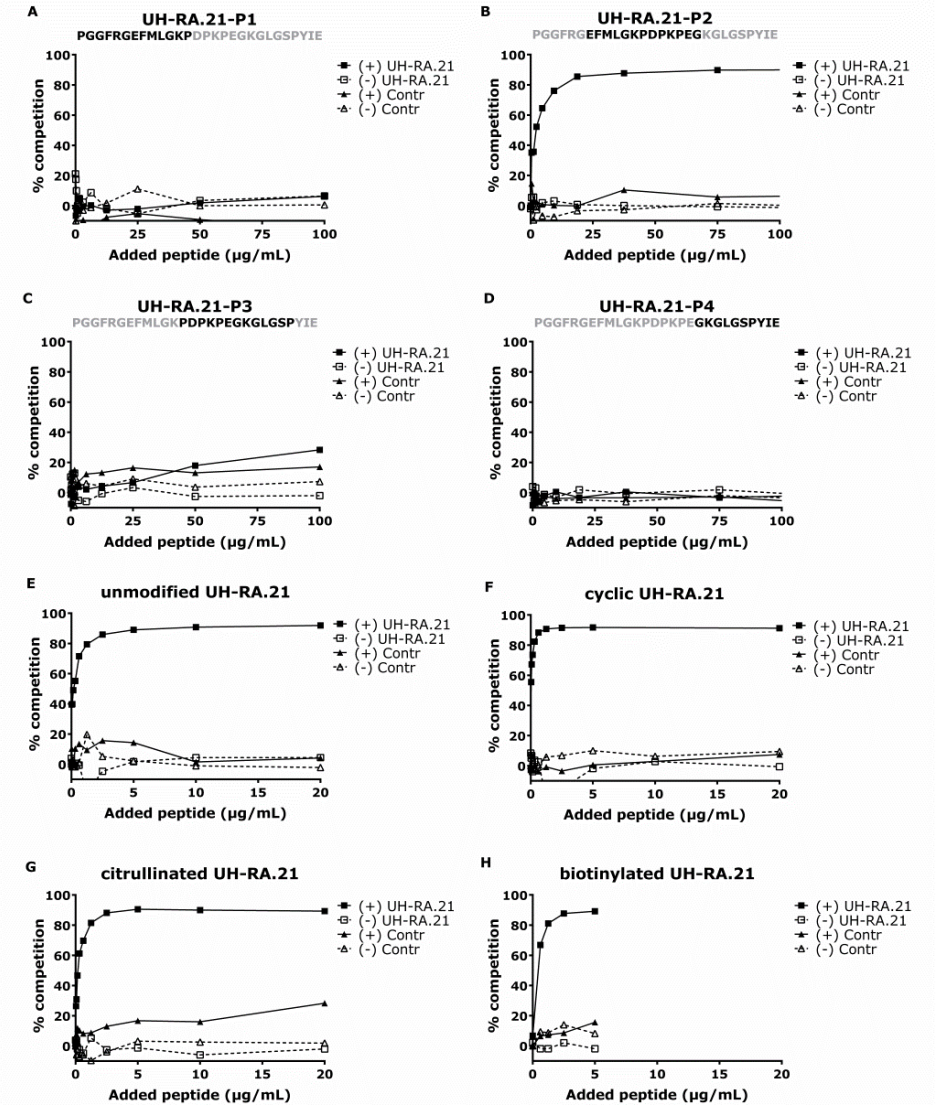

Figure 2.3. Competition assays for peptides related to UH-RA.21. The specificity of the anti-UH-RA.21 antibody interaction with phage-displayed peptides and corresponding synthetic peptides, was confirmed in competition enzyme-linked immunosorbent assays (ELISA), in which prior incubation with specific synthetic peptides was shown to decrease the signal in the phage ELISA, in contrast to prior incubation with irrelevant control peptide (Contr). Synthetic peptides were tested corresponding to overlapping sequences (A-D) and the full length peptide either unmodified (E), cyclized (F), citrullinated (G) or biotinylated (H). Both antibody-positive (+) and –negative (-) plasma samples were applied in competition assays.

Also for the other candidate biomarkers, competition assays were performed (data not shown). For the short sequences UH-RA.1 (9 aa) and UH-RA.14 (7 aa), eight additional aa from the phage vector backbone were included. Successful competition was accomplished for UH-RA.1, UH-RA.9 and UH-RA.14. No competition was observed using UH-RA.10 peptide, even after the addition of a part of the phage vector backbone sequence.

2.3.2 Solid-phase peptide ELISA

We demonstrated that the full length sequence of UH-RA.21 and one of its fragments (P2) were successful in blocking the phage ELISA signal by binding their corresponding antibody targets. Next, they were applied in a solid-phase peptide ELISA. We evaluated their accuracy in the measurement of samples with a negative, low-positive and high-positive signal in the phage ELISA for anti-UH-RA.21 antibodies. Concurrently, the samples were also tested for non-specific reactivity (control peptide). Although UH-RA.21-P2 was able to detect samples with high-positive reactivity, no signal was observed for low-positive samples (Figure 2.4A) even at higher peptide coating concentrations ranging up to 8 µg/mL (not depicted on graph). In contrast, the full length peptide detected both low- and high-positive reactivity (Figure 2.4B), at lower peptide concentrations (starting from 0.5 µg/mL), with a slightly higher signal obtained using the cyclic peptide (Figure 2.4C).

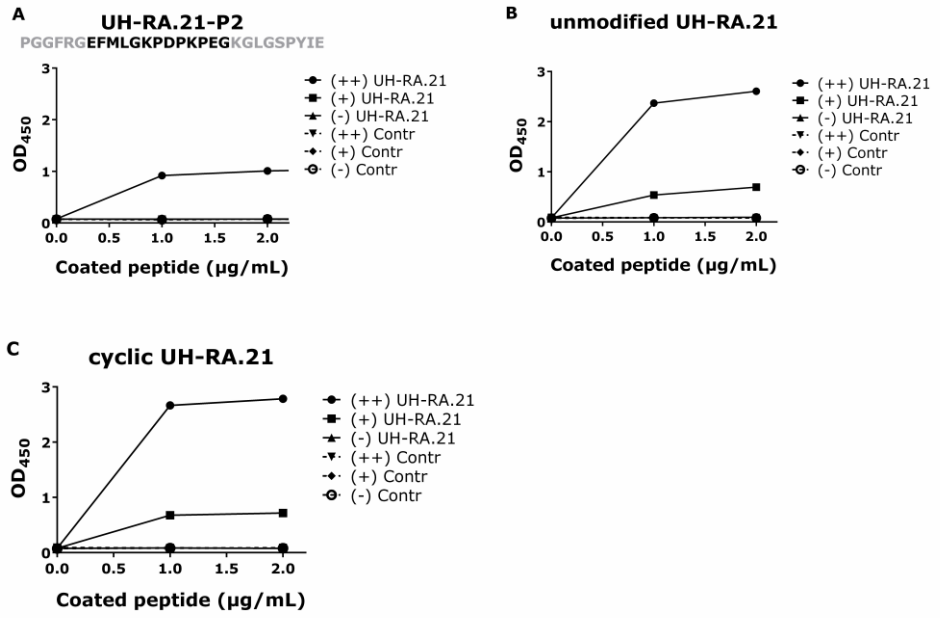

Figure 2.4. Implementation of competitive peptides in a solid-phase enzyme-linked immunosorbent assay (ELISA). The sensitivity of the competitive peptides in detecting specific antibody reactivity was tested in plasma samples high-positive (++), low-positive (+) and negative (-) for antibodies against UH-RA.21 according to phage ELISA. Reactivity was measured against the peptide fragment UH-RA.21-P2 (A), the unmodified full length sequence (B) and the cyclic full length peptide (C) coated at different concentrations. The specificity of the antibody reactivity was verified by simultaneous measurement of reactivity against a control peptide (Contr). Antibody reactivity is expressed as the optical density (OD) signal at a wavelength of 450 nm.

Based on these preliminary tests, the full length peptide thus appeared most effective in detecting antibody reactivity directed against UH-RA.21. To further evaluate the performance of the peptide ELISA format, we tested 100 patient samples selected from previous phage screenings. We also investigated whether cyclization of the peptide or the use of a biotinylated peptide on streptavidin-coated plates, improved antibody binding. Results shown in Figure 2.5 demonstrate that, compared to phage ELISA, the signal is stronger with peptide testing. Specific and non-specific signals were more easily distinguished. The specific signal is higher when a cyclic or biotinylated peptide is used. Notably,

higher non-specific reactivity and slightly more polyreactive samples were encountered using the biotinylated format (data not shown).

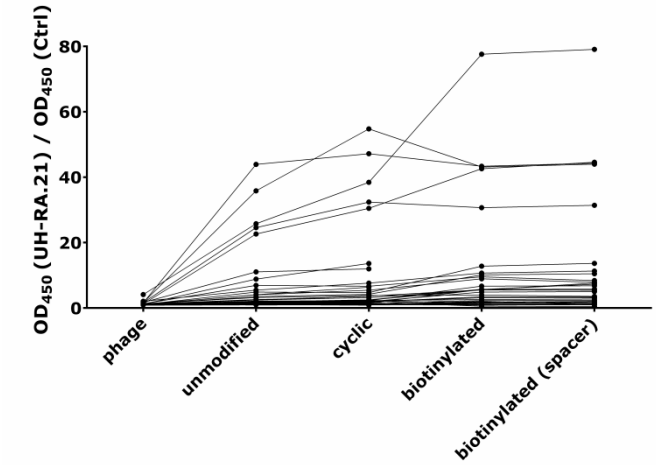

Figure 2.5. Comparison of signal detection between phage and peptide enzyme-linked immunosorbent assays (ELISA) for antibody reactivity against UH-RA.21. The performance of phage and peptide ELISA for the detection of antibodies against UH-RA.21, was compared in 40 rheumatoid arthritis patients and 60 controls. Antibody reactivity is expressed as the ratio of the optical density (OD) signal at 450 nm for the UH-RA.21 sequence to control reactivity.

Besides UH-RA.21, also UH-RA.1, UH-RA.9 and UH-RA.14 were successfully applied in solid-phase peptide ELISA and the protocols were optimized and harmonized into the final protocol described in Section 2.2.3.

2.4 Discussion

Novel candidate biomarkers for RA have been identified using a technique based on phage display, the phages express peptides which are targeted by antibodies present in RA patients. Previously, screenings were performed by phage ELISA in which the target-expressing phage was captured onto a surface using an anti-phage antibody [267]. However, specific signals measured by phage ELISA are low and often accompanied by relatively high non-specific signals. Moreover, inter- and intra-assay variability is generally high. Phage ELISA are therefore not convenient for screenings on large sample populations. The labor intensiveness that comes along with the use of phages, together with specific safety measures further debilitates its suitability in clinical laboratories. Therefore, we aimed to transform the phage ELISA into a more reproducible, sensitive peptide ELISA. This was accomplished by testing synthetic peptides corresponding to phage-displayed peptides in competition assays and applying the successful peptides in a solid-phase format. For the first clone UH-RA.21, successful competition was demonstrated using different peptides. Pre-incubation of plasma samples with the full length sequence, either unmodified or cyclized, or a partial sequence UH-RA.21-P2, blocked most of the signal in the phage ELISA. Competition by the peptide sequence UH-RA.21-P2, located in the middle of the full sequence, reveals interesting information on role of this particular sequence in the antigen-antibody interaction. Nevertheless, the fragment is unlikely to contain the entire epitope targeted by anti-UH-RA.21 antibodies, since huge amounts of peptide were required to cause competition in the phage ELISA. Moreover, in a solid-phase format UH-RA.21-P2 failed to detect samples with low-positive antibody reactivity according to phage ELISA. The full length on the other hand, was able to measure both high and low antibody reactivity. Cyclization and biotinylation of the peptide slightly increased the specific signals. Given the higher occurrence of non-specific reactivity and the high costs associated with streptavidin-coated plates, the full length sequence was preferably selected for further optimization, in its unmodified simplest form. Whereas the unmodified peptide is convenient for measuring antibody reactivity in plasma samples of patients and controls on a large scale, the cyclic peptide can be easily applied when higher signals are preferred, and in a similar approach as the one currently optimized.

Although not extensively elaborated in this chapter, the development of peptide ELISA has also been successfully accomplished for the other clones UH-RA.1, UH-RA.9 and UH-RA.14. Interestingly, all peptide ELISA work according to the same protocol which facilitates simultaneous testing. For UH-RA.10 no competition was observed, even after elongation with eight aa from the phage vector backbone. The peptide sequences applied, probably did not cover the entire epitope.

On its way into clinic, the peptide ELISA require further optimization and extensive validation, with well-defined reference values and quality control, but the current assays are found suitable as non-commercial robust test assays in order to measure antibodies in a research setting. The finding that antibodies against different targets can be detected using harmonized protocols, favors the feasibility to combine different markers into one multiplex biomarker panel.

To conclude, we report the development and optimization of sensitive non-commercial peptide ELISA for the measurement of plasma antibodies directed against UH-RA.1, UH-RA.9, UH-RA.14 and UH-RA.21. Compared to the original phage ELISA, the new assays are less time consuming, can better discriminate between specific and non-specific reactivity, and generate reproducible results. These new assays are convenient for large-scale screenings and can serve as a useful semi-quantitative tool to validate the diagnostic potential of the candidate biomarkers in the RA population.

3

AUTOANTIBODIES TO TWO NOVEL PEPTIDES IN SERONEGATIVE AND EARLY RHEUMATOID ARTHRITIS

Based on:
De Winter L, Hansen W, van Steenbergen H, Geusens P, Lenaerts J, Somers K, Stinissen P, van der Helm-van Mil A, Somers V
Autoantibodies to two novel peptides in seronegative and early rheumatoid arthritis
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Abstract

Despite recent progress in biomarker discovery for rheumatoid arthritis (RA) diagnostics, still over one third of RA patients – and even more in early disease – present without rheumatoid factor (RF) or antibodies against citrullinated proteins (ACPA). Here we report large-scale screening of previously identified autoantibodies to novel Hasselt University (UH) peptides in early and seronegative RA. Screening for antibodies against novel UH peptides UH-RA.1, UH-RA.9, UH-RA.14 and UH-RA.21, was performed in two large independent cohorts. Peptide enzyme-linked immunosorbent assays (ELISA) were developed to screen for the presence of antibodies to UH-RA peptides. First, 292 RA patients (including 39 early RA patients), 90 rheumatic (RC) and 97 healthy controls (HC) of UH were studied. Antibody reactivity to two peptides (UH-RA.1 and UH-RA.21) was also evaluated in 600 RA patients, 309 patients with undifferentiated arthritis (UA) and 157 RC from the Leiden Early Arthritis Clinic (EAC) cohort. In both cohorts, 38% of RA patients were seronegative for RF and ACPA. Testing for autoantibodies to UH-RA.1 and UH-RA.21 reduced the serological gap from 38% to 29% in the UH cohort and from 38% to 32% in the EAC cohort. Furthermore, 19-33% of early RA patients carried antibodies to these peptides. The specificities of the peptides in RC ranged from 82-96%. Whereas antibodies against UH-RA.1 were related to remission, anti-UH-RA.21 antibodies were associated with inflammation, joint erosion, and higher tender and swollen joint counts. This study validates the presence of antibody reactivity to novel UH-RA peptides in seronegative and early RA. This might reinforce current diagnostics and improve early diagnosis and intervention in RA.

3.1 Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease, characterized by a chronic inflammation of synovial joints, which may result in joint destruction [3]. The association between rheumatoid factor (RF) and RA has been known since decades, and antibodies against citrullinated proteins (ACPA) are known since the late 1990s. Whereas ACPA is more RA-specific than RF, sensitivities of both autoantibodies are similar (around 70%) [3, 220, 222, 256]. Both markers are less sensitive in early disease than in established RA [222, 257]. Also, they often occur together leaving one-third of RA patients behind, the so-called serological gap. The current lack of other markers for this seronegative subgroup and the major role of RF and ACPA serology in the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) RA classification criteria from 2010, impede diagnosis of this RA subtype. This is accompanied by a therapeutic delay further bolstered by the (mis)conception that seronegative RA is a mild disease. Ajeganova and Huizinga recently expounded on these features of seronegative disease, emphasizing the importance of accurate and timely intervention in seronegative RA patients [33]. The question remains whether these RF-negative ACPA-negative patients are truly autoantibody-negative or whether they harbor other not yet identified RA-related autoantibodies. Unraveling novel autoantibodies is not only relevant for early diagnosis and prognosis, but also for a better understanding of disease-related processes, especially for seronegative disease of which we currently have least comprehension.

Our research group previously identified autoantibodies to novel peptides in RA using serological antigen selection based on cDNA phage display [266, 267]. An RA synovial cDNA library was screened for antibody reactivity in two pools of RA patients: seronegative patients and patients with early disease. A first panel of four peptides – UH-RA.1, UH-RA.9, UH-RA.14, UH-RA.21 – was selected for further exploration based on prevalence in the original cohort and presence in early and/or seronegative RA.

The aim of the present study was to validate autoantibody reactivity to these peptides, especially in early and seronegative RA. Therefore, a large-scale screening was performed, of RA patients included in two European cohorts.

3.2 Materials and Methods

3.2.1 Study population

Cohort from Hasselt University

This study was approved by the Medical Ethical Committee of Hasselt University (UH) and informed consent was obtained from all subjects. A total of 382 patients, of which 292 UH-RA patients and 90 rheumatic controls (RC), were included at three rheumatology clinics located near Hasselt, Belgium. Samples were collected between 2003 and 2012. RA diagnosis was based on fulfillment of the 1987 criteria for RA [17]. Within the RA group, 39 patients were diagnosed not more than one year ago and classified as early RA patients. Also, a group of 97 healthy controls (HC) was included, aged older than 18 years, in good health at sampling date and without any underlying chronic illness. Samples were stored in the University Biobank Limburg. RF-serology was evaluated with the RF Latex Reagent (Olympus/Beckman Coulter, Analis SA, Suarlée, Belgium; upper limit of normal (ULN) 14 units/mL), the RF-II Cobas C system (Roche, Vilvoorde, Belgium; ULN 14 units/mL), or by means of the Serodia RA test (Fujirebio Europe NV, Ghent, Belgium). ACPA-testing was performed using the Phadia EliA CCP assay (CCP2; Thermo Scientific, Erembodegem, Belgium) or the QUANTA Lite CCP3 IgG ELISA (INOVA Diagnostics Inc, San Diego, USA; ULN 19 units).

Leiden Early Arthritis Clinic cohort

The antibody response against UH-RA.1 and UH-RA.21 was further investigated by blinded screening of a second cohort, consisting of 600 RA patients, 309 undifferentiated arthritis (UA) patients and 157 RC from the Leiden Early Arthritis Clinic (EAC) cohort [272]. Stored samples obtained at baseline were used. The patients included in the EAC cohort had clinical arthritis of >1 joint and a symptom duration <2 years. All RA patients fulfilled the 1987 ACR criteria for RA within one year of follow up, 55% fulfilled the RA criteria already at baseline. Presence of RF was demonstrated by means of ELISA, ACPA-testing was performed using the anti-CCP2 test (Euro-Diagnostica, Nijmegen, the Netherlands; ULN 25 units/mL). The overlap was examined between antibody reactivity to UH-RA peptides and anti-CarP antibodies [75].

3.2.2 Screening for antibody reactivity with peptide ELISA

Screening for antibodies against UH-RA.1 (GLQEFGTREKRQEITTE) and UH-RA.21 (PGGFRGEFMLGKPDPKPEGKGLGSPYIE) was performed by a peptide-based ELISA. All samples were tested on both the specific peptide (P) and the irrelevant control peptide (C). Synthetic peptides were coated overnight at room temperature (RT) at 1 µg/mL in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 130 mM NaCl) in ELISA plates (polystyrene flat-bottom microplates, Greiner Bio-One, Wemmel, Belgium). After washing, plates were blocked with 200 µL/well of PBS containing 2% (w/v) skimmed milk powder (MPBS) for 2 hours (h) shaking at 37°C. Plates were washed during 5 minutes (min) for three consecutive times. Samples (diluted 1:100 in 2% MPBS) and a serial dilution of a positive sample were incubated for 2 h at RT (100 µL/well, shaking). After washing, antibody binding was detected using a polyclonal rabbit anti-human IgG secondary antibody conjugated to horseradish peroxidase (HRP; Dako, Heverlee, Belgium), 100 µL/well diluted 1:2,000 in 2% MPBS for 1 h shaking at RT. Following washing, staining was performed in the dark with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific). After 6 min the reaction was stopped with 50 µL 2N H_2SO_4 and results were read at 450 nm (Microplate reader Infinite M1000 Pro, TECAN, Männedorf, Switzerland). Washing steps were performed with PBS containing 0.05% (v/v) Tween-20 (VWR, Leuven, Belgium). All samples were tested in duplicate within one experiment and experiments were performed twice. A serial dilution of a positive sample was included in each experiment as a reference in assigning arbitrary units (AU) for comparison of antibody levels, and to control for interassay variability: assays outside the range of mean AU ± three times the standard deviation (SD) of total assays were excluded from analyses.

3.2.3 Data analysis

Optical density (OD) signals from specific (P) and control (C) peptides were log-transformed, and background was subtracted from each measurement. Individual linear equations were calculated based on the serial dilution and all samples were interpolated and assigned AU (relative antibody levels). Samples were considered positive when (1) OD_P-OD_C was >0.1, and (2) the AU exceeded the cutoff value defined by two times the SD above the mean AU of the HC

group after exclusion of outliers using the same formula. All AU were normalized to the cutoff so that a sample with AU >1.0 is considered positive.

Chi-Square (χ^2) or Fischer's Exact testing (expected count less than 5) was used to compare proportions and to evaluate the additional diagnostic value of the UH-RA peptides to RF and ACPA. Continuous variables were analyzed using Mann-Whitney U testing or the Kruskal Wallis test followed by Dunn's multiple comparison test. Spearman's rho (ρ) correlations were applied to study associations between continuous data. For all statistical tests, a p-value <0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism 5 (Graph Pad software, La Jolla, California, USA) and IBM SPSS Statistics for Windows, version 22.0 (IBM Corp. Armonk, New York, USA). Samples with AU values not fulfilling the second criterion for a positive outcome (OD_P-OD_C >0.1) but exceeding the cutoff were excluded from analyses and graphs based on antibody titers.

3.3 Results

3.3.1 Antibodies to UH-RA peptides in RF-negative ACPA-negative RA patients and in early disease

Two independent study populations (patient demographic and serological data provided in Table 3.1) were screened for the presence of antibodies against four UH-RA peptides. Results reported here focus on two of these peptide targets (UH-RA.1 and UH-RA.21), as they emerged as the most sensitive antibody responses. Antibody levels observed are depicted in Figure 3.1.

Table 3.1. Characteristics of patients and controls used in this study

UH COHORT Diagnosis	N	Mean age[a]	Gender[b]	Disease duration[c]	RF-[d]	ACPA-[e]	RF-ACPA-[f]
RA	292	60.0 ± 12.1	69	8.6 ± 9.4	45	53	38
early RA	39	59.6 ± 13.6	67	0.5 ± 0.3	41	33	26
est. RA	253	60.1 ± 11.8	70	9.6 ± 7.7	45	56	40
RC	90	49.7 ± 11.6	41	10.3 ± 7.8	88	67	60
HC	97	38.9 ± 15.0	64	NA	NA	NA	NA
EAC COHORT							
Diagnosis	N	Mean age[a]	Gender[b]	Symptom duration[c]	RF-[d]	ACPA-[e]	RF-ACPA-[f]
RA							
early RA	600	57.0 ± 16.3	66	0.6 ± 0.9	42	50	38
UA	309	51.2 ± 16.4	62	0.5 ± 0.7	81	86	77
RC	157	51.2 ± 17.3	47	0.5 ± 0.6	90	91	85

[a] Mean age in years ± standard deviation

[b] % females

[c] Mean disease/symptom duration in years ± standard deviation; symptom duration was missing for 40 RA patients and 43 RC from the EAC cohort although all were <2 years

[d] % negative patients; RF status was missing for 30 RC from the UH cohort

[e] % negative patients; ACPA status was missing for 30 RC from the UH cohort and 1 RA patient from the EAC cohort

[f] % negative patients

ACPA, antibodies against citrullinated proteins; EAC, Early Arthritis Clinic; est, established; NA, not available; RA, rheumatoid arthritis; RC, rheumatic controls; RF, rheumatoid factor; UH, Hasselt University

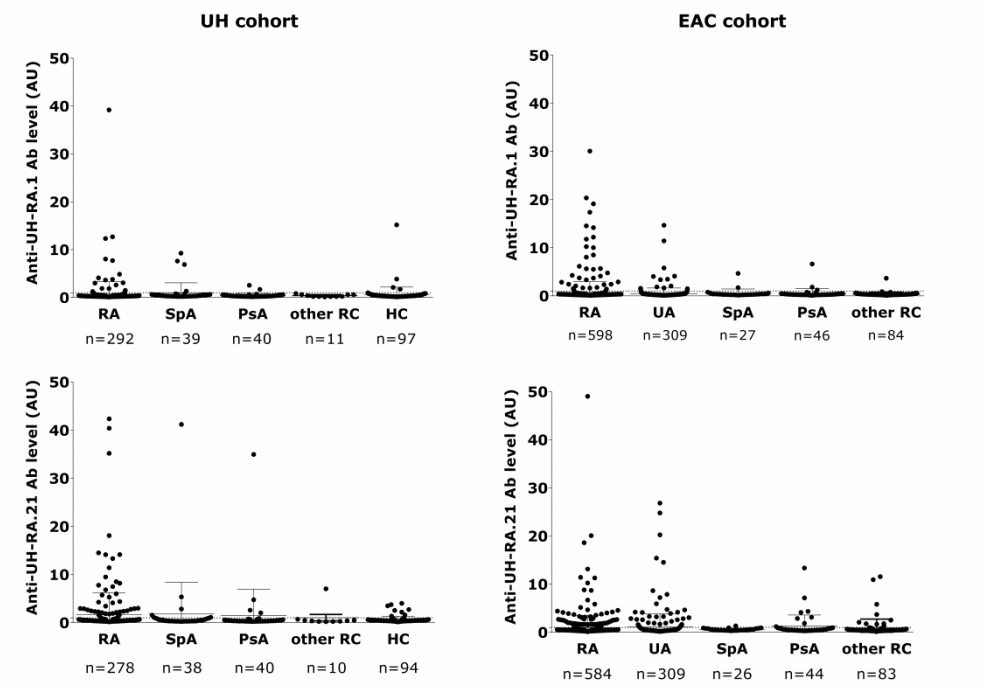

Figure 3.1. Antibody reactivity against UH-RA.1 (upper) and UH-RA.21 (bottom) in the Hasselt University (UH, left) and Early Arthritis Clinic (EAC, right) cohort. The presence of plasma antibodies against UH-RA.1 and UH-RA.21 was measured using peptide ELISA. Samples were tested in duplicate and experiments were performed twice. Optical density (OD) signals were transformed into arbitrary units (AU, relative antibody levels). Samples were considered positive when 1/ the OD_P-OD_C was >0.1, and 2/ the AU exceeded the cutoff value, normalized to 1 (dashed line). Samples not fulfilling both criteria are not depicted on the figure. Ab, antibodies; HC, healthy controls; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RC, rheumatic controls; SpA, spondyloarthropathy; UA, undifferentiated arthritis.

In both cohorts, the proportion of seronegative RA patients was 38%. Detailed antibody reactivity presented in Table 3.2 and Table 3.3 show that within the UH cohort, 23% of these seronegative RA patients could be identified by testing for autoantibodies against UH-RA.1 (6%) and UH-RA.21 (17%), combined as UH-RA.PANEL2, reducing the serological gap with 9% (p=0.029, OR=1.469; Figure 3.2A left panel). Within the 39 early patients from the UH cohort, a similar reduction of the serological gap was reached: 26% of the early patients was seronegative and this fraction was reduced to 18% by testing for autoantibodies against UH-RA.PANEL2 (p=0.411, OR=1.576).

Presence of the autoantibodies in early and/or seronegative RA was further studied in the EAC cohort. Within the 229 seronegative RA patients enrolled in this cohort, antibodies against UH-RA.1 and UH-RA.21 were observed in 6% and 14%, respectively, with combined sensitivity of 18%, leading to a reduction of the serological gap of 6% in early RA (p=0.011, OR=1.364; Figure 3.2A, right panel). Additionally, anti-CarP status appeared to be positive for 12% of these patients reducing the serological gap from 38% to 34% (p=0.116, OR=1.209), or when combined with UH-RA.PANEL, from 38% to 27% (p<0.0001, OR=1.623).

Table 3.2. Antibody reactivity within the UH and the EAC cohort

UH COHORT UH-RA biomarker	Sens all RA (n=292)	Sens early RA (n=39)	Sens RF-/ACPA-RA (n=111)	Spec based on reactivity in RA (n=292) vs RC (n=90)[a]
UH-RA.1	6 (18)	10 (4)	6 (7)	93
UH-RA.21	18 (53)	23 (9)	17 (19)	88
UH-RA.PANEL2[b]	24 (70)	33 (13)	23 (25)	82
RF[c]	55 (162)	59 (23)	0 (0)	88
ACPA[d]	47 (137)	67 (26)	0 (0)	67
RF or ACPA	62 (181)	74 (29)	0 (0)	60
EAC COHORT UH-RA biomarker		Sens early RA (n=600)	Sens RF-/ACPA-RA (n=229)	Spec based on reactivity in early RA (n=600) vs early RC (n=157)[a]
UH-RA.1		7 (41)	6 (13)	96
UH-RA.21		13 (77)	14 (32)	88
UH-RA.PANEL2[b]		19 (113)	18 (42)	84
RF[c]		58 (347)	0 (0)	90
ACPA[d]		50 (298)	0 (0)	91
RF or ACPA		62 (371)	0 (0)	85

Sensitivity (sens) data are presented as percentage (absolute number), specificity (spec) is presented as percentage.

[a] Calculation of diagnostic specificity based on RC. Additional calculations including UA patients and HC are provided in Table 3.5. Prevalence of antibody-positive controls is provided in Table 3.4.

[b] UH-RA.PANEL2 combines antibody reactivity towards UH-RA.1 and UH-RA.21.

[c] RF status was missing for 30 RC from the UH cohort.

[d] ACPA status was missing for 30 RC from the UH cohort, and for 1 RA patient from the EAC cohort.

ACPA, antibodies against citrullinated proteins; EAC, Early Arthritis Clinic; HC, healthy controls; RA, rheumatoid arthritis; RC, rheumatic controls; RF, rheumatoid factor; UA, undifferentiated arthritis

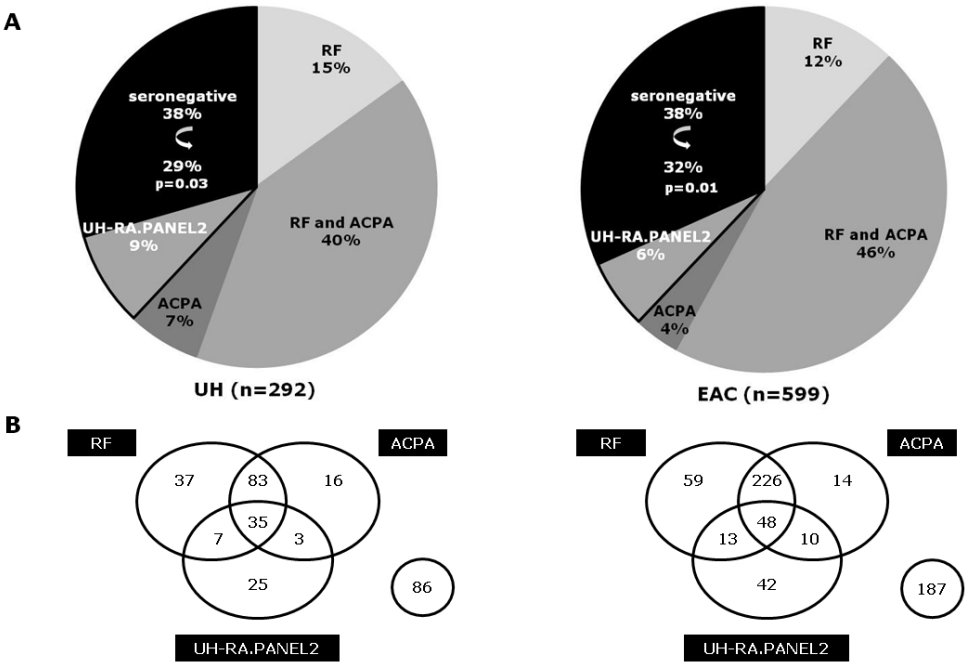

Figure 3.2. Reducing the serological gap in rheumatoid arthritis (RA) patients in the Hasselt University (UH) and Early Arthritis Clinic (EAC) cohort. [A] Addition of UH-RA.PANEL2 (antibodies towards UH-RA.1 and UH-RA.21) to RF and ACPA reduced the serological gap from 38% to 29% in the UH cohort (p=0.03), and from 38% to 32% in the EAC cohort (p=0.01). The proportions of seronegative and seropositive patients with or without addition of UH-RA.PANEL2 to diagnostic testing based on RF and ACPA, was compared by Chi Square testing. A p-value <0.05 was considered statistically significant. [B] Graphical representation of overlapping antibody reactivity (absolute numbers). Within the EAC cohort, ACPA status was missing for one patient. ACPA, antibodies against citrullinated proteins; RA, rheumatoid arthritis; RF, rheumatoid factor

In both study populations, antibody reactivity to the UH-RA peptides was neither restricted to seronegative patients (Table 3.3), nor correlated with RF or ACPA as there was no notable overlap between the different autoantibodies (Figure 3.2B).

Within patients with UA (n=309, Table 3.4), antibody reactivity to the UH-RA peptides was found in 60/309 (19%) of patients, as were RF (60/309 or 19%) and ACPA (43/307 or 14%), but anti-UH-RA antibodies were not predictive for the development of RA.

Table 3.3. Antibody reactivity towards the novel UH-RA peptides in different serological subpopulations of RA

UH COHORT				
UH-RA biomarker	RF- / ACPA - n=111	RF- / ACPA+ n=19	RF+ / ACPA- n=44	RF+ / ACPA+ n=118
UH-RA.1	6 (7)	11 (2)	5 (2)	6 (7)
UH-RA.21	17 (19)	5 (1)	11 (5)	24 (28)
UH-RA.PANEL2[a]	23 (25)	16 (3)	16 (7)	30 (35)
EAC COHORT[b]				
UH-RA biomarker	RF- / ACPA- n=229	RF- / ACPA+ n=24	RF+ / ACPA- n=72	RF+ / ACPA+ n=274
UH-RA.1	6 (13)	17 (4)	10 (7)	6 (17)
UH-RA.21	14 (32)	25 (6)	8 (6)	12 (33)
UH-RA.PANEL2[a]	18 (42)	42 (10)	18 (13)	18 (48)

Data are presented as percentage (absolute number).

[a] UH-RA.PANEL2 combines antibody reactivity towards UH-RA.1 and UH-RA.21

[b] Within the EAC cohort, ACPA status was missing for 1 patient

ACPA, antibodies against citrullinated proteins; EAC, Early Arthritis Clinic; RF, rheumatoid factor; UH, Hasselt University

3.3.2 Antibody reactivity to UH-RA peptides in the general RA population and other arthritides

Table 3.4 shows a detailed overview of antibody reactivity in the control populations included in the screenings of the UH and EAC cohorts. Within the UH cohort, antibodies against UH-RA.PANEL2 resulted in the identification of 24% of all UH-RA patients with an RA-specificity of 82% based on reactivity in RC (Table 3.2). Sensitivities and specificities for RA, of the individual peptides, were 6% and 93% (UH-RA.1) and 18% and 88% (UH-RA.21), respectively.

The EAC cohort included, next to early RA patients and patients with UA, also 157 RC. Based on antibody reactivity within RC, similar specificities were observed, being 96%, 88% and 84% for UH-RA.1, UH-RA.21 and UH-RA.PANEL2, respectively (Table 3.2). Specificity for RA based on reactivity in HC (UH cohort) or UA patients (EAC cohort) is provided in Table 3.5.

Table 3.4. Prevalence of the anti-UH-RA peptide antibodies, RF and ACPA in control groups

	UH (n=187)[a,b]						EAC (n=477)[c]					
	UH-RA.1	UH-RA.21	UH-RA.PANEL2	RF	ACPA	RF or ACPA	UH- RA.1	UH-RA.21	UH-RA.PANEL2	RF	ACPA	RF or ACPA
I. RC												
PsA	2/40	4/40	6/40	6/32	13/32	17/32	3/46	8/46	11/46	3/46	5/46	6/46
SS	0/3	0/3	0/3	NA	NA	NA	-	-	-	-	-	-
(Infl.[d]) OA	0/8	1/8	1/8	0/8	4/8	4/8	0/25	2/25	2/25	5/25	3/25	7/25
SpA	4/39	6/39	9/39	1/20	3/20	3/20	2/27	1/27	3/27	1/27	3/27	3/27
React. arthr.	-	-	-	-	-	-	0/11	2/11	2/11	2/11	1/11	2/11
Lyme arthr.	-	-	-	-	-	-	1/1	0/1	1/1	0/1	0/1	0/1
PM arthr.	-	-	-	-	-	-	0/4	0/4	0/4	1/4	0/4	1/4
Gout	-	-	-	-	-	-	0/24	3/24	3/24	1/24	1/24	2/24
Pseudo-gout	-	-	-	-	-	-	0/1	0/1	0/1	0/1	0/1	0/1
Sarcoidosis	-	-	-	-	-	-	0/1	0/1	0/1	0/1	0/1	0/1
MCTD, vasc.	-	-	-	-	-	-	0/7	1/7	1/7	2/7	1/7	3/7

Table 3.4 *(continued)*

RS3PE	-	-	-	-	-	-	0/10	2/10	2/10	0/10	0/10	0/10
TOTAL	6/90	11/90	16/90	7/60	20/60	24/60	6/157	19/157	25/157	15/157	14/157	24/157
II. UA												
UA	-	-	-	-	-	-	15/309	48/309	60/309	60/309	43/307	71/308
III. HC												
HC	4/97	8/97	12/97	NS	NS	NS	-	-	-	-	-	-

[a] In the UH cohort, two additional biomarkers were tested – not depicted in this table: 1/292 RA patients, 1/97 HC and 2/39 SpA patients did also show antibody reactivity towards UH-RA.9 resulting in a sensitivity of <1% with an associated specificity of 98% for RA. Antibody reactivity against UH-RA.14 was 100% specific for RA with a sensitivity of 6/292 (2%).

[b] In the UH cohort, data on RF and ACPA status were not available for all HC and some RC.

[c] The diagnoses in the Leiden EAC were established after 1 year of disease.

[d] All OA patients from the EAC cohort presented with inflammatory OA, inflammatory status was not provided for OA patients from the UH cohort.

ACPA, antibodies against citrullinated proteins; arthr, arthritis; EAC, Early Arthritis Clinic; HC, healthy controls; infl, inflammatory; NA, not available; OA, osteoarthritis; MCTD, mixed connective tissue disease; PM, paramalignant; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RC, rheumatic controls; RF, rheumatoid factor; RS3PE, remitting seronegative symmetrical synovitis with pitting edema; SpA, spondyloarthropathy; SS, Sjögren syndrome; UA, undifferentiated arthritis; UH, Hasselt University; vasc, vasculitis

Table 3.5. RA-specificity of antibodies to UH-RA peptides within the UH and the EAC cohort

UH-RA biomarker	Spec (%) based on reactivity in HC (n=97) in the UH cohort[a]	Spec (%) based on reactivity in UA (n=309) in the EAC cohort
UH-RA.1	96	95
UH-RA.21	92	84
UH-RA.PANEL2[b]	87	81
RF	NA	81
ACPA	NA	86
RF or ACPA	NA	77

Antibody positivity was compared between RA patients and HC (UH cohort) or between RA patients and UA patients (EAC cohort). Calculations based on RC are provided in Table 3.2.

[a] In the UH cohort, data on RF and ACPA status were not available for HC.

[b] UH-RA.PANEL2 combines antibody reactivity towards UH-RA.1 and UH-RA.21.

ACPA, antibodies against citrullinated proteins; EAC, Early Arthritis Clinic; NS, not available; RA, rheumatoid arthritis; RF, rheumatoid factor; UA, undifferentiated arthritis; UH, Hasselt University

3.3.3 Associations between antibody reactivity to UH-RA peptides and disease outcome

Putative associations between clinical parameters and the presence of anti-UH-RA.1 and anti-UH-RA.21 antibodies were investigated. The former appeared to be related to a better outcome as within the EAC cohort, antibodies against UH-RA.1 were associated with sustained DMARD-free remission as defined by the sustained absence of synovitis for at least one year after the discontinuation of therapy with DMARDs (37% of the antibody-positive group vs 21% of the antibody-negative group, p=0.016) [273]. Within the RA patients of the UH cohort, positive for anti-UH-RA.1 antibodies, 5/10 patients (50%) were in remission (p=0.301), while in the antibody-negative group only 68/214 (32%) were in remission (disease activity score based on 28 joints (DAS28) < 2.6 at time of sampling). The presence of anti-UH-RA.21 antibodies on the other hand, was

associated with a negative outcome. In the UH cohort, antibody reactivity towards UH-RA.21 was significantly associated with increased inflammation (increased erythrocyte sedimentation rate (ESR) level in 17/49 or 35% of the positive patients vs 37/224 or 17% of the negative patients, p=0.004). Moreover, also an association was observed with the presence of joint erosion, prior to sampling or within the first three years of follow-up (31/51 or 61% of the positive patients vs 89/223 or 40% of the negative patients, p=0.006). This association between anti-UH-RA.21 antibody reactivity and erosions was also found within seronegative RA: 9/18 (50%) of the anti-UH-RA.21 antibody-positive patients presented with erosions compared to 17/77 (22%) of the antibody-negative patients (p=0.017). In the EAC cohort of early RA patients, no link between baseline anti-UH-RA.21 antibody reactivity and inflammation (CRP, ESR) or radiological progression was found when looking at the Sharp-van der Heijde score or its partitioned erosion score over 7 years of follow-up. However, the presence of anti-UH-RA.21 antibodies was associated with higher swollen and tender joint counts (SJC, 12.8 ± 12.7 in the antibody-positive group vs 9.4 ± 7.1 in the antibody-negative group, p=0.010, and TJC, 11.9 ± 14.3 vs 8.4 ± 5.8, p=0.019). Finally, no further associations were observed between antibody reactivity and HAQ or DAS28, nor with smoking.

3.4 Discussion

Although RA diagnostics has greatly improved during the last decades, current serological biomarkers are absent in one third of RA patients and even more in early disease. Previously, novel UH-RA peptides were identified by serological antigen selection, a phage display method in which an RA synovial cDNA library was screened for reactivity with antibodies present in plasma pools of early and seronegative RA patients [267]. Exploratory screenings were performed using phage ELISA. For this validation study, the phage ELISA was successfully translated into a peptide-based ELISA which is more reproducible and allows large-scale screenings and application in clinical laboratories, offering a testing method similar to the ACPA testing kit.

Antibody reactivity to novel UH-RA peptides was studied in two large independent study cohorts to validate presence in early and seronegative RA. Of four peptides tested, UH-RA.1 and UH-RA.21 were most promising regarding the highest prevalence and their presence in early disease. Up to one third of early RA patients from the UH cohort – diagnosed <1 year ago – tested positive for at least one of these autoantibodies. Their presence in early disease was further validated in 600 early RA patients from the Leiden EAC cohort. From these patients with symptoms for <2 years at time of sampling, 19% tested positive confirming the peptides' ability to detect reactivity in early disease. Early disease markers are of utmost importance when aiming at preventing joint damage and disability [20-22, 25, 29].

Next to the detection of the autoantibodies in early disease, our major observation was that the so-called seronegative, i.e. RF-negative ACPA-negative, RA patients are not truly autoantibody-negative. The proportion of seronegatives was 38% in both cohorts, and testing for antibody reactivity to UH-RA.1 and UH-RA.21 reduced this serological gap to 29% (UH cohort) and 32% (EAC cohort). These results indicate a role for combined antibody testing, of which our novel peptides could be part of, in order to increase the diagnostic window and eventually close the serological gap. This approach was further illustrated by involving the anti-CarP antibodies in the EAC cohort, solely reducing the gap from 38% to 34% but when combined with autoantibodies to UH-RA.1 and UH-RA.21 even down to 27%.

Exploration of the prognostic potential of the UH-RA antibody markers, pointed towards a better outcome linked to anti-UH-RA.1 antibodies (sustained DMARD-free remission in the EAC cohort) while for anti-UH-RA.21 antibodies associations with inflammation and erosions were found in the UH cohort but could not be confirmed in the EAC cohort. However, in the EAC cohort, patients with anti-UH-RA.21 antibodies presented with higher numbers of swollen and tender joint counts further supporting a link between anti-UH-RA.21 antibodies and a worse prognosis for the disease course of RA.

In conclusion, a validation screening in two independent cohorts has confirmed the presence of autoantibodies towards novel UH-RA peptides in early and seronegative RA. Full diagnostic potential and predictive values will be determined in further prospective screenings. Additionally, the identification and characterization of these antibody responses and their targets will provide insight into their biological relevance and role in RA pathogenesis.

4

CHANGES IN LEVELS OF ANTI-CCP3 ANTIBODIES, RF, ANTI-UH-RA.1 AND ANTI-UH-RA.21 ANTIBODIES IN RHEUMATOID ARTHRITIS PATIENTS: A FOLLOW-UP STUDY OF 17 MONTHS

Based on:
De Winter L, Hansen W, Stinissen P, Vanhoof J, Geusens P, Somers V
Changes in levels of anti-CCP3 antibodies, rheumatoid factor and antibodies against two novel UH-RA peptides in rheumatoid arthritis patients: a follow-up study of 17 months
Submitted

Abstract

Only limited data are available on changes in RF and ACPA levels during follow-up, and for the novel autoantibodies repeated testing has not been performed so far. Therefore, the aim of this study was to investigate changes in plasma levels of anti-CCP3 antibodies, RF and antibodies against UH-RA.1 and UH-RA.21 during follow-up in RA patients on a treat-to-target strategy in daily practice. The antibody levels were tested in 96 patients with definite RA, during a follow-up of 17 months on average with 6-months intervals. Changes from positive to negative (seroreversion) and from negative to positive (seroconversion) were addressed, as well as changes in titers within seropositive patients. Observed serological patterns were related to clinical measures of outcome and to the requirement of a biological. Six patients were treated with a biological at study entry and an additional 17 patients switched to a biological during follow-up.

At baseline, the number of seropositive patients was 78 for anti-CCP3 antibodies, 52 for RF, 7 and 35 for antibodies against UH-RA.1 and UH-RA.21 respectively. Seroreversion was barely observed, except for anti-UH-RA.21 antibodies, mostly reverting from low-positive to negative titers. Changes from a negative to a positive status (seroconversion) occurred mainly for anti-CCP3 antibodies (12/18) and was also rather restricted to low-positive titers (10/12). Changes in antibody levels were observed in patients persistently positive and treated with DMARD for the total duration of the study, with for anti-CCP3 antibodies an increase in titers, while for anti-UH-RA.21 antibodies a decline in titers. Thereby, the decrease in anti-UH-RA.21 antibodies was only observed in treatment responders. Changes in serology were not associated with clinical parameters. Finally, patients under biological treatment at study entry had significantly higher baseline levels of anti-CCP3 antibodies and RF compared to patients continuing DMARD treatment.

To conclude, seroconversion and increased reactivity for a third-generation CCP3 assay was observed in a considerable number of RA patients on a treat-to-target strategy in daily practice suggesting a possible benefit of repeated testing in seronegative or low-positive patients. The magnitude of baseline titers of RF and anti-CCP3, but not changes in antibody levels, were linked with worse outcome reflected by clinical measures and the requirement of a biological. Finally, anti-UH-RA.21 antibodies may decrease under effective DMARD treatment.

4.1 Introduction

In rheumatoid arthritis (RA), rheumatoid factor (RF) and antibodies against citrullinated proteins (ACPA) are established diagnostic markers [8]. However, up to one third of the RA patients is not recognized using these current serological markers – the seronegative subpopulation [220, 256]. In a previous study, 23% of the seronegative patients appeared to carry antibodies against two novel peptide targets (UH-RA.1 and UH-RA.21), candidate biomarkers for RA which were also detected in up to one third of early RA patients (Chapter 3) [267]. To which extent these novel antibody levels fluctuate prior to or during disease course has not been addressed so far. Additionally, no consensus exists regarding the occurrence of seroconversion and the effect of fluctuations in levels of RF and ACPA during the course of RA.

It is often proposed that seroconversion of autoantibodies is most likely to be seen in the upcoming years of disease onset. Both RF and ACPA can be present years before RA onset, and seroconversion of RF was shown to continue during the first years after symptom onset [274]. However, results from studies in early inflammatory arthritis are inconsistent [275-279]. Furthermore, the majority of studies in which changes in serum titers of RF or ACPA were investigated, have been performed in treatment cohorts. Treatment with conventional disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate appears to partly eliminate circulating RF and ACPA only in treatment responders, and reductions in serum titers were closely linked to disease activity measures [280, 281]. Antibody titers did seldom decrease to such extent that patients became negative. The majority of treatment cohorts in which serum fluctuations of RF and ACPA are studied, focus on tumor necrosis factor α-inhibiting agents. Since these biologicals have variable responses in RA patients, it is interesting to evaluate the antibodies' potential as prognostic marker for responsiveness to treatment. Studies in which stable antibody levels were reported initially included non-responders [282, 283]. Several other studies demonstrated reductions in titers of RF or ACPA following treatment with infliximab [282, 284-289], adalimumab [283, 290] or etanercept [291], often related to treatment response [282, 283, 286, 288-291]. Also treatment with rituximab, a B lymphocyte depleting therapy has been shown to gradually reduce RF levels [190].

Regardless which treatment used, RF titers seem more vulnerable for treatment effects than ACPA. This has been demonstrated in many studies in which reductions in RF levels were observed while not in ACPA [190, 285, 286, 292]. Also, infliximab-induced reductions in ACPA levels have been reported to return to baseline levels after a longer period of follow-up [284]. Furthermore, RF levels decreased in larger extent and in a higher proportion of patients than ACPA following DMARD intervention [281]. Since RF is often more closely linked to markers of the acute phase response [190, 281, 286, 288], higher seroreversion rates observed for RF during early disease might be attributed to early intervention.

Changes in serum levels or seropositivity in consecutive RA patients outside the context of particular treatment strategies are barely reported and it remains unclear whether repeated testing is useful in daily practice and whether fluctuations in antibody biomarkers can be applied in prognostic evaluation of the disease course [293].

The primary objective of this study was to investigate fluctuations in plasma levels of the UH-RA antibodies, RF and anti-CCP3 antibodies during follow-up in RA patients on a treat-to-target strategy in daily clinical practice. In a 17-month follow-up study, we examined whether antibody levels decreased in patients being treated by DMARDs and biologicals, whether changes in antibody levels were different with particular therapies, and whether these changes were linked to clinical measures of disease activity or inflammation. This study is among the first to report longitudinal ACPA data measured by the third-generation anti-CCP assay (QUANTA Lite CCP3, INOVA Diagnostics, Inc., San Diego, California) [244, 294].

4.2 Materials and methods

4.2.1 Patient selection and assessment

Patients were selected from a prospective study cohort in which consecutive RA patients were re-assessed every 6 months [295]. Diagnosis of RA was based on the 1987 classification criteria [17]. The study was approved by the Medical Ethical Committee of Hasselt University. Informed consent was obtained from all subjects. Previously, patients were screened for the presence of antibodies against UH-RA.1 and UH-RA.21 (Chapter 3). For this study, similar numbers of patients – with random serology for RF and anti-CCP3 antibodies – were selected from the anti-UH-RA.1 or -21 antibody positive and negative subpopulation. A final population of 96 RA patients was enrolled in this study, with a total of 384 samples. Demographical data provided were age, gender and disease duration. The presence of any erosion was evaluated by radiographic bone-damage assessment of hands and feet and recorded as either present or absent. (Re-)assessment of patients and blood sample collection was performed at first sampling and follow-up with intervals from 6 months or more. Samples were stored in the University Biobank Limburg. Clinical data consisted of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), and the outcome of a health assessment questionnaire (HAQ) together with disease activity. The latter was registered by a 28-joint disease activity score (DAS28) integrating measures of physical examination (TJC and SJC, tender/swollen joint count), ESR and patient self-assessment on a visual analogue score (VAS) [34]. Treatment with DMARDs and biologicals was registered at study entry and during follow-up.

4.2.2 Measurement of plasma autoantibody levels

(IgM-)RF-serology was determined in routine clinical testing with the RF-II Cobas C system (Roche, Vilvoorde, Belgium). The upper limit of normal (ULN) for RF titers is 14 units (U) per mL.

ACPA-testing was performed using the QUANTA Lite CCP3 IgG ELISA (INOVA Diagnostics Inc, San Diego, USA). Anti-CCP3 antibody values have a cutoff for positive samples at 20U according to the manufacturer's guidelines. ACPA positivity was also verified by CCP2 assessment at routine clinical analyses

(Phadia EliA CCP assay, Thermo Scientific, Erembodegem, Belgium) with an ULN of 9.

Screening for antibodies against UH-RA.1 (GLQEFGTREKRQEITTE) and UH-RA.21 (PGGFRGEFMLGKPDPKPEGKGLGSPYIE) was performed by a peptide-based ELISA. Briefly, all samples were tested on both the specific peptide (P) and the irrelevant control peptide (C). Synthetic peptides were coated overnight at room temperature (RT) at 1 µg/mL in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 130 mM NaCl) in ELISA plates (polystyrene flat-bottom microplates, Greiner Bio-One, Wemmel, Belgium). After washing, plates were blocked with 200 µL/well of PBS containing 2% (w/v) skimmed milk powder (MPBS) for 2 hours (h) shaking at 37°C. Plates were washed during 5 minutes (min) for three consecutive times. Samples (diluted 1:100 in 2% MPBS) and a serial dilution of a positive sample were incubated for 2 h at RT (100 µL/well, shaking). After washing, antibody binding was detected using a polyclonal rabbit anti-human IgG secondary antibody conjugated to horseradish peroxidase (HRP; Dako, Heverlee, Belgium), 100 µL/well diluted 1:2,000 in 2% MPBS for 1 h shaking at RT. Following washing, staining was performed in the dark with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific). After 6 min, the reaction was stopped with 50 µL 2N H_2SO_4 and results were read at 450 nm (Microplate reader Infinite M1000 Pro, TECAN, Männedorf, Switzerland). Washing steps were performed with PBS containing 0.05% (v/v) Tween-20 (VWR, Leuven, Belgium). A serial dilution of a positive sample was included in each experiment as a reference in assigning arbitrary units (AU) for comparison of antibody levels, and to control for interassay variability. The antibody concentration was estimated by interpolation from linear equations based on the standard serial dilution. The ULN for antibodies against UH-RA.1 and UH-RA.21 was 21 and 8 units, respectively. For measurement of ACPA and antibodies against UH-RA.1 and -21, longitudinal samples obtained from one patient were tested on the same assay and measurements were performed in duplicate. For anti-UH-RA antibody screenings, assays were performed twice.

Antibody reactivity for the four biomarkers of interest were categorized as negative, (low-)positive (>ULN) or high-positive (>3*ULN) according to the definition applied in the RA classification criteria defined by the American College of Rheumatology and the European League Against Rheumatism [8].

4.2.3 Statistical analyses

Patients were assigned trajectories for each antibody: always positive, always negative, seroconverting (from negative to positive) or seroreverting (from positive to negative) based on antibody status at first and final visit. Differences in continuous variables between groups were analyzed using Mann-Whitney U testing (MWU) or the Kruskal Wallis test (KW) followed by Dunn's multiple comparison test. Proportions were compared by Chi Square (χ^2) or Fischer's Exact testing (expected count less than 5). Spearman's rho (ρ) correlations were applied to study associations between continuous data. For analysis of serial measurements, linear mixed models (LMM) were implemented with continuous response variables and random intercepts and slopes. For all statistical tests, a p-value <0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism version 5 (Graph Pad software, La Jolla, California, USA), IBM SPSS Statistics for Windows, version 22.0 (IBM Corp. Armonk, New York, USA) and JMP Version Pro 11.2 (SAS Institute Inc, Cary, North Carolina, USA).

4.3 Results

4.3.1 Patient characteristics

Patient demographics (provided in Table 4.1) were as follows: mean (SD) age was 62.7 (11.1) years, 62/96 (65%) was female and mean (SD) disease duration of RA, i.e. time since diagnosis, was 9.0 (8.2) years. Anti-CCP3 antibody and RF positivity at first visit were 78/96 (81%) and 52/96 (54%), respectively, whereas 7/96 (7%) and 35/96 (36%) were positive for antibodies against UH-RA.1 and UH-RA.21. Median DAS28 of 3.2 at study entry demonstrates moderate disease activity for the RA study population for which patients were treated according to a treat-to-target strategy: around 20% of the patients continued glucocorticoid intake during the study and >93% was on DMARD therapy. Moreover, 6% of the RA patients was treated with a biological at study entry and another 18% initiated biological treatment during follow-up.

Table 4.1. Patient demographics at study entry

	RA study population (n=96)
Age (mean ± SD)	62.7 ± 11.1
Female, n (%)	62 (65%)
Disease duration (mean ± SD), in years	9.0 ± 8.2
Erosions, n (%)	41 (43%)
DAS28, median (IQR)[a]	3.2 (1.8)
HAQ, median (IQR)[a]	14 (25)
VAS, median (IQR), in mm[a]	20 (28)
SJC, median (IQR)[a]	1 (0-4)
TJC, median (IQR)[a]	2 (0-8)
ESR, median (IQR), in mm/h[a]	9 (16)
CRP, median (IQR), in mg/dL[a]	0.0 (1.1)
Treated with DMARD, n (%)	94 (98%)
Treated with biological, n (%)	6 (6%)
Anti-CCP3 antibody positive, n (%)	78 (81%)
RF positive, n (%)	52 (54%)
Anti-UH-RA.1 antibody positive, n (%)	7 (7%)
Anti-UH-RA.21 antibody positive, n (%)	35 (36%)

[a] Patient characteristics remained stable across time according to linear mixed models analyses.

CRP, C-reactive protein; CCP, cyclic citrullinated peptides; ESR, erythrocyte sedimentation rate; DAS28, disease activity score based on 28 joints; DMARD, disease-modifying anti-rheumatic drug; HAQ, health assessment questionnaire; IQR, interquartile range; n, number; RA, rheumatoid arthritis; RF, rheumatoid factor; SD, standard deviation; SJC, swollen joint count; TJC, tender joint count; UH, Hasselt University; VAS, visual analogue scale

4.3.2 Changes in plasma levels of autoantibodies

The titers of anti-CCP3, RF, anti-UH-RA.1 and anti-UH-RA.21 antibodies were evaluated during an average follow-up period of 17 months (range 11-34) with 6-month intervals. Changes from a positive to a negative status – seroreversion – were barely observed (Figure 4.1A): of the seropositive patients, 1/78 patient became negative for anti-CCP3 antibodies and 5/52 for RF. Additionally, 0/7 patients seroreverted for anti-UH-RA.1 antibodies. For antibodies against UH-RA.21, 8/35 patients seroreverted during follow-up and patients with low-positive baseline titers (n=7) were more likely to become negative than patients with high-positive baseline titers (n=1) (Fischer's p=0.018). Changes from a negative to a positive status – seroconversion – occurred in 12/18 patients for anti-CCP3, 5/44 for RF, 2/89 for anti-UH-RA.1 and 5/61 for anti-UH-RA.21 antibodies (Figure 4.1B). For anti-CCP3 antibodies, 10/12 patients seroconverted to low-positive titers while only 2/12 patients converted to high-positive titers (Fischer's p=0.010).

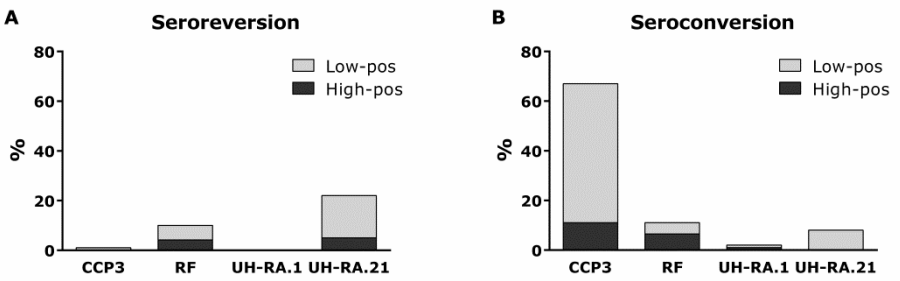

Figure 4.1. Rates of seroreversion and seroconversion for anti-CCP3 antibodies, RF and antibodies against UH-RA.1 and UH-RA.21 from first to last visit during a 17-month follow-up study. Bars represent the proportion of patients that switched from positive at first visit to negative at last visit (seroreversion) (A) or vice versa (seroconversion) (B). The majority of change rates were attributable to low-positive titers (light) rather than high-positive titers (dark). CCP, cyclic citrullinated peptides; RF, rheumatoid factor; UH, Hasselt University

Next to changes in antibody status, changes in antibody levels were also observed in patients persistently positive. The highest variation was observed for anti-CCP3 antibodies: from 32 patients with low-positive titers at study entry, 5 evolved towards high-positive titers (3*ULN) during follow-up. An additional 22 patients evolved from low-positive to moderate-positive titers (>2*ULN, according to the manufacturer's guidelines). Taken together, at last visit, levels of anti-CCP3 antibodies had increased with 69% of the baseline titers (LMM $p<0.0001$, Figure 4.2A). For three quarters (n=72/96) of the study population, levels of anti-CCP3 antibodies increased with an average of 96% (range 0.1-1243). In the remaining patients, an average decrease in baseline titers of 17% (range 0.4-86) was observed. Changes in anti-CCP3 antibodies were not accompanied by changes in clinical measures of inflammation or disease activity, neither did they coincide with a switch in treatment. This was also true for antibodies against UH-RA.21, for which a decline in time was observed (LMM $p=0.0124$ after correction for age and anti-CCP3 antibody titers, Figure 4.2D). Levels of RF and anti-UH-RA.1 antibodies remained stable during follow-up (Figure 4.2B and C, respectively).

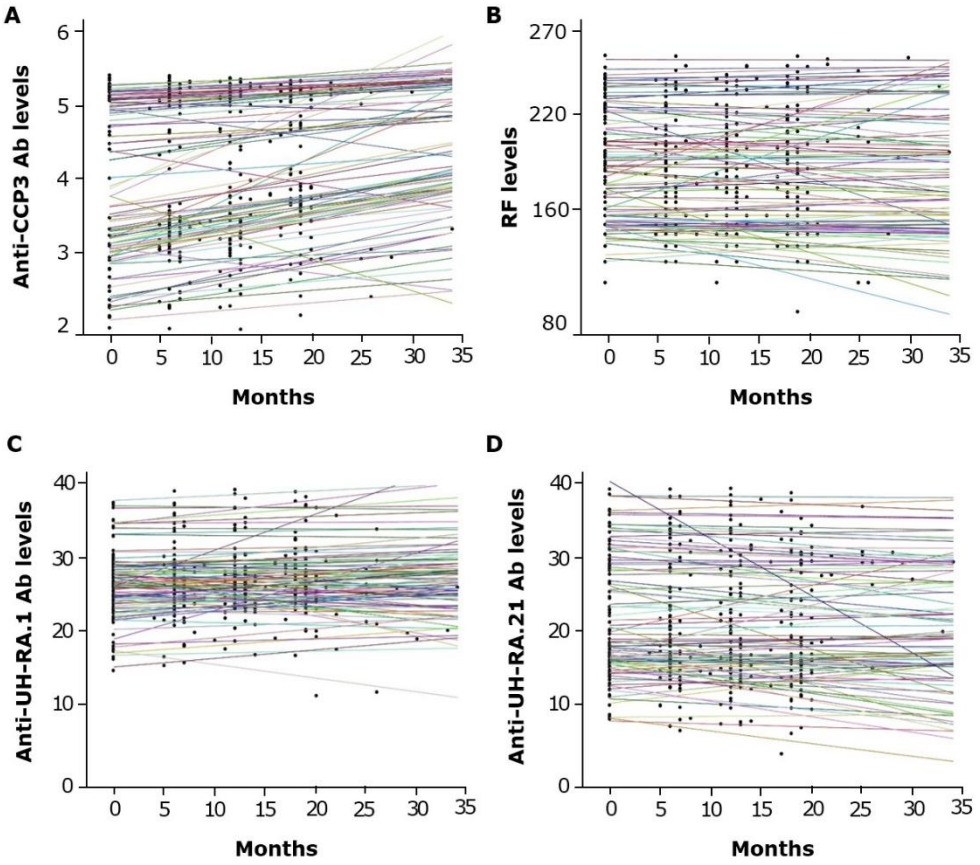

Figure 4.2. Course of anti-CCP3 antibody levels, RF levels and levels of anti-UH-RA.1 and -UH-RA.21 antibodies during follow-up. Linear mixed model analyses were conducted to study changes over time in levels of antibodies against CCP3 (A), RF (B), anti-UH-RA.1 antibodies (C) and anti-UH-RA.21 antibodies (D) within 96 RA patients. A p-value <0.05 was considered statistically significant. Each line represents individuals' regression line for change over time in antibody level, depicted on the Y axis according to Box-Cox transformation (not expressed in original units). Levels of anti-CCP3 antibodies increased over time (p<0.0001, A), while levels of RF (B) and antibodies against UH-RA.1 (C) overall remained constant. Levels of anti-UH-RA.21 antibodies showed a decline over time after correction for age and anti-CCP3 antibody titers (p=0.0124, D).

4.3.3 Antibody reactivity at baseline and during follow-up under different treatment strategies

The vast majority of the study population is treated with DMARDs (n=73). However, 6 patients were already treated with a biological when they entered the study (group B) and an additional 17 patients switched to a biological during follow-up (group D-B). We evaluated whether the observed changes in antibody levels were related to particular treatment groups.

At baseline, a significant difference in anti-CCP3 antibody titers was observed between patients treated with a biological (group B) and patients continuing DMARDs (group D) (KW p=0.001, Figure 4.3A). Baseline titers within patients treated with a biological at study entry, were high-positive in 6/6 (100%) of the patients. Within patients switching to a biological during follow-up, high-positive levels were found in 10/17 (59%) of patients, and two subgroups of patients could be distinguished as anti-CCP3 antibody levels were explicitly either high-positive, or low-positive or negative. Comparing these two subgroups showed that patients with high-positive anti-CCP3 antibody titers had higher anti-UH-RA.21 antibody levels (MWU p=0.019), higher RF titers (MWU p=0.001) and higher ESR levels (MWU p=0.014). Finally, within patients continuing DMARD therapy, only 27/73 (37%) of patients had high-positive anti-CCP3 antibody levels. At the end of follow-up, this pattern of anti-CCP3 levels among the treatment groups had not changed (Figure 4.3B). However, in the meantime anti-CCP3 levels increased in patients on continuous DMARD treatment (LMM p<0.0001, Figure 4.4A) while for patients from the other two treatment groups no significant trend in time was observed (Figure 4.4B and C). Also for RF, patients treated with a biological at study entry, had higher baseline (KW p=0.003, Figure 4.3C) and final (KW p=0.004, Figure 4.3D) titers, compared to patients continuing DMARD treatment. In addition, patients switching from DMARD to biological during follow-up, had higher baseline titers than patients continuing DMARD treatment (KW p=0.019, Figure 4.3C). Baseline or final titers of antibodies against UH-RA.1 or UH-RA.21 were not different amongst patients grouped by their need for a biological (Figure 4.3E-H). Furthermore, patients treated with a biological during the entire study or switching during follow-up, showed no changes in antibody levels (Figure 4E and F). However, patients continuing DMARD treatment showed a significant decrease in anti-UH-RA.21

antibody levels during follow-up (LMM p=0.0334, after correction for age and anti-CCP3 antibody titers, Figure 4.4D). This decrease was only observed in patients continuing the same DMARD (LMM, n=62, p=0.0112), while not in patients switching from one DMARD to another (LMM, n=11, p=0.5142) suggesting that anti-UH-RA.21 antibody levels only decrease in patients responding to current therapy.

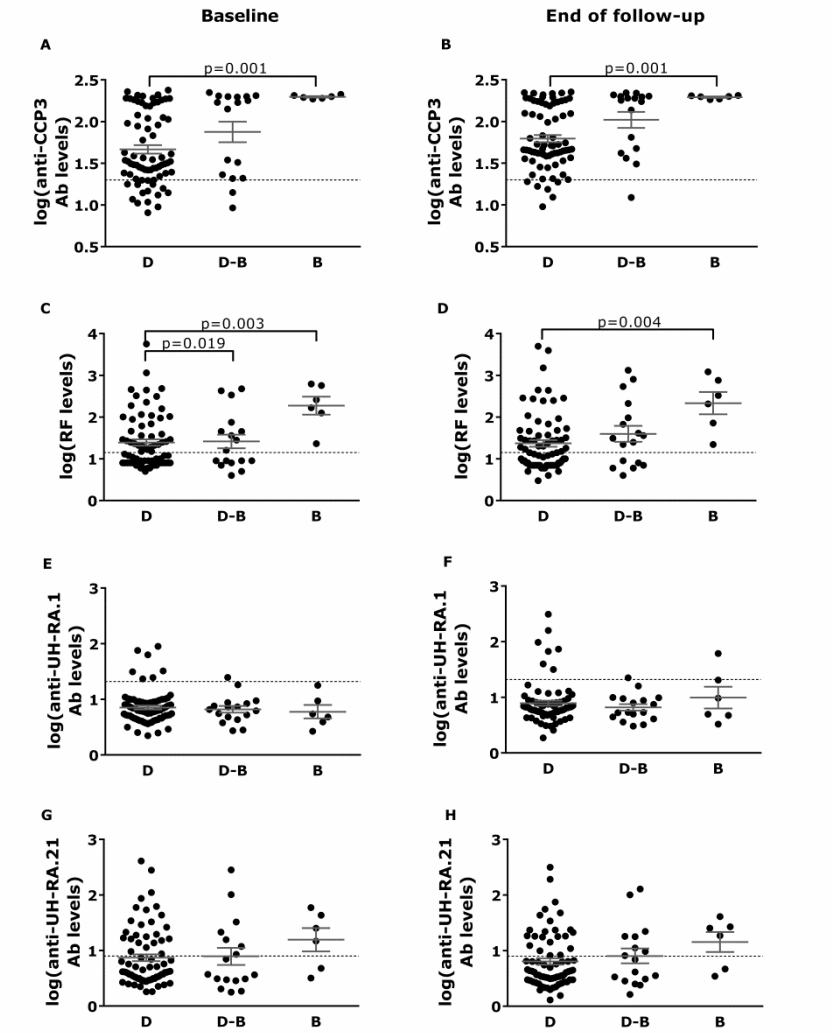

Figure 4.3. Levels of anti-CCP3 antibodies, RF and antibodies against UH-RA.1 and UH-RA.21 at first and last visit according to different treatment regimens. The levels of anti-CCP3 antibodies (A, B), RF (C, D) and antibodies against UH-RA.1 (E, F) and UH-RA.21 (G, H) were measured within 96 patients at baseline (left; A, C, E, G) and end (right; B, D, F, H) of a 17-month follow-up study in patients treated with DMARD (D), switching from DMARD to a biological during follow-up (D-B) and treated with a biological for the total duration of the study (B). Antibody levels were compared by the Kruskal Wallis test followed by Dunn's multiple comparison test. A p-value <0.05 was considered statistically significant. Ab, antibody; CCP, cyclic citrullinated peptides; RA, rheumatoid arthritis; RF, rheumatoid factor; UH, Hasselt University

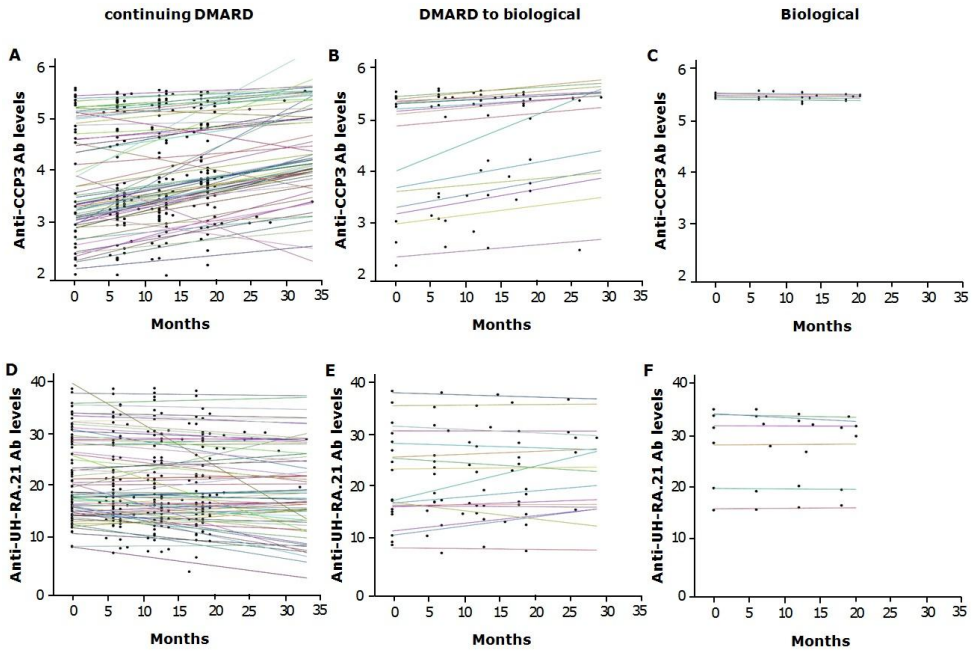

Figure 4.4. Course of anti-CCP3 and anti-UH-RA.21 antibody levels during follow-up. Linear mixed model analyses were conducted to study changes over time in levels of antibodies against CCP3 (A-C) and UH-RA.21 (D-F), within patients treated with DMARD (A, D), switching from DMARD to a biological during follow-up (B, E) and treated with a biological for the total duration of the study (C, F). A p-value <0.05 was considered statistically significant. Each line represents individuals' regression line for change over time in antibody level, depicted on the Y axis according to Box-Cox transformation (not expressed in original units). Within patients continuing DMARD treatment, anti-CCP3 antibody levels rose significantly during follow-up ($p<0.0001$, A) while titers of anti-UH-RA.21 antibodies declined ($p=0.0334$, D). Any observed change in antibody titer could not be linked to clinical measures in time. Ab, antibody; CCP, cyclic citrullinated peptides; DMARD, disease-modifying anti-rheumatic drug; RA, rheumatoid arthritis; UH, Hasselt University

4.3.4 Baseline autoantibody levels and clinical measures

Putative associations were addressed between the magnitude of baseline titers, and clinical parameters such as acute-phase response variables (CRP and ESR), measures of disease activity (DAS28, HAQ, VAS) and clinical assessment (TJC and SJC), and the presence of erosions (Table 4.2).

Firstly, a strong correlation was found between anti-CCP3 antibody levels and RF levels (ρ=0.677, p<0.0001), and for both markers plasma levels were higher in patients with erosive disease (anti-CCP3 antibody levels: MWU=698, p=0.002); RF levels: MWU=829, p=0.036). Furthermore, both markers were associated with inflammation as reflected by higher levels of ESR (anti-CCP3 antibody levels: ρ=0.349, p=0.001; RF levels: ρ=0.343, p=0.001) and CRP (anti-CCP3 antibody levels: ρ=0.267, p=0.009; RF levels: ρ=0.345, p=0.001). Regarding RF, baseline titers were also correlated with a higher disease activity indicated by DAS28 levels (ρ=0.304, p=0.003) and a higher number of swollen joints (SJC, ρ=0.224, p=0.032).

For antibodies against UH-RA.21, the presence of erosions was associated with a positive antibody status: erosions were found in 33% of antibody-negative patients and 62% of antibody-positive patients (χ^2=7.47, p=0.006). This association was also reflected by higher antibody levels within the erosive subpopulation (MWU=795, p=0.019). Additionally, the link with worse outcome was further supported by a correlation between anti-UH-RA.21 antibody titers and ESR levels (ρ=0.204, p=0.048).

Antibodies against UH-RA.1 on the other hand, appeared to be associated with a better outcome since an inverse relation to the presence of erosions was found: none of the seven patients positive for the UH-RA.1-specific antibodies presented with erosions compared to 47% of the antibody-negative patients (n=89, Fischer's p=0.018).

When evaluating clinical measures at the end of this study's follow-up, baseline levels of anti-CCP3 antibodies and RF maintained their correlation with ESR and CRP measured at final visit (anti-CCP3 antibodies: ρ=0.320, p=0.002 for ESR and ρ=0.318, p=0.003 for CRP; RF: ρ=0.332, p=0.002 for ESR, and ρ=0.348, p=0.001 for CRP). Additionally, a positive status for anti-UH-RA.1 antibodies at baseline was associated with lower disease activity, expressed in DAS28 levels (MWU=73.5, p=0.005) or defined as remission by DAS28<2.6 (Fischer's

p=0.003): all patients positive at study entry were in remission at the end of the follow-up in contrast to only 36% of the 89 patients initially negative for anti-UH-RA.1 antibodies.

Table 4.2. Baseline autoantibodies and clinical measures of disease activity and outcome

	Anti-CCP3	RF	Anti-UH-RA.1	Anti-UH-RA.21
Erosions[a]	P=0.002	P=0.036	P=0.018[c]	P=0.019
ESR[b]	P=0.001	P=0.001	ns	P=0.048
CRP[b]	P=0.009	P=0.001	ns	ns
DAS28[b]	ns	P=0.003	ns	ns
SJC[b]	ns	P=0.032	ns	ns

[a] Autoantibody levels at study entry were compared between patients with/without erosions by Mann-Whitney U testing. For antibodies to UH-RA.1, proportions of positives were analyzed by Fischer's Exact test.

[b] Spearman's rho correlations were applied to study associations between auto-antibody levels at study entry and clinical measures of inflammation and disease activity: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), disease activity score based on 28 joints (DAS28) and swollen joint count (SJC). No further associations were observed for health assessment questionnaire (HAQ), tender joint count (TJC), visual analogue score (VAS) or disease duration.

For all tests, a p-value <0.05 was considered statistically significant.

[c] All observed associations were positively directed, except for anti-UH-RA.1 antibody status and erosions.

CCP, cyclic citrullinated peptides; ns, not statistically significant; RA, rheumatoid arthritis; RF, rheumatoid factor; UH, Hasselt University

4.4 Discussion

In this study, we investigated changes in serology of current and novel candidate biomarkers for RA: anti-CCP3 antibodies, RF and antibodies against UH-RA.1 and UH-RA.21. The latter two were first described in Somers et al. 2011 [267] and their presence in early and seronegative RA was confirmed in Chapter 3). The current study included 96 RA patients re-assessed every 6 months with a total follow-up of 17 months on average. All patients were actively treated towards remission with glucocorticoids, DMARDs and for some patients also biologicals. We investigated whether plasma levels of anti-CCP3 antibodies, RF and antibodies against UH-RA.1 and UH-RA.21 fluctuated in time and whether changes could be linked to clinical measures of disease activity or inflammation. Next, we evaluated whether these changes were different for particular treatments.

At baseline of this longitudinal study, the number of seropositive patients was 78 for anti-CCP3 antibodies, 52 for RF, 7 and 35 for antibodies against UH-RA.1 and UH-RA.21 respectively. During follow-up, changes from positive to negative (seroreversion) were barely observed for ACPA, RF and anti-UH-RA.1 antibodies. A higher seroreversion rate (8/35 or 23%) was observed for anti-UH-RA.21 antibodies, but in 6/8 seroreverting patients the antibody levels were limited to low-positive reactivity. Changes in the other direction, from negative to positive (seroconversion), were primarily encountered for anti-CCP3 antibody reactivity (12/18 or 67%). Here again, antibody levels within these seroconverting patients were restricted to low-positive titers in 10/12. Notably, in our study, only moderate- or high-positive anti-CCP3 reactivity was generally in agreement with the CCP2 results from routine diagnostics (data not shown). Indeed, CCP3 has been previously reported to be more sensitive than CCP2 [244-246]. The use of a more sensitive assay may explain higher seroconversion rates than usually reported for CCP2 – ranging from 0 to 11% and highly variable for different cohorts and clinical settings [275, 278, 279, 284, 293]. Although repeated testing did not seem to add value to baseline prognostic information, it might be useful in patients with negative or low-positive anti-CCP3 reactivity based on the relatively high seroconversion rate in our study. Once positive, patients presumably remain positive. As mentioned above, we barely observed seroreversion and additionally, therapy-induced reductions in ACPA levels have

been described but not to such extent that they drop below cutoff. Thus, lack of seroreversion does not imply that ACPA levels remain constant. The majority of the patients in this study showed limited within-subject variation, but surprisingly, we observed an overall increase in anti-CCP3 levels during follow-up and a decline in levels of anti-UH-RA.21 antibodies. For none of the biomarkers, changes in antibody levels could be linked to clinical measures of disease activity or inflammation. However, we found that this increase in anti-CCP3 antibody levels and decrease in anti-UH-RA.21 antibody levels were particularly present in patients treated with DMARDs during the entire study. The effect was absent in patients switching from DMARDs to biologicals, or in patients treated with biologicals already at baseline. These results for anti-CCP3 should be taken with precaution, as within these two groups, a considerable number of patients have high-positive anti-CCP3 antibody levels, reaching up to the upper detection limit of the assay. Therefore, we cannot exclude the possibility that variations in samples with high-positive antibody levels were missed. High-positive baseline levels of anti-CCP3 antibodies were found in all six patients already treated with a biological, in 59% of patients switching from DMARD to biological during follow-up, and in 37% of patients continuing DMARD treatment. An increase in anti-CCP3 reactivity in time suggests a correlation between anti-CCP3 levels and disease duration as recently described [296], but we were not able to confirm this association. As we are among the first to apply the third-generation CCP assay in a longitudinal setting, we believe conformational studies are required to verify our findings. The decline in anti-UH-RA.21 titers in time might be caused by DMARD treatment, as the decreasing trend was only observed in patients continuing treatment with the initial DMARD (suggesting patients respond well) in contrast to patients switching to another DMARD (non-responders). As this is the first time anti-UH-RA.21 antibodies were measured in consecutive RA samples, further research is warranted to study the behavior of this new autoantibody system under different treatments, but results from this study carefully suggest that anti-UH-RA.21 antibodies could reflect DMARD response.

Patients requiring biological therapy, did not show fluctuations in titers of autoantibodies studied here, but the high baseline anti-CCP3 antibody levels in these patients reflected disease severity as correlations were found with

measures of inflammation and disease activity. The well-established association of anti-CCP3 and RF with worse outcome was confirmed, and also for antibodies against UH-RA.21 we confirmed the link with erosions and inflammation. Anti-UH-RA.1 antibodies on the other hand were associated with a better outcome as all seven antibody-positive patients were in remission at the end of the follow-up in contrast to only 36% of the 89 patients initially negative. These findings confirm our results reported in Chapter 3 based on overlapping patient cohorts.

To summarize, we report seroconversion and increased reactivity for a third-generation CCP3 assay in a considerable number of RA patients on a treat-to-target strategy. Based on these results, repeated testing may be useful in seronegative patients and patients with low-positive titers. Patients persistently positive for anti-CCP3 antibodies showed an overall increase in plasma titers during follow-up, particularly in patients treated with DMARDs, but serological changes could not be linked to clinical measures of disease activity or inflammation. Nevertheless, the magnitude of baseline titers of anti-CCP3 antibodies and RF were linked with worse outcome. Whereas levels of RF and anti-UH-RA.1 antibodies remained fairly constant in consecutive samples, we cautiously raised evidence that anti-UH-RA.21 antibody titers decline after DMARD treatment. This potential theranostic feature of anti-UH-RA.21 antibodies needs further investigation in well defined treatment cohorts.

5

THE ISOTYPE DISTRIBUTION OF ANTIBODIES AGAINST UH-RA.1 AND UH-RA.21

Based on:
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The isotype repertoire of antibodies against novel UH-RA peptides in rheumatoid arthritis
Submitted

Abstract

Previously, measurement of autoantibodies against UH-RA.1 and UH-RA.21 focused on the IgG response, the most abundant antibody isotype in serum, and therefore most often used in clinical diagnostics. However, we hypothesized that measurement of other isotypes might improve the performance of diagnostic testing for the antibodies of interest. In addition, assigning the isotype profile might provide valuable information on effector functions of the antibody systems. In this chapter, we determined the isotype profile of antibodies against UH-RA.1 and UH-RA.21. The IgG, IgM and IgA classes, together with the four different IgG subclasses, were determined in 285 RA patients, 88 rheumatic controls (RC) and 90 healthy controls (HC). Anti-UH-RA.1 antibodies were primarily of the IgM isotype, twice as prevalent as IgG and IgA, and IgG3 was the dominant IgG subclass. Testing for anti-UH-RA.1 IgM was shown to be superior to testing for the IgG isotype regarding sensitivity for RA. Within antibodies against UH-RA.21, the IgG and IgA classes were more common than IgM. Different anti-UH-RA.21 IgG subclasses were found, with the highest prevalence for IgG2. Combined testing for IgG and IgA increased the RA-sensitivity of UH-RA.21-specific antibody testing from 23% to 27% compared to solely testing for IgG. Importantly, the full antibody isotype usage was demonstrated in early and seronegative RA. Although the exact mechanisms by which the different antibody isotypes act still have to be unraveled, the isotype distribution of anti-UH-RA.1 and -UH-RA.21 antibodies was successfully outlined.

5.1 Introduction

Upon first antigen encounter, B cells produce antibodies of the immunoglobulin (Ig)M isotype. Once activated by T cells, B cells start producing antibodies in which the antigen recognition is retained but the constant region of the antibody has changed in order to elicit different effector functions. This process is known as isotype switching or class switching, and leads to the secretion of high-affinity IgG, IgA and IgE [210]. IgG is the most abundant antibody isotype in serum, therefore it is most often used in clinical diagnostics. However, in immunodiagnostics and –pathogenicity, also other Ig isotypes have proven their utility. Testing for rheumatoid factor (RF), the first known antibody in rheumatoid arthritis (RA), relies on the presence of IgM rather than IgG or IgA although all isotypes are present prior to diagnosis and have been shown to be associated with disease severity and radiological outcome [8, 30, 297]. Also for the other antibody system currently included in RA diagnostics, antibodies against citrullinated proteins (ACPA), the isotype repertoire has been investigated and next to IgG, also IgM and IgA isotypes were frequently encountered [189, 228, 298, 299]. RA patients present with more different ACPA isotypes than their family members, indicating a difference in isotype usage between health and disease [228]. Years before RA onset, ACPA of the IgG and IgA class are present and predict the development of RA [300]. The ACPA isotype repertoire expands towards RA development and in the early arthritis course [189, 228, 230]. Besides the presence of ACPA, also a broader range of ACPA isotypes predicts a higher risk for radiographic damage [301]. Measurement of isotype-specific autoantibodies can thus provide valuable information related to RA diagnosis and prognosis. The autoantibody isotypes might give information on the source of the antigen-recognition, the major effector functions involved and the pathogenicity of the antibodies.

Previously, the presence of autoantibodies against UH-RA.1 and UH-RA.21 – two novel peptides – was demonstrated in early and seronegative RA patients (Chapter 3). Antibodies against UH-RA.1 were associated with sustained disease-modifying anti-rheumatic drug (DMARD)-free remission. Anti-UH-RA.21 antibodies on the other hand, were linked with worse outcome as associations with the presence of erosions, inflammation and higher tender and swollen joint counts were found.

The primary aim of this study is to explore the isotype usage within anti-UH-RA.1 and -UH-RA.21 antibodies. RA patients are cross-sectionally tested for antibodies of IgG and all of its subclasses (IgG1-4), IgM and IgA. The results from this study might provide insight into the biological role of the circulating autoantibodies as Ig isotypes differ in localization and biological properties. Moreover, presence of multiple isotypes within the antibody response might also have implications for diagnostic and prognostic use.

5.2 Materials and methods

5.2.1 Patient material

This study was approved by the Medical Ethical Committee of Hasselt University and informed consent was obtained from all participants. Plasma samples were used from 285 RA patients, 88 RC and 90 HC from the Hasselt University (UH)-cohort. Samples from RA and RC patients were collected between 2003 and 2012 in three rheumatology clinics located near Hasselt, Belgium. RA diagnosis was based on fulfillment of the 1987 criteria for RA [17], and for 36 patients samples were collected within the first year of diagnosis (early patients). HC were electable when minimum 18 years old and healthy without any underlying chronic illness. Samples were stored in the University Biobank Limburg.

5.2.2 Clinical data

The presence of erosions is registered as either present or absent. Additional clinical data retrieved from patients' records are erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), the outcome of a health assessment questionnaire (HAQ) together with disease activity registered by a 28-joint disease activity score (DAS28) integrating measures of physical examination (TJC and SJC, tender/swollen joint count), ESR and patient self-assessment on a visual analogue scale (VAS). RF-serology was evaluated in routine clinical laboratory testing with the RF Latex Reagent (Olympus/Beckman Coulter, Analis SA, Suarlée, Belgium; upper limit of normal (ULN) 14 units/mL), the RF-II Cobas C system (Roche, Vilvoorde, Belgium; ULN 14 units/mL), or by means of the Serodia RA test (Fujirebio Europe NV, Ghent, Belgium). ACPA-testing was performed using the Phadia EliA CCP assay (CCP2; Thermo Scientific, Erembodegem, Belgium) or the QUANTA Lite CCP3 IgG enzyme-linked immunosorbent assay (ELISA) (INOVA Diagnostics Inc, San Diego, USA; ULN 19 units).

5.2.3 Peptide ELISA

Plasma samples were tested for antibodies of the IgG, IgM and IgA isotype. Patients positive for IgG were further tested on subclasses 1 to 4. Samples were

tested on both the specific peptide (P) – UH-RA.1: GLQEFGTREKRQEITTE and UH-RA.21: PGGFRGEFMLGKPDPKPEGKGLGSPYIE – and an irrelevant control peptide (C) – WTKTPDGNFQLGGTEP. Synthetic peptides were coated overnight at room temperature (RT) at 1 µg/mL in phosphate-buffered saline (PBS; 1.5 mM KH2PO4, 5 mM Na2HPO4, 130 mM NaCl) in ELISA plates (polystyrene flat-bottom microplates, Greiner Bio-One, Wemmel, Belgium). After washing, plates were blocked with 200 µL/well of PBS containing 2% (w/v) skimmed milk powder (MPBS) for 2 hours (h) shaking at 37°C. Plates were washed during 5 minutes (min) for three consecutive times. Samples (diluted 1:50 in 2% MPBS) were incubated for 2 h at RT (100 µL/well, shaking). After washing, antibody binding was detected using rabbit anti-human IgG secondary antibody (1:2,000) (Dako, Heverlee, Belgium), monoclonal mouse anti-human IgG1, -2, -3 or -4 secondary antibody (1:1,000) (Life Technologies, Ghent, Belgium), rabbit anti-human IgA secondary antibody (1:500) (Dako) or goat anti-human IgM secondary antibody (1:5,000) (Sigma-Aldrich, Diegem, Belgium), all conjugated to horseradish peroxidase (HRP) and diluted in 2% MPBS. 100 µL/well was applied for 1 h shaking at RT. Following washing, staining was performed in the dark with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific). The reaction was stopped with 50 µL 2N H_2SO_4 and results were read at 450 nm (Microplate reader Infinite M1000 Pro, TECAN, Männedorf, Switzerland). Washing steps were performed with PBS containing 0.05% (v/v) Tween-20 (VWR, Leuven, Belgium). All samples were tested in duplicate within one experiment and experiments were performed independently twice at least. A positive sample was included in each experiment to control for interassay variability.

5.2.4 Statistical analyses

Antibody reactivity against UH-RA.1 and UH-RA.21 is expressed by the ratio of the specific optical density (OD) signal to the non-specific OD signal. For each test, the cutoff value was set at a 90% specificity based on reactivity in the HC group. Proportions were compared by Chi Square (χ^2) or Fischer's Exact testing (expected count less than five), while continuous data were compared between groups using the Mann-Whitney U test (MWU, two groups) or Kruskal Wallis test (KW, more than two groups). Spearman rho (ρ) correlations were applied to

study associations between continuous data. For all statistical tests, a p-value <0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism version 5 (Graph Pad software, La Jolla, California, USA), IBM SPSS Statistics for Windows, version 22.0 (IBM Corp. Armonk, New York, USA) and JMP Version Pro 11.2 (SAS Institute Inc, Cary, North Carolina, USA).

5.3 Results

5.3.1 The isotype distribution of anti-UH-RA.1 and anti-UH-RA.21 antibodies

The presence of anti-UH-RA.1 and -UH-RA.21 antibodies of the IgG, IgM and IgA isotype was investigated in 285 RA patients, 88 RC and 90 HC from the UH cohort. Population characteristics are provided in Table 5.1.

Table 5.1. Characteristics of patients and controls tested for IgG, IgM and IgA isotypes of antibodies against UH-RA.1 and UH-RA.21

Diagn	N	Mean age[a]	Gender[b]	Disease duration[a]	RF[d]	ACPA[d]	RF/ACPA[d]
RA	285	60.1 ± 12.1	68	8.4 ± 7.8	56	48	63
RC	88	49.5 ± 11.6	42	10.2 ± 7.9[e]	NA	NA	NA
HC	90	38.7 ± 15.1	63	-	NA	NA	NA

[a] mean ± standard deviation, in years

[b] % female

[d] % positive

[e] unknown for 17 RC

ACPA, antibodies against citrullinated proteins; Diagn, diagnosis; HC, healthy controls; Ig, immunoglobulin; NA, not available; RA, rheumatoid arthritis; RC, rheumatic controls; RF, rheumatoid factor

The contribution of individual Ig classes of the IgG, IgM and IgA type, to total reactivity of anti-UH-RA.1 and anti-UH-RA.21 antibodies was investigated.
Regarding antibodies against UH-RA.1, all three isotypes were present and IgM was most common, as it was found in almost twice as many anti-UH-RA.1 antibody-positive patients than IgG and IgA (IgM 76/130 (58%) vs IgG 44/130 (34%) and IgA 40/130 (31%), Figure 5.1A). The distribution of the different isotypes was similar amongst RA patients and RC (Figure 5.1B). Twenty-nine IgG-positive individuals – of which 19 RA, 6 RC and 4 HC – were further subtyped for IgG1, -2, -3 and -4. IgG reactivity was mainly attributable to the IgG3 subclass (Figure 5.1A and C): IgG3 was present in 17/19 IgG-positive RA

patients and in all of the IgG-positive controls. IgG1 en IgG2 were RA-specific but with a limited prevalence of 2/19 and 1/19, respectively (Figure 5.1C). Anti-UH-RA.1 antibodies of the IgG4 subclass were not detected.

Up to 26/130 or 20% of the anti-UH-RA.1 antibody-positive patients harbored more than one antibody isotype: two different antibody isotypes were found in 14 RA patients, 3 RC and 5 HC, while positivity for 3 different isotypes was found in 2 RA patients and 2 RC. When patients harbored two different antibody isotypes, mainly the combination IgG/IgA (11/22) or IgA/IgM (9/22) was found while IgG/IgM was less common (2/22). This pattern is also reflected by correlations between the levels of the different antibody isotypes (Figure 5.1D). IgG levels were correlated with IgA levels (Spearman's rho (ρ)=0.254, $p<0.0001$) and IgA levels were also correlated with IgM levels (ρ=0.269, $p<0.0001$). No correlation was found between IgG and IgM ($p=0.984$). Finally, due to the dominant role of IgG3, only two RA patients carried more than one IgG subclass.

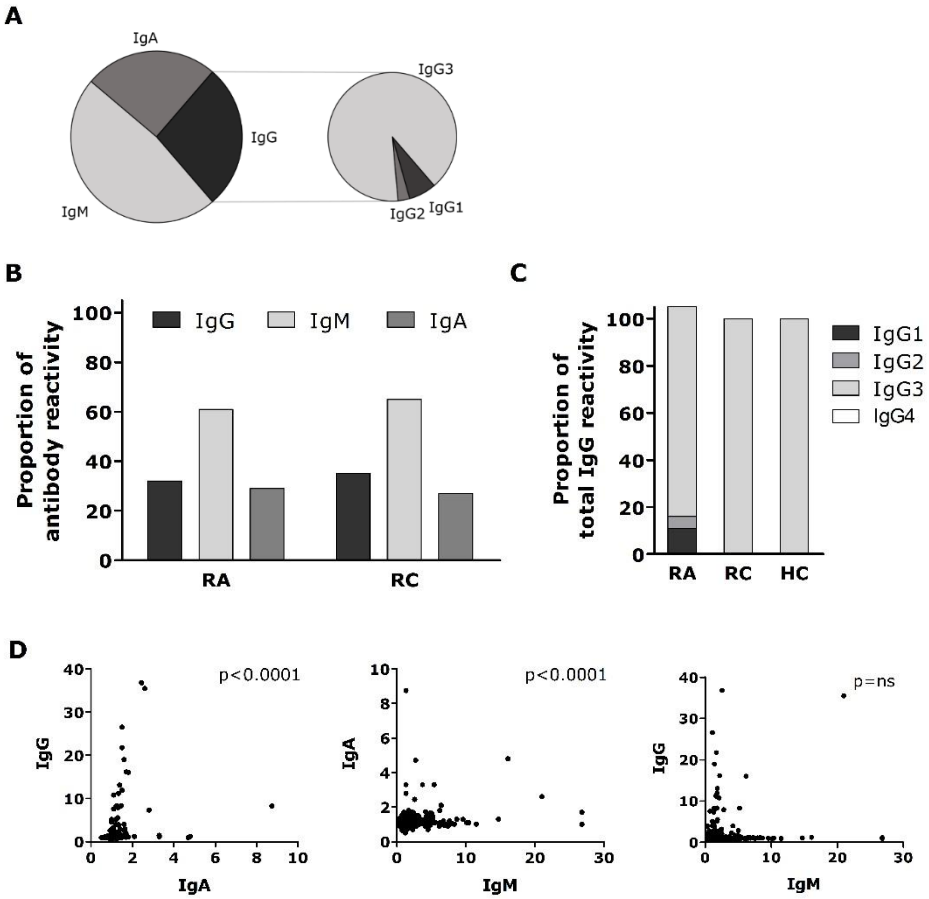

Figure 5.1. Prevalence of the IgG, IgM and IgA (sub)classes within anti-UH-RA.1 antibodies. [A] Anti-UH-RA.1 antibodies exist of the IgG, IgM and IgA isotype with IgM being the most prevalent Ig class. [B] The proportion of IgG, IgM and IgA was similar in RA patients and RC. [C] Subtyping of IgG-positive patients for IgG1-4 pointed towards IgG3 as the dominant subclass in all patient and control groups. IgG1 and IgG2 were RA-specific but less prevalent. No anti-UH-RA.1 antibodies from the IgG4 isotype were detected. Bars represent the proportion of the respective isotype to the total antibody reactivity. Sums of individual proportions within the same patient group can exceed 100% because patients can carry more than one (sub)class. [D] Correlation between anti-UH-RA.1 IgG, IgM and IgA levels within the total study population. HC, healthy controls; Ig, immunoglobulin; RA, rheumatoid arthritis; RC, rheumatic controls.

In contrast to the antibody system against UH-RA.1, IgM was less redundant within antibodies against UH-RA.21, IgG and IgA were both twice as prevalent (IgM 35/158 (22%) vs IgG 85/158 (54%) and IgA 86/158 (54%), Figure 5.2A). Although not significant, the presence of IgG seemed higher in RA patients compared to RC in which IgM and IgA appeared slightly more (Figure 5.2B). IgG-subtyping was performed in 67 IgG-positive patients (51 RA, 10 RC and 6 HC). IgG2 was the most dominant isotype (30/51 or 59%) followed by IgG3 (11/51 or 22%) and IgG1 (4/51 or 8%) (Figure 5.2A and C). The latter two were only present in RA and RC whereas in HC only IgG2 was found. Anti-UH-RA.21 IgG4 antibodies were detected in one RA patient (Figure 5.2C).

For anti-UH-RA.21 antibodies, 36/158 individuals carried two different antibody isotypes (27 RA, 6 RC and 3 HC). Co-occurrence of two isotypes was dominated by IgG/IgA (26/36) while IgA/IgM (7/36) and IgG/IgM (3/36) were less frequently observed. Three individuals carried 3 different antibody isotypes (4 RA, 1 RC and 1 HC). Correlation plots for the IgG, IgM and IgA isotype levels are provided in Figure 5.2D. IgA levels were correlated with both IgG levels ($\rho=0.236$, $p<0.0001$) and IgM levels ($\rho=0.209$, $p<0.0001$), while IgG and IgM levels were not correlated ($p=0.247$). The correlation between IgA and IgG levels was only present in the RA group.

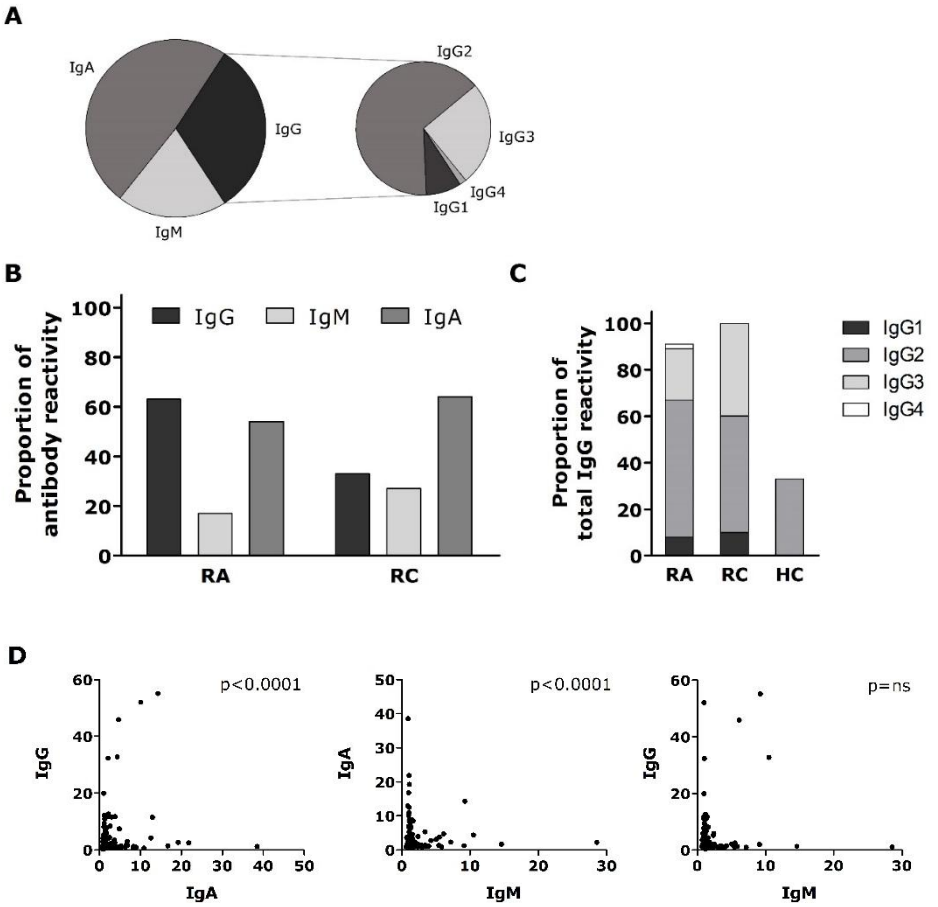

Figure 5.2. Prevalence of the IgG, IgM and IgA (sub)classes within anti-UH-RA.21 antibodies. [A] Anti-UH-RA.21 antibodies exist of the IgG, IgM and IgA isotype with IgA being the most prevalent Ig class. [B] The proportion of IgG, IgM and IgA was not statistically different between RA patients, RC and HC. [C] IgG is mostly represented by IgG2, followed by IgG3 and IgG1. The latter two were less prevalent but not found in HC. Anti-UH-RA.21 antibodies from the IgG4 isotype were detected in one RA patient. Bars represent the proportion of the respective isotype to the total antibody reactivity. Sums of individual proportions within the same patient group can exceed 100% because patients can carry more than one (sub)class. [D] Correlation between anti-UH-RA.21 IgG, IgM and IgA levels within the total study population. HC, healthy controls; Ig, immunoglobulin; RA, rheumatoid arthritis; RC, rheumatic controls.

5.3.2 Implications of antibody isotype profiling for rheumatoid arthritis diagnostics

The levels of isotype-specific antibodies against UH-RA.1 and UH-RA.21 are depicted in Figure 5.3. Depending on the antibody isotype, anti-UH-RA.1 antibody levels were significantly higher in RA patients compared to RC (IgG, IgA) or HC (IgM) (Figure 5.3A). Regarding UH-RA.21, RA patients showed significantly higher levels of IgG and IgA compared to HC, whereas RA patients and RC had significantly different IgM level and IgA levels (Figure 5.3B).

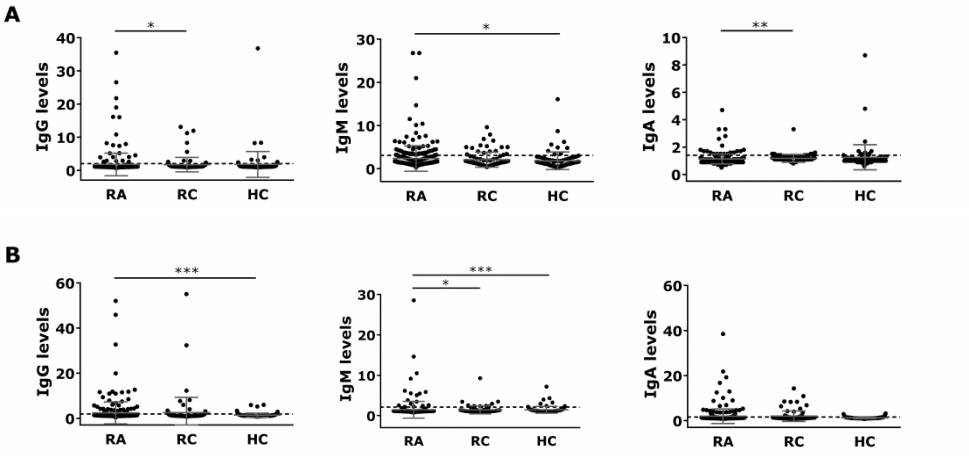

Figure 5.3. Levels of anti-UH-RA.1 (A) and -UH-RA.21 (B) antibody isotypes in rheumatoid arthritis patients and controls. The dashed line represents the cutoff value set at 90% based on reactivity in healthy controls. Antibody levels were compared by Kruskal Wallis testing. *p<0.05, **p<0.01, ***p<0.001. HC, healthy controls; RA, rheumatoid arthritis; RC, rheumatic controls.

Diagnostic sensitivity of the anti-UH-RA.1 and –UH-RA.21 antibodies previously reported was 6% and 18%, respectively, with associated specificities of 93% and 88% (Chapter 3). These were established using a detection antibody directed against IgG, with only minimal cross-reactivity to other isotypes. However, since antibody reactivity is not only represented by IgG but also by IgM and IgA, we evaluated if isotype-specific testing could improve the diagnostic performance of anti-UH-RA.1 and anti-UH-RA.21 antibodies.

Cutoff values based on reactivity in HC and set at 90% specificity, resulted in a sensitivity for RA of 9% and 8% for anti-UH-RA.1 IgG and IgA, respectively (Figure 5.4A). Highest sensitivity for anti-UH-RA.1 antibody testing was achieved by testing for IgM (18%). Even combining two or three antibody isotypes did not exceed this sensitivity observed for IgM. IgM together with IgG or IgA resulted in an RA-sensitivity of 13% and 16%, respectively. Because of the strong correlation between IgG and IgA (Figure 5.3A), combined testing did not perform better than testing for both isotypes individually. The three antibody isotypes together ended up with a sensitivity of 15%.

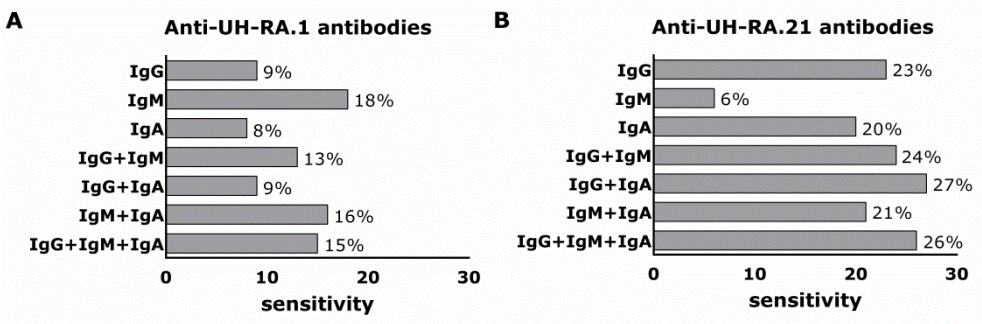

Figure 5.4. Sensitivity of isotype-specific testing for antibodies against UH-RA.1 (A) and UH-RA.21 (B) in rheumatoid arthritis (RA) patients with an associated specificity of 90%. Based on reactivity in healthy controls, cutoff values were determined and specificity was set at 90%. Bars represent the proportion of positive patients, and corresponding sensitivity for RA is provided.

So far, testing for anti-UH-RA.21 antibodies has always been conducted using an anti-IgG antibody, in this study resulting in an RA-sensitivity of 23% which was slightly improved by combined testing with IgM (24%), IgA (27%) or both (26%) (Figure 5.4B). Testing for anti-UH-RA.21 IgM or IgA yielded individual sensitivities of 6% and 20%, respectively, or a combined sensitivity of 21%.

5.3.3 Antibody isotypes in early and seronegative rheumatoid arthritis

The study population included 285 RA patients, of which 105 RF-/ACPA-, 19 RF-/ACPA+, 43 RF+/ACPA- and 118 RF+/ACPA+ patients. Within all serological subgroups, the full isotype usage was found for antibodies against UH-RA.1 and UH-RA.21. Levels of anti-UH-RA.1 IgG, IgM or IgA were not different between these serological subgroups (Figure 5.5A). Sensitivity of the antibody isotypes within seronegative RA was 9%, 20% and 9% for IgG, IgM and IgA, respectively, which is similar as for the total RA population. Also for antibodies against UH-RA.21, no differences were observed in antibody levels between serological subgroups or sensitivities of individual isotype testing (Figure 5.5B). Sensitivity of anti-UH-RA.21 IgG, IgM or IgA within seronegative RA was 24%, 8% and 17%, respectively.

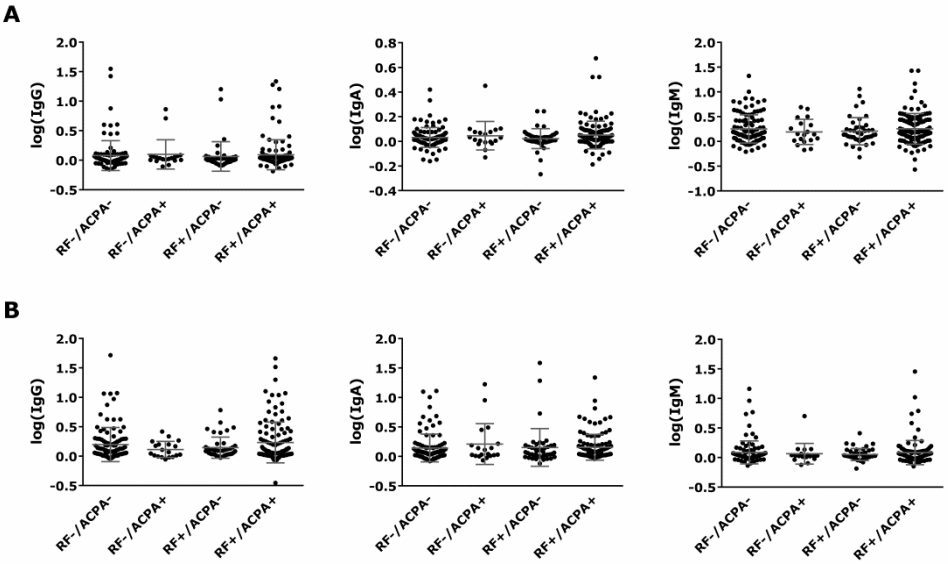

Figure 5.5. Levels of anti-UH-RA.1 and -UH-RA.21 antibodies in different serological subgroups of rheumatoid arthritis. Levels of anti-UH-RA.1 (A) and –UH-RA.21 (B) antibodies were compared between patients positive or negative for rheumatoid factor and/or antibodies against citrullinated proteins by Kruskal Wallis testing. ACPA, antibodies against citrullinated proteins; Ig, immunoglobulin; RF, rheumatoid factor

Another diagnostically challenging subpopulation of RA patients, involves patients in early disease. This study population contained 36 RA patients who were diagnosed not more than one year prior to sampling and therefore classified as early RA. While for both antibody systems (UH-RA.1 and UH-RA.21) all antibody isotypes were found in early stages of the disease, the levels of IgG, IgM or IgA isotypes were similar between early and established RA (Figure 5.6).

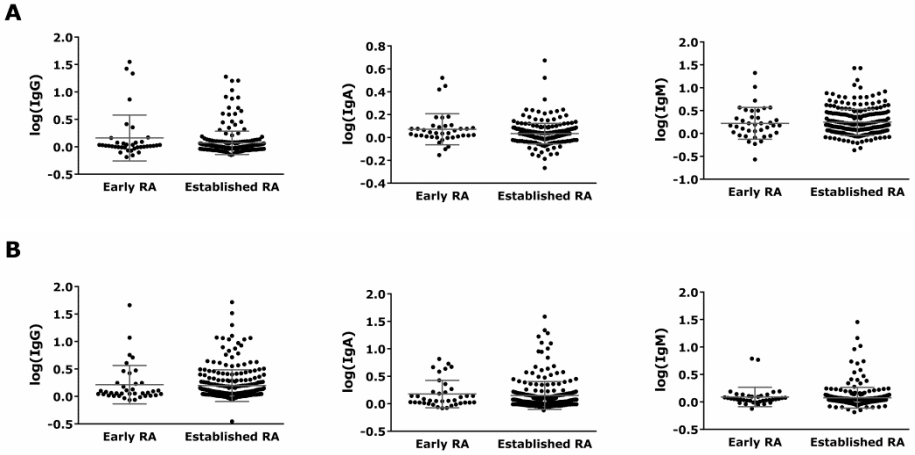

Figure 5.6. Levels of anti-UH-RA.1 and -UH-RA.21 antibodies in rheumatoid arthritis patients with early and established disease. Levels of anti-UH-RA.1 (A) and –UH-RA.21 (B) antibodies were compared between early and established rheumatoid arthritis patients using the Mann-Whitney U test. Ig, immunoglobulin; RA, rheumatoid arthritis

5.3.4 Antibody isotypes in other joint-related diseases

Previously, antibody reactivity against UH-RA.1 and UH-RA.21 has already been reported in other rheumatic disorders such as ankylosing spondylitis (SpA) and psoriatic arthritis (PsA). Figure 5.7 presents the prevalence of the specific IgG, IgM and IgA isotypes within these patient groups. The pattern of isotype-specific testing is similar in RC compared to RA.

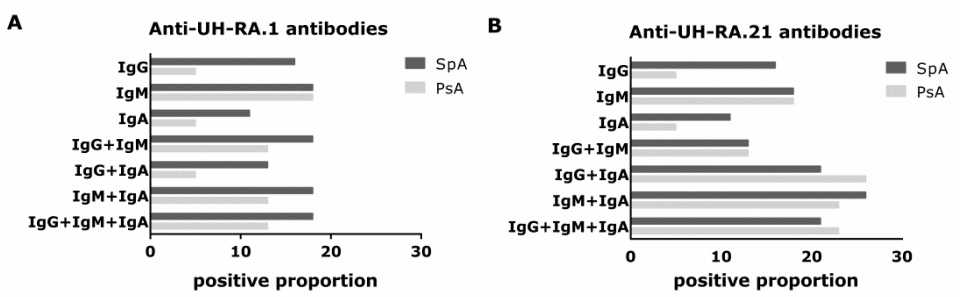

Figure 5.7. Prevalence of anti-UH-RA.1 and anti-UH-RA.21 antibody isotypes in other joint-related diseases. Based on reactivity in healthy controls, cutoff values were determined and specificity was set at 90%. Bars represent the proportion of positive patients when testing specific antibody isotypes within anti-UH-RA.1 antibodies (A) or anti-UH-RA.21 antibodies (B) in the control population including patients with ankylosing spondylitis (SpA, n=38) and psoriatic arthritis (PsA, n=39) (patients with osteoarthritis (n=8) and Sjögren syndrome (n=3) are not depicted because of low sample size). Ig, immunoglobulin; PsA, psoriatic arthritis; SpA, ankylosing spondylitis

5.3.5 Prognostic information based on antibody (sub)class testing

Antibody isotypes interact differently with effector molecules and Fc receptors (FcR). Therefore, they can have different pathogenic potential. Apart from their biological properties, possible associations with clinical data were investigated to explore prognostic information based on isotype distribution. Within this study, a link was found between smoking and the presence of anti-UH-RA.1 antibodies of the IgA class: IgA was found in 4/16 smokers and 0/77 non-smokers (Fischer's $p=0.001$). No other prognostic information could be deduced from testing for anti-UH-RA.1 antibodies. Regarding antibodies against UH-RA.21, an association was found between the presence of erosions and levels of IgG (MWU $p=0.028$) or IgG2 (MWU $p=0.033$). For both (sub)class, also associations were observed for ESR (IgG MWU $p=0.010$ and χ^2 $p=0.045$; IgG2 χ^2 $p<0.0001$). Additionally, ESR was associated with a positive test for anti-UH-RA.21 IgM (χ^2 $p=0.004$).

5.4 Discussion

In this study, we report the contribution of individual Ig classes of the IgG, IgM and IgA type, to total reactivity of novel autoantibodies against UH-RA.1 and UH-RA.21. Both antibody systems are represented by the full isotype repertoire and the isotype profile is similar in RA patients and RC. The major difference in isotype distribution between the two antibody systems is the contribution of IgM. For antibodies against UH-RA.1, IgM accounted for half of the reactivity, whereas for anti-UH-RA.21 antibodies the other two isotypes were twice as prevalent. IgM is the first antibody produced during the primary humoral immune response [302]. Presence of IgM is therefore associated with recent antigen exposure. If the antigenic stimulus persists, the μ chain is changed to γ (IgG) or α (IgA) [210]. The presence of the latter isotypes thus points towards a secondary antibody response. Because IgG is typical for a persistent immune response and for its relative abundance, IgG is often the first choice in diagnostic testing for chronic conditions. However, as the presence of IgM is indicative for recent antigen exposure, we can presume that the antibody response against UH-RA.1 (and in lesser extent also UH-RA.21) is marked by an ongoing immune response and a continuous (re)activation of the immune system. It is not fully understood how IgM production is sustained in the presence of IgG against the same antigen, but similar observations have been reported for ACPA [303]. IgA then, is the key component of the humoral response in mucosal tissue such as the lungs or the gut [304]. Its presence in the mucosa of the lungs to protect the epithelial surface, might explain the higher participation of anti-UH-RA.1 IgA in smokers compared to non-smokers as has also been observed for RF and ACPA. Pre-RA patients who were smokers, were significantly more often IgA RF positive [305]. Furthermore, IgA ACPA appeared earlier in smokers than in non-smokers [228, 299, 300].

Regarding the IgG-subclasses, the majority of anti-UH-RA.1 antibody reactivity was attributable to IgG3, the most pro-inflammatory and pathogenic subclass as it is a potent activator of the complement cascade [306, 307]. This role is shared with IgG1, also found within the anti-UH-RA.1 antibody response. The dominance of the IgG3 subclass in the anti-UH-RA.1 antibody system seems not in line with previous findings of an association between the presence of the antibodies and the achievement of sustained DMARD-free remission (Chapter 3).

However, IgG antibodies can act in anti-inflammatory ways as well through the engagement of type II Fc receptors rather than type I FcR [308]. This is mediated by the glycan core structure of IgG, and modifications such as galactosylation and sialylation have been shown to shift the activity from pro- to anti-inflammatory [309-313]. Sialylated IgG-Fc, for instance, upregulates the inhibitory receptor FcγRIIB, increasing the activation threshold of innate effector cells to immune complexes [314].

For IgG subclasses directed against UH-RA.21, IgG2 was the most abundant subclass, followed by IgG3 and IgG1. IgG2 is considered less pathogenic than IgG3 and IgG1 and so far the IgG2 isotype is less comprehensively understood. The short hinge between the constant regions of the IgG2 prevents binding of the complement component C1q and affects the binding of the isotype to specific FcR [315]. In humans, IgG2 binds with high affinity to the neonatal Fc receptor (FcRn) and with low affinity to the FcγRIIA [316]. Binding to FcRn in acidic conditions prolongs the life span of IgG by recycling it back into the circulation instead of directing it towards degrading lysosomes [317-320]. FcRn is ubiquitously expressed in adult tissues and has the ability to transport the different IgG subclasses within and across cells, and may therefore contribute to the spread of the antibodies across the different joints affected in RA [305, 321-323]. FcγRIIA is the most widely expressed FcγR on myeloid cells but not on lymphocytes, and binding activates these FcγRIIA-expressing cells via its own immunoreceptor tyrosine-based activation motif [316]. Further characterization of the biological properties (e.g. glycan modifications) of the anti-UH-RA.1 and -UH-RA.21 antibody isotypes and their binding to different FcR will further clarify the significance of the isotypes in the pathophysiology of RA.

The usage of multiple antibody isotypes raised the question whether isotype-specific testing could be of added value to the diagnostic performance of the antibodies. Previously, antibody reactivity against UH-RA.1 and UH-RA.21 has been measured using an anti-IgG detection antibody. In this report, isotype-specific testing for anti-UH-RA.1 antibodies suggested an improvement by testing for IgM rather than IgG, as the sensitivity in RA patients was twice as high (18% vs 9%) with an associated specificity of 90%. Also the levels of anti-UH-RA.1 IgM were significantly higher in RA patients than in RC or HC. Although IgG1 and IgG2 were RA-specific compared to RC and HC, their prevalence was

too low to consider them for isotype-specific diagnostic testing. For anti-UH-RA.21 antibody testing, the sensitivity was shown to increase when adding IgA to the current IgG detection. Importantly, the full antibody isotype usage was already present in early stages of the disease. Furthermore, they were all detected in seronegative RA. These findings further support the promising role of the antibody systems in the diagnosis of early and seronegative RA patients.

In conclusion, we examined the isotype distribution of antibodies against UH-RA.1 and UH-RA.21. Since effector functions differ between antibody classes and subclasses, the study of the isotype profile is important to understand the pathophysiological role of the antibody systems. At present, we can only speculate about the exact mechanisms via which anti-UH-RA.1 and -UH-RA.21 antibodies work but we have outlined the isotype profile for both antibodies. The exact mechanisms by which the isotypes act need further investigation. The impact of the full usage of the antibody isotype repertoire was also evaluated for diagnostic application, and interestingly, the sensitivity of anti-UH-RA.1 antibodies was shown to increase considerably when measuring IgM instead of IgG. Full antibody isotype usage in early and seronegative RA is important in the validation of the candidate biomarkers for these diagnostically challenging RA subpopulations.

6

EFFECTS OF THE ANTI-UH-RA ANTIBODIES ON DISEASE SEVERITY IN *IN VIVO* MODELS OF RHEUMATOID ARTHRITIS

Abstract

In this study, in collaboration with Ghent University (UGhent), two *in vivo* mouse models were used in an antibody passive-transfer design to study the effect of the anti-UH-RA.1 and -UH-RA.21 antibodies in arthritic disease onset and progression. The first model was collagen-induced arthritis (CIA) in which arthritis is induced by immunization with collagen type II (CII). In these mice, antibodies against UH-RA.1 and UH-RA.21 were passively transferred to study the effects on disease severity and incidence. A pilot experiment demonstrated a disease-exacerbating effect of anti-UH-RA.21 antibodies in CIA. Subsequent experiments were conducted to validate these findings for UH-RA.21, and also to study the role of anti-UH-RA.1 antibodies in a similar setup. Although a trend was observed for higher disease severity in mice injected with anti-UH-RA.21 antibodies in subsequent experiments, the significant disease-exacerbating effect from the piloty study could not be confirmed, at least not on the level of visual clinical arthritis scores. However, examining subclinical bone resorption by means of micro-computed tomography indicated that mice injected with anti-UH-RA.21 antibodies suffered more erosion of the tibial condyles compared to mice injected with control antibodies. For antibodies against UH-RA.1, no disease-modifying effect was observed.

A second mouse model for RA that was applied, is based on the pathogenic properties of anti-CII antibodies, the collagen antibody-induced arthritis (CAIA). Using this model we studied the arthritogenic nature of UH-RA.21-specific antibodies. Antibodies against UH-RA.21 failed to induce arthritis in naïve mice and did not exacerbate arthritogenicity of the anti-CII antibodies. In conclusion, results from these *in vivo* models suggest the absence of arthritogenic properties of antibodies against UH-RA.21, but a putative role in subclinical events taking place in eroding bone lesions.

6.1 Introduction

Exploration of the prognostic relevance of the anti-UH-RA biomarkers suggested a role as 'the good and the bad' for antibodies against UH-RA.1 and UH-RA.21: the former were associated with sustained DMARD-free remission in an early arthritis cohort while the latter were linked to inflammation and the presence of erosions, inflammation and higher tender and swollen joint counts (Chapter 3 and 4). These findings increased our interest in the biological role of the UH-RA antibodies in rheumatic and arthritic pathology. Animal models are a valued research tool for studying disease mechanisms. Two principal models of choice, in terms of obtaining more insight into the underlying disease processes of rheumatoid arthritis (RA), are collagen-induced arthritis (CIA) and collagen antibody-induced arthritis (CAIA) in mice [138, 139, 149-151]. Both models rely on arthritis elicited by antibodies against collagen type II (CII). CII is exclusively expressed in the joints as major constituent of the cartilage, and in RA it is a known target for autoantibodies [140]. Immunization of mice with heterologous CII causes a chronic and progressive form of arthritis strongly resembling RA in humans as the inflammatory arthritis affects the synovial joints leading to cartilage damage and bone erosions. As RA susceptibility is linked to specific major histocompatibility (MHC) II molecules, susceptibility to CIA is attributed to the q haplotype of the mouse MHC ($H\text{-}2^q$) [145, 146]. Based on their $H\text{-}2^q$ background, DBA/1 mice are generally chosen for CIA which is induced by immunization with CII in Complete Freund's Adjuvant (CFA) [141]. The pathophysiological properties of CII-specific antibodies enable the use of transfer models such as CAIA, in which anti-CII antibodies are passively transferred to mice in order to study the antibody-mediated pathways in later stages of the disease – after disease initiation. CAIA is characterized by an acute, rapid-onset arthritis induced by anti-CII antibodies and enhanced by lipopolysaccharide (LPS) [152]. Optimal results are obtained when mice are administered anti-CII antibodies directed against different epitopes spread over the entire CII protein (antibody cocktail) [153].

The primary aim of this study is to investigate the effect of antibodies against UH-RA.1 or UH-RA.21 on the induction and exacerbation of arthritic processes based on the CIA and CAIA mouse models. Anti-UH-RA.1 and -UH-RA.21 autoantibodies, will be applied in a passive transfer in CIA mice after which

arthritis incidence and severity is evaluated. For anti-UH-RA.21 antibodies previously linked with the presence of erosions, putative subclinical effects on bone resorption are further examined by micro-computed tomography (micro-CT). Furthermore, we will study the effect of anti-UH-RA.21 antibodies in naïve DBA/1 mice without prior immunization by (antibodies to) CII, to evaluate the potential arthritogenic nature of anti-UH-RA.21 antibodies analogous to the arthritis induction by anti-CII antibodies in the CAIA model. Whether anti-UH-RA.21 antibodies enhance the arthritogenicity of anti-CII antibodies in CAIA, will also be investigated. Results from this study will provide insight into the biological relevance of circulating anti-UH-RA.1 and anti-UH-RA.21 antibodies in the disease course of RA.

6.2 Materials and methods

6.2.1 Affinity-purification of human polyclonal antibodies

By means of affinity chromatography, polyclonal antibodies against UH-RA.21 (PGGFRGEFMLGKPDPKPEGKGLGSPYIE) were purified from pooled plasma obtained from a heterogeneous population of rheumatic patients testing positive on an in-house UH-RA.21 peptide enzyme-linked immunosorbent assay (ELISA). HiTrap™ N-hydroxysuccinimide (NHS)-activated High-Performance (HP) 1 ml columns (GE Healthcare, Diegem, Belgium) were covalently coupled with synthetic UH-RA.21 peptide (>95% purity, Eurogentec, Seraing, Belgium) using the peristaltic pump P-10 (GE Healthcare) according to manufacturer's guidelines. Briefly, after washing out the isopropanol from the column with 1 mM ice-cold HCl, 1 mg UH-RA.21 peptide was applied in 1 mL coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and incubated for 1.5 hour (h) at room temperature (RT). Any excessive active groups were deactivated by 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3. Non-specifically bound ligands were washed out by 0.1 M acetate, 0.5 M NaCl, pH 4. Coupling efficiency was verified by acidification and spectrophotometry (adsorption at 280nm, SmartSpec™ Plus, Bio-Rad, Nazareth, Belgium).
Control antibodies were purified from plasma of healthy controls testing negative on UH-RA.21 peptide ELISA, using Pierce™ Protein A/G Chromatography Cartridges (Thermo Scientific).
An ÄKTA Prime plus device (GE Healthcare) was used for the purification of antibodies. Plasma material was cleared by centrifugation, diluted in binding buffer and filtered (0.45 µm). The samples were then applied to the peptide-coupled resin after an initial equilibration step with binding buffer (0.1 M Na_3PO4, 0.15 M NaCl, pH 7.2). Bound antibodies were eluted with an acidic elution buffer (pH 2.8; Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) and immediately neutralized with 1 M Tris pH 9.0. In-between washing steps were performed with binding buffer and all buffers were passed through a 0.22 µm filter prior to application on the column. Control immunoglobulin (Ig) yield was determined by spectrophotometry (adsorption at 280nm, SmartSpec™ Plus, Bio-Rad or NanoDrop 2000 Spectrophotometry, Thermo Scientific). Presence of specific anti-UH-RA.21 antibodies in elution

fractions was verified by UH-RA.21 peptide ELISA and quantified by spectrophotometry.

6.2.2 Peptide ELISA

Synthetic cyclized UH-RA.21 peptides (>85% purity, Eurogentec, and GL Biochem, Shanghai, China) were coated overnight at RT at 1 µg/mL in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 130 mM NaCl) in polystyrene flat-bottom microplates (Greiner Bio-One, Wemmel, Belgium). After washing, plates were blocked with 200 µL/well of PBS containing 2% (w/v) skimmed milk powder (MPBS) for 2 h shaking at 37°C. Plates were washed during 5 minutes (min) for three consecutive times. Plasma samples (diluted 1:100 in 2% MPBS) were incubated for 2h at RT (100 µL/well, shaking). After washing, antibody binding was detected using a polyclonal rabbit anti-human IgG or rabbit anti-mouse Ig secondary antibody conjugated to horseradish peroxidase (HRP; Dako, Heverlee, Belgium), 100 µL/well diluted 1:2,000 in 2% MPBS for 1 h shaking at RT. Following washing, staining was performed in the dark with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific). After 6 min, the reaction was stopped with 50 µL 2N H_2SO_4 and results were read at 450 nm (Microplate reader Infinite M1000 Pro, TECAN, Männedorf, Switzerland). Washing steps were performed with PBS containing 0.05% (v/v) Tween-20 (VWR, Leuven, Belgium). Samples are considered positive when the optical density (OD) signal of reactivity to UH-RA.21 peptide was at least twice as to an irrelevant control peptide (WTKTPDGNFQLGGTEP).

6.2.3 Generation of rabbit polyclonal antibodies

Purified polyclonal rabbit antibodies were purchased from Eurogentec. Briefly, rabbits were challenged by two boost injections with synthetic UH-RA.1 or UH-RA.21 peptides (produced by Eurogentec) coupled to 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS)-conjugated keyhole limpet hemocyanin (KLH) carrier protein, in order to raise peptide-specific antibodies. A non-Freund adjuvant was used to stimulate the immune response and reactivity was verified by ELISA. Specific antibodies were

purified by means of AF-Amino Toyopearl affinity chromatography (Tosoh Bioscience, Darmstadt, Germany).
Control Ig were isolated from pre-immune rabbit sera using protein A resin. The specificity of the purified antibodies was also verified on our in-house peptide ELISA for UH-RA.1 and UH-RA.21.

6.2.4 Antibody preparation

Anti-UH-RA.21 antibodies purified from human samples were dissolved in PBS (1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 130 mM NaCl). Rabbit antibodies against UH-RA.1 and UH-RA.21 were dissolved in PBS (1.5 mM KH_2PO_4, 137 mM NaCl, 27 mM Na_2HPO_4, 2.5 mM KCl, pH 7.4) containing 0.01% thimerosal, and bovine serum albumin (BSA) in a concentration of 0.27% (CAIA) or 0.1% (CIA). For antibody concentration and buffer exchange, Pierce Concentrators 9K were used (Thermo Fisher Scientific). After pre-rinsing the concentrator with vehicle buffer for glycerin removal, the antibody pools were placed in the upper sample chamber and centrifuged. The concentrated sample was recovered and vehicle buffer was added to the concentrator once more for additional sample recovery. Centrifugation steps were performed at 4°C. Antibody concentration was measured using NanoDrop 2000 Spectrophotometry (Thermo Scientific).

6.2.5 Collagen-induced arthritis

Animal experiments were performed at Ghent University (UGhent) with the approval of the Animal Ethical Committee from UGhent. Male DBA/1 Rj (H-2q background) mice were purchased from Janvier Labs (Saint Berthevin Cedex, France) and at age of 9 weeks immunized intradermally at the base of the tail with 200 µg of chicken CII (in 0.1 M acetic acid; Morwell Diagnostics GmbH, Zurich, Switzerland) emulsified in CFA containing 150 µg mycobacterium Tuberculosis H37RA from Difco (Lawrence, KS, USA). Twenty-one days after immunization, mice were re-challenged with a booster injection of CII in Incomplete Freund's Adjuvant (IFA). Starting from day 18, mice were monitored for clinical symptoms of arthritis by two trained laboratory personnel acting independently and blinded to treatment regimen. Clinical severity was graded as described in Table 6.1. The total score for visual clinical

arthritis was based on all four paws averaged from two investigators. At day 48 after immunization, all mice were sacrificed.

Table 6.1. Scoring system for clinical severity in collagen-induced arthritis and collagen antibody-induced arthritis

Visual arthritis score	Clinical severity
0.0	No erythema or edema
0.5	Erythema and edema in one digit
1.0	Erythema and mild edema of the footpad or ankle or two to five digits
2.0	Erythema and moderate edema of two joints (footpad, ankle, two to five digits)
3.0	Erythema and severe edema of the entire paw
4.0	Reduced swelling and deformation leading to incapacitated limb

Clinical severity is graded for each paw and the total score for four paws is calculated to obtain a final visual arthritis score per mouse

Passive transfer of human antibodies

In two CIA experiments, a passive transfer of the purified human polyclonal antibodies directed against UH-RA.21 was conducted to investigate putative disease-exacerbating effects. In the pilot experiment, 500 µg of affinity-purified human anti-UH-RA.21 antibodies, control antibodies or PBS in a volume of 170 µl was injected intraperitoneally in CIA mice (n=5 per group) when an individual arthritis score equal to or higher than 0.5 for more than one day was observed. Blood was collected at day -1, 7, 19, 33 and 42 of the CIA induction.

In a subsequent validation experiment, mice with an individual clinical score of ≥1 for two consecutive days were treated with affinity-purified human anti-UH-RA.21 antibodies, human control Ig or vehicle (PBS). Treatment was administered once with 500 µg of antibodies in a volume of 300 µl, injected intraperitoneally. Blood was collected at day -1 of the CIA induction, and at day 1, 5, 10, 15 and 23 after treatment.

The presence of anti-UH-RA.21 antibodies in the collected blood samples was evaluated by means of peptide ELISA.

Passive transfer of rabbit antibodies

In a second experiment, polyclonal rabbit antibodies against either UH-RA.1 or UH-RA.21 were injected intraperitoneally in CIA mice. Control mice received only vehicle, PBS or no treatment. After the appearance of the first signs of arthritis at day 28, mice were randomly assigned and administered a treatment. A weekly dose of 200 µg of antibodies was injected for three weeks on a row. Knee joints were collected for histochemical analyses, scoring of inflammation, and micro-CT.

6.2.6 Collagen antibody-induced arthritis

Animal experiments were performed at Ghent University (UGhent) with the approval of the Animal Ethical Committee from UGhent. Male DBA/1 Rj mice were purchased from Janvier Labs. At age of 9 weeks mice were intravenously injected with 2 mg ArthritoMab^{TM} antibody (MAB) cocktail (MD Biosciences Inc, St Paul, MN, USA), followed by an intraperitoneal injection with 100 µg LPS three days later. Starting from day 2 mice were monitored for clinical symptoms of arthritis by two trained laboratory personnel acting independently and blinded to treatment regimen. Grading of clinical severity and calculation of visual clinical arthritis scores was performed analogous to CIA methods. At day 10 all mice were sacrificed.

Passive transfer of rabbit antibodies

To study the arthritogenic nature of anti-UH-RA.21 antibodies, 2 mg polyclonal rabbit antibodies was injected solely or in combination with the MAB cocktail. Control mice received vehicle, PBS or only MAB cocktail. Knees were collected for histochemical analyses, scoring of inflammation, and micro-CT.

6.2.7 Bone analysis by micro-CT

Micro-CT images were obtained at the Centre for X-ray tomography of Ghent University, Belgium (UGCT). The primary regions of interest for bone resorption are the tibial and femoral condyles right above the epiphysial plate, where histological scoring is usually performed and erosions are most likely to be seen in CIA. Bone erosion was analyzed by micro-CT. Optimal scanner settings were

selected based on the sample size and composition. The samples were scanned on the High-Energy CT system Optimized for Research (HECTOR) [324], using a directional X-ray source set at 100 kV, 1 mm Aluminum filtration, 10 Watt beam power. The detector was a Perkin-Elmer flat panel measuring 40 x 40 cm, with a pixel pitch of 200 µm. The magnification was set at 40 times resulting in a voxel pitch inside the sample of 5 µm. A total of 2,000 projections of 1 second exposure time each was recorded. The data were then reconstructed using Octopus, a commercial software originally developed by the UGCT which uses a custom implementation of the standard FDK-algorithm for reconstruction of cone-beam CT data [325, 326]. Three-dimensional (3D) visualizations and calculations were made using the commercial rendering software VG Studio MAX (Volume Graphics, Heidelberg, Germany) or Fiji (Image J) [327].

6.2.8 Statistical analyses

Visual clinical arthritis scores are depicted as mean ± standard error of the mean (SEM). Mean scores were compared by the non-parametric Mann-Whitney U test for two groups and Kruskal-Wallis one-way analysis of variance (ANOVA) for more than two groups. Longitudinal clinical scores were analyzed using linear mixed models. A p-value less than 0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism version 5 (Graph Pad software, La Jolla, California, USA), IBM SPSS Statistics for Windows, version 22.0 (IBM Corp. Armonk, New York, USA) and JMP Version Pro 11.2 (SAS Institute Inc, Cary, North Carolina, USA).

6.3 Results

6.3.1 Effect of passive transfer of anti-UH-RA.21 antibodies in collagen-induced arthritis

To investigate the effect of anti-UH-RA.21 antibodies on the arthritic disease process, we purified human polyclonal anti-UH-RA.21 antibodies from patient plasma and passively transferred them into CIA mice shortly after symptom onset.

Results from a pilot experiment demonstrated disease-exacerbation in the anti-UH-RA.21 antibody-injected mice compared to animals injected with purified healthy control Ig (n=5) (Figure 6.1). From day 3-6, the visual clinical arthritis scores were significantly higher in the mice injected with anti-UH-RA.21 antibodies (day 3 p=0.0269, day 4 p=0.0153, day 5 p=0.0153, day 6 p=0.0189).

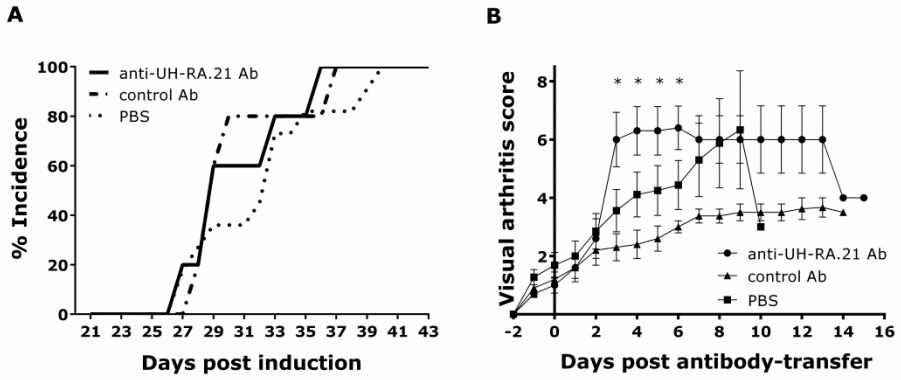

Figure 6.1. Effect of antibodies against UH-RA.21 after passive transfer in collagen-induced arthritis (CIA) in a pilot experiment. Mice were injected with human affinity-purified polyclonal antibodies (Ab) against UH-RA.21 (n=5), control antibodies (n=5) or phosphate-buffered saline (PBS) (n=11) shortly after symptom onset. [A] Incidence (%). [B] Visual arthritis score (mean ± SEM). *p<0.05

In a subsequent validation experiment, this passive transfer was repeated in larger treatment groups. 0.5 mg of human anti-UH-RA.21 antibodies was administered in DBA/1 CIA mice shortly after symptom onset, which ranged from day 33 to 35 after the first immunization (Table 6.2). Mean (SD) clinical scores at time of antibody injection were 2.1 (1.3), 1.8 (0.8) and 1.9 (0.9) for the anti-UH-RA.21 antibodies, control antibodies and PBS group, respectively. Furthermore, at the peak of the disease, clinical scores of 5.8 (2.0), 6.2 (3.5) and 5.1 (2.2) were reached for the respective groups.

Table 6.2. Incidence of CIA, days post induction and visual arthritis scores at time of passive transfer of human anti-UH-RA.21 antibodies

	Anti-UH-RA.21 Ab	**Control Ab**	**PBS**
No. of animals	14	15	14
Incidence of CIA (%)	100	93	100
Days post induction at time of passive transfer, mean±SD	35.1 ± 5.6	33.1 ± 5.7	33.4 ± 5.1
Clinical score at time of passive transfer, mean ± SD	2.1 ± 1.3	1.8 ± 0.8	1.9 ± 0.9

Ab, antibodies; CIA, collagen-induced arthritis; PBS, phosphate-buffered saline; SD, standard deviation

No difference in CIA incidence was observed between the experimental groups (Figure 6.2A). Furthermore, the anti-UH-RA.21 antibodies did not increase disease severity ($p=0.4621$, Figure 6.2B) when compared to control antibodies or vehicle (PBS). The first mice developed arthritis symptoms around day 25 and the number of affected mice increased gradually until a high incidence rate was reached ranging from 93% to 100% at 43 days after the first immunization (Table 6.2).

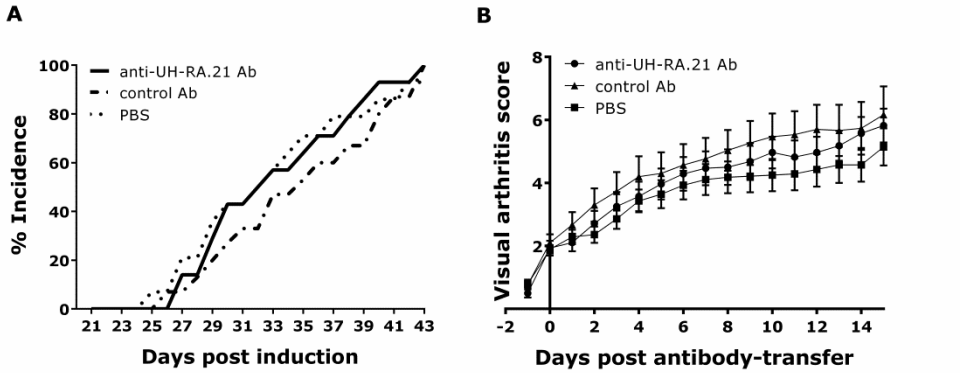

Figure 6.2. Effect of passive transfer of antibodies against UH-RA.21 on the disease severity of collagen-induced arthritis (CIA). Mice were injected with human affinity-purified polyclonal antibodies (Ab) against UH-RA.21 (n=14), control antibodies (n=15) or phosphate-buffered saline (PBS) (n=14) shortly after symptom onset. [A] Incidence (%). [B] Visual arthritis score (mean ± SEM).

Circulating anti-UH-RA.21 antibodies after antibody administration were assessed by peptide ELISA. Within two mice from the pilot experiment, the injected anti-UH-RA.21 antibodies could be detected: for one mouse antibody levels at day 1 had a ratio of specific to non-specific target of 62, for the other mouse a ratio of 44 was observed at day 4. In the validation experiment, the passive transfer of antibodies was generally accompanied by high levels at day 1 after antibody injection, followed by a decrease at day 5. From day 10 onwards, no antibodies could be detected anymore in the serum samples (Figure 6.3). Intrinsic mouse anti-UH-RA.21 antibodies were not detected.

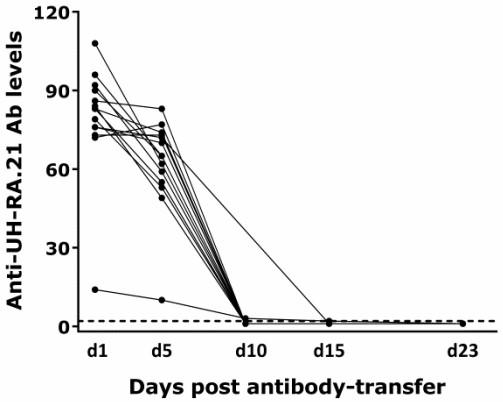

Figure 6.3. Serum levels of anti-UH-RA.21 antibodies after passive transfer to mice with collagen-induced arthritis (CIA). Serum levels of anti-UH-RA.21 antibodies (Ab) are shown after passive transfer in CIA mice shortly after disease onset. Antibody levels were measured by peptide enzyme-linked immunosorbent assay and expressed as the ratio between reactivity to the specific UH-RA.21 peptide and a control peptide. The cutoff represents the cutoff for an antibody-positive sample.

To reduce variability in antibodies, anti-UH-RA.1 and -UH-RA.21 antibodies were generated in rabbits and also used for passive transfer in CIA mice to study the effect on the arthritic disease process. When the first mice started to develop arthritis symptoms at day 28, all mice were treated simultaneously with 0.2 mg of antibodies for three weeks on a row (day 28, day 35 and day 42). Mean visual clinical arthritis scores for each group at time of treatment are depicted in Table 6.3.

Table 6.3. Incidence and average visual arthritis score at times of treatment in a passive-transfer experiment in collagen-induced arthritis

	Anti-UH-RA.21 Ab	Anti-UH-RA.1 Ab	Pre-imm Ab	Vehicle	PBS
No. of animals	10	12	13	13	12
Day 28					
Inc of CIA (%)	23	21	14	21	21
Clinical score	0.5 ± 1.1	0.5 ± 1.3	0.4 ± 0.9	0.6 ± 1.3	0.6 ± 1.1
Day 35					
Inc of CIA (%)	62	50	71	50	43
Clinical score	2.4 ± 3.2	1.9 ± 3.4	1.5 ± 1.8	2.1 ± 2.6	1.5 ± 2.2
Day 42					
Inc of CIA (%)	699	77	93	86	71
Clinical score	3.5 ± 4.0	2.7 ± 3.3	3.1 ± 2.7	3.5 ± 3.3	2.7 ± 2.8

Clinical scores are presented as mean ± standard deviation.
Ab, antibodies; CIA, collagen-induced arthritis; Inc, incidence; Pre-imm, pre-immune; PBS, phosphate-buffered saline

Neither antibodies against UH-RA.1, nor antibodies against UH-RA.21, modified disease onset (p=0.8607, Figure 6.4A) or clinical disease score (p=0.2762, Figure 6.4B) after passive transfer to CIA mice. Mean (SD) clinical scores at the peak of CIA were lower compared to the passive-transfer experiment using human polyclonal antibodies: 4.8 (4.8) and 4.2 (4.8) for antibodies directed against UH-RA.21 and UH-RA.1, respectively, 4.4 (3.5) for pre-immune antibodies, 4.5 (3.6) for vehicle and 3.8 (2.9) for PBS.

Although no significant differences were observed in CIA incidence and disease scores between the different treatments, we noticed that mice injected with anti-UH-RA.21 antibodies tended to have the highest average clinical score (Figure 6.4B) despite the lowest incidence (Figure 6.4A). Therefore, we evaluated the effects of the different treatments in clinically arthritic mice only. However, within diseased mice, anti-UH-RA.21 antibodies also had no significant effect on disease severity in CIA (p=0.3487, Figure 6.4C).

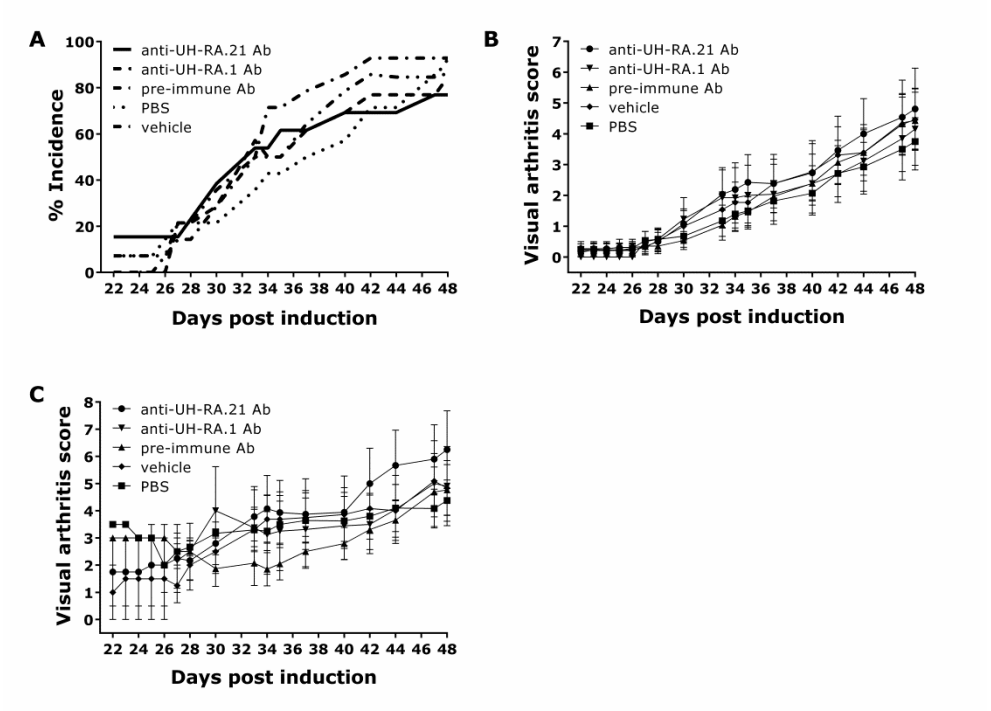

Figure 6.4. Effect of passive transfer of antibodies against UH-RA.1 or UH-RA.21 on the disease process of collagen-induced arthritis (CIA). DBA/1 CIA mice were injected with rabbit affinity-purified polyclonal antibodies (Ab) against UH-RA.1, UH-RA.21, pre-immune control Ab, vehicle (phosphate-buffered saline (PBS) with 0.01% thimerosal and 0.27% bovine serum albumin) or PBS shortly after the appearance of the first arthritic symptoms (day 28). [A] Incidence (%). [B] Visual arthritis score (mean ± SEM). [C] Visual arthritis score (mean ± SEM) for clinically arthritic mice only.

6.3.2 Effect of anti-UH-RA.21 antibodies on bone resorption in collagen-induced arthritis

In humans, the presence of anti-UH-RA.21 antibodies has been linked to the presence of erosions. Therefore, we investigated subclinical features such as increased bone resorption in knee in more detail. This was performed by means of micro-CT analysis on knees from 6 mice treated with anti-UH-RA.21 antibodies and 6 mice treated with control antibodies (Table 6.4).

Table 6.4. Visual clinical arthritis scores of hind legs and erosion scores of knees selected for bone analysis by micro-computed tomography after passive antibody transfer in collagen-induced arthritis

Mouse	Experimental group	Visual arthritis score	Semi-quantitative visual erosion score
#1	anti-UH-RA.21 Ab	0	0.0
#2	anti-UH-RA.21 Ab	1	0.0
#3	anti-UH-RA.21 Ab *	1	2.7
#4	anti-UH-RA.21 Ab	3	1.7
#5	anti-UH-RA.21 Ab	4	2.3
#6	anti-UH-RA.21 Ab *	4	2.3
#7	pre-immune Ab	0	0.0
#8	pre-immune Ab	1	0.0
#9	pre-immune Ab *	1	1.3
#10	pre-immune Ab	3	2.0
#11	pre-immune Ab *	4	1.3
#12	pre-immune Ab	4	2.3

Erosions were semi-quantitatively scored based on visual erosion scores independently assigned by three experienced researchers. *3D-images of affected knee provided in Figure 6.5. Ab, antibodies.

The primary region of interest was the tibial condyle. 3D-images of tibial condyles from mice treated with anti-UH-RA.21 antibodies and control antibodies are shown in Figure 6.5. Erosions were assessed for mice with mild arthritis (clinical score of 1) and severe incapacitating disease (clinical score of 4). Visual assessment of the bone surface suggests more erosion after injection of anti-UH-RA.21 antibodies compared to control antibodies in mice with the same clinical score (Figure 6.5).

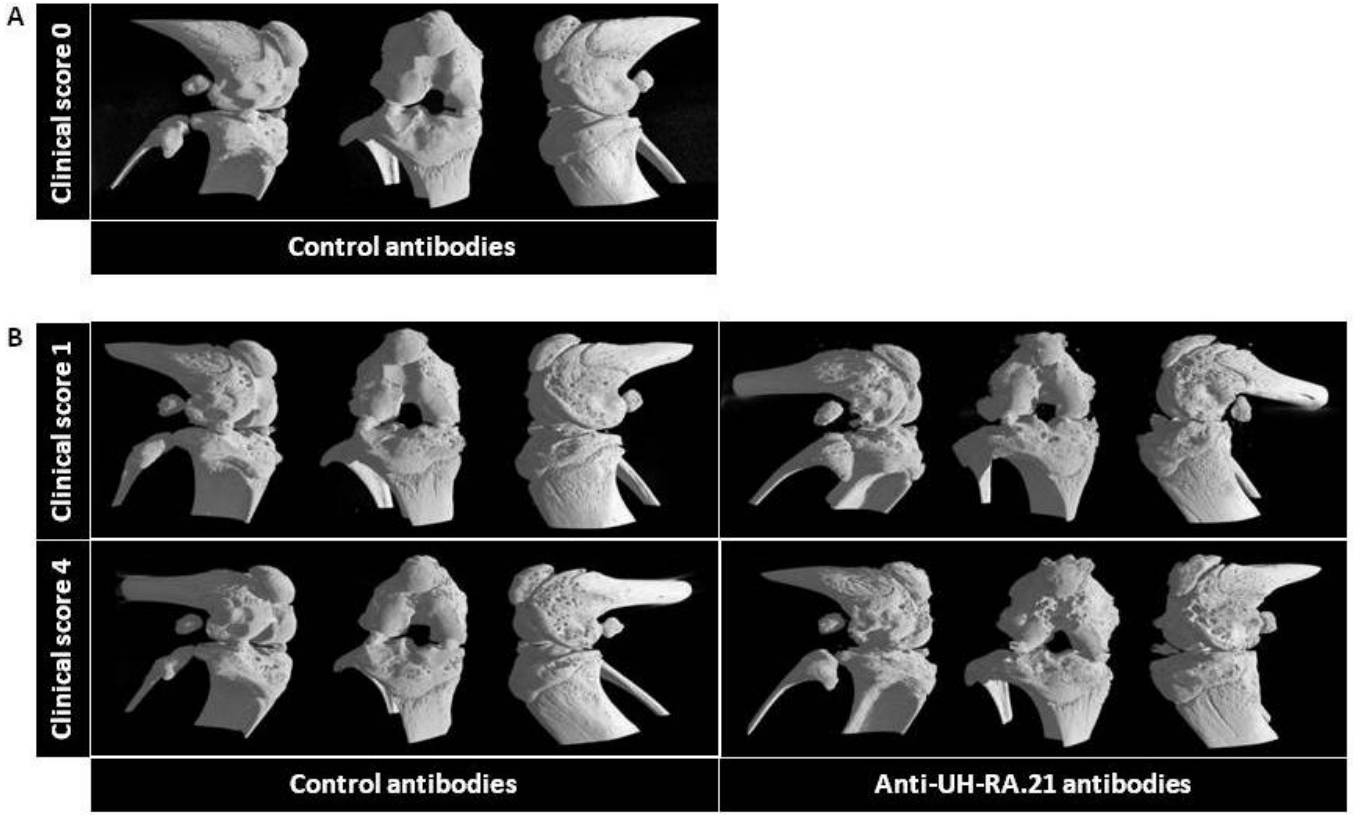

Figure 6.5. 3D micro-computed tomography (micro-CT) bone analysis of collagen-induced arthritis (CIA) mice after passive transfer of anti-UH-RA.21 antibodies. [A] Knee bone from CIA mice without clinical arthritis symptoms after administration of control antibodies. [B] Knee bone from CIA mice with clinical arthritis (score 1 and 4) after administration of either control or anti-UH-RA.21 antibodies.

6.3.3 Effect of anti-UH-RA.21 antibodies in naïve DBA/1 mice and collagen antibody-induced arthritis

In the CAIA mouse model, the injection of a cocktail containing four arthritogenic monoclonal antibodies to CII (MAB cocktail) causes acute arthritis in DBA/1 mice. To investigate the arthritogenic nature of antibodies against UH-RA.21, they were injected in DBA/1 mice solely or in combination with the MAB cocktail, at 2 mg per mouse. Arthritis induced by the MAB cocktail emerged rapidly with a 100% incidence (Figure 6.6A, n=6), and was not significantly modified by the presence of antibodies against UH-RA.21 or control treatments (p=0.6650, Figure 6.6B, n=5 per group). Furthermore, anti-UH-RA.21 antibodies on their own did not have an arthritogenic effect in DBA/1 mice as none of the mice developed arthritic symptoms (Figure 6.6C, n=5 per group).

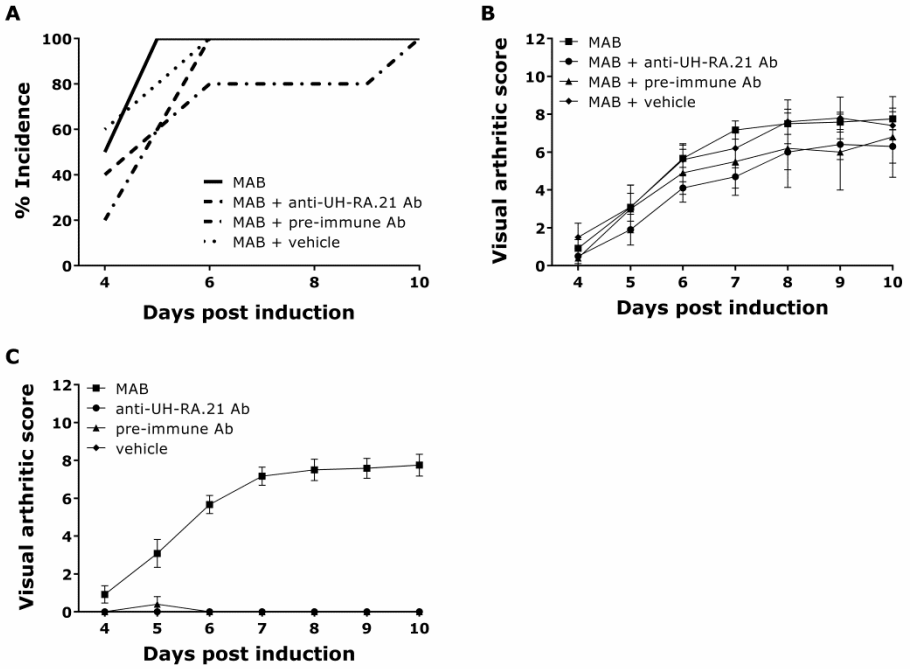

Figure 6.6. Passive transfer of antibodies against UH-RA.21 in naïve DBA/1 mice or in combination with an arthritogenic antibody cocktail (collagen antibody-induced arthritis or CAIA). Arthritis was successfully induced by a monoclonal antibody cocktail (MAB) [A, B]. The administration of polyclonal rabbit antibodies against UH-RA.21 did not exacerbate MAB-induced arthritis symptoms [B] and failed to induce arthritis in naïve DBA/1 mice [C].

6.4 Discussion

In humans, antibodies against UH-RA.1 and UH-RA.21 were shown to possess prognostic information. While anti-UH-RA.1 antibodies were linked to remission, patients carrying anti-UH-RA.21 antibodies were more likely to present with erosions and more severe inflammation (Chapter 3). To elucidate the role of the anti-UH-RA antibody responses in the arthritis disease course, *in vivo* experiments were performed using two standard mouse models for RA – CIA and CAIA. These models allow us to study disease-modifying effects of the antibodies and to assess the putative arthritogenic nature.

A pilot experiment based on the passive transfer of anti-UH-RA.21 antibodies in CIA mice, suggested a disease-exacerbating effect of the specific antibodies. The mean visual clinical arthritis score was significantly higher in the anti-UH-RA.21 antibody group at day 3-6. To confirm these findings, a validation experiment was designed similar to the pilot experiment: affinity-purified UH-RA.21-specific antibodies were administered to CIA mice shortly after the appearance of the first clinical symptoms. In contrast to the pilot experiment, anti-UH-RA.21 antibodies did not modify arthritis clinical scores or incidence this time when compared to control antibodies. Reasons for the inconsistent results might be attributable to the use of donor samples for the isolation of antibodies. In view of availability, either different donors were used or samples from same donors were collected on various time points – antibody characteristics might vary considerably between patients and can change during disease course. Furthermore, the timing of antibody injection is slightly different and what happens with the antibodies after injection is also not fully understood. If they were to exacerbate clinical arthritis, they presumably leave the blood stream and move to the joints. Whereas in the pilot experiment, the injected antibodies were detected in two out of four mice at day 1 or 4 of treatment, and irrespective of their clinical score, in the validation study all mice presented with much higher antibody levels – although an effect of long-term storage of the samples cannot be ruled out: serum samples from CIA mice from the pilot experiment had been stored at -80°C for a considerably longer time period than samples from the validation experiment, when they were tested simultaneously for the presence of anti-UH-RA.21 antibodies. Serum samples from CIA mice were also tested for the presence of mouse anti-UH-RA.21 antibodies, but we

could not detect any intrinsic antibodies as has been demonstrated for ACPA [328]. The distribution of the injected antibodies towards the (inflamed) joints or other organs, could be studied by a radiolabelling procedure using ^{99m}Tc labeling and gamma camera imaging which has already been successfully employed by our research collaborators [329]. This would allow to interpret the action and distribution of injected antibodies in function of the affected joints.

Antibody isolation from human material is limited and prone to donor variability, so polyclonal rabbit antibodies were generated directed against UH-RA.21 and an additional target, UH-RA.1. This approach allows higher reproducibility and antibody production on larger scale. For injection of CIA mice with rabbit anti-UH-RA.1 antibodies, no effect on disease scores was observed. Passive transfer of rabbit anti-UH-RA.21 antibodies in CIA mice did not lead to a disease-exacerbating effect on clinical level. Additionally, no arthritogenic properties could be attributed to anti-UH-RA.21 antibodies as they were not arthritogenic on their own and they did not enhance the arthritogenic effect of CII-specific antibodies in CAIA. Despite the lack of an effect on clinical scores, the anti-UH-RA.21 antibodies might have influenced bone resorption in CIA on microscopic level: 3D micro-CT analyses suggested more bone erosion after the injection of UH-RA.21-specific antibodies compared to control antibodies. It is important to note that mice with a clinical score of 1 can have an affected footpad, ankle or two or more digits – which might escort different subclinical features of the knees. Nevertheless, a different degree of bone erosions was clearly visible on mice with most severe arthritis accompanied by incapacitated limbs. These findings may suggest evidence for a subclinical role of UH-RA.21-specific antibodies in bone damage in the inflammatory arthritic pathology. Quantification of bone resorption in the affected knees is currently ongoing and will provide a more objective measure of the visual erosions. Further expansion of these preliminary data on bone resorption, and immunohistochemical evaluation of the affected knees, will help to elucidate the role of the antibodies in the underlying pathology. In future experiments, additional subclinical effects can also be determined by measuring the severity of inflammation more objectively, either by performing histological evaluation of the targeted joints or by measuring CII-specific T cell response [141, 330, 331]. Also, use of different strains will assess the role of the genetic background.

In conclusion, the first exploration of *in vivo* effects of anti-UH-RA.1 antibodies showed no disease-modifying effects. Moreover, the disease-exacerbating effects of anti-UH-RA.21 antibodies on clinical disease scores in CIA according to a pilot experiment, could not be confirmed in subsequent validation experiments. However, micro-CT images of affected knees suggest higher bone resorption after passive transfer of anti-UH-RA.21 antibodies. While these findings require further investigation, the identification of the corresponding *in vivo* targets of UH-RA.1 and UH-RA.21, will also open a broad range of opportunities to study and decipher the pathophysiological role of the antibody responses. The generation of antibodies specifically directed against the *in vivo* targets might yield more precise antibodies with distinct properties, likely to resemble true *in vivo* conditions more accurately and provide important information on the role of the targets and antibodies of interest in RA pathogenesis.

7

SUMMARY, GENERAL DISCUSSION & FUTURE PERSPECTIVES

7.1 Summary and general discussion

Rheumatoid arthritis (RA) is a chronic disabling disease for which no cure exists so far. Persistent inflammation attacks synovial joints and destroys joint cartilage and bone [3, 4]. Moreover, systemic features lower patients' life expectancy [13]. Currently, available treatments suppress symptoms and disease progression with varying efficacy and it is of utmost importance to initiate treatment as early as possible [23-29]. Therefore, an early and accurate diagnosis of RA is necessary. The diagnosis of RA is made clinically by fulfillment of classification criteria [8]. These criteria involve the presence of two serological biomarkers, rheumatoid factor (RF) and antibodies against citrullinated proteins (ACPA). Unfortunately, up to one third of RA patients test negative on these biomarkers hampering RA diagnosis in these patients [220, 256]. Novel biomarkers may improve RA diagnostics, and help to establish an early diagnosis and intervention. In this thesis, we aimed to validate and characterize novel candidate biomarkers, previously identified by serological antigen selection (SAS), a powerful high-throughput screening technology based on phage display [265-267]. The initial phage-based research test was translated into a sensitive and reproducible test format, allowing large-scale screenings. Two large independent European cohorts (n=479 and n=1066) were screened for the presence of two novel candidate markers – antibodies against UH-RA.1 and UH-RA.21 – in order to evaluate their diagnostic and prognostic value. Furthermore, putative pathogenic properties and the biological relevance of the antibody markers were addressed. In the following paragraphs, the main findings are summarized and discussed.

PART 1: Optimization of the testing method

The presence of novel autoantibodies was previously measured by means of enzyme-linked immunosorbent assays (ELISA) based on phages expressing the antibody targets, a format emerging from the SAS procedure by which the novel targets were identified. Unfortunately, the intensive labor and specific safety measures that come along with the use of phages, make this approach not suitable for clinical laboratories. Furthermore, phage ELISA are characterized by relatively high background reactivity, high inter- and intra-assay variability and

therefore low reproducibility. To this end, the initial research test was first of all translated into a noncommercial clinical test. Synthetic peptides corresponding to the antibody-binding part expressed on the phages was directly bound on a solid-phase ELISA. The specificity of the antibody interaction with the phage-displayed peptides and the corresponding synthetic peptides was confirmed in competition ELISA, in which prior incubation with specific synthetic peptides was shown to decrease the signal in the phage ELISA, in contrast to prior incubation with irrelevant control peptide. The accuracy of the newly-developed peptide ELISA was verified by comparing exploratory screenings with phage ELISA results. These results proved that the phage ELISA were successfully translated into peptide ELISA. Thereby, background reactivity was reduced, inter- and intra-assay variability was reduced and the peptide ELISA were found to be more reproducible allowing large-scale screenings. As also experienced in subsequent screenings described in several chapters of this dissertation, peptide ELISA were successfully developed and optimized for UH-RA.1, UH-RA.9, UH-RA.14 and UH-RA.21, all constituting a relatively small phage-displayed peptide and selected based on the presence of specific autoantibodies in early and seronegative RA [267]. Interestingly, one standard protocol works for the detection of these different autoantibody systems. This facilitates simultaneous testing and is of particular interest when working towards a multiplex biomarker approach.

PART 2: The diagnostic and prognostic potential of the novel candidate biomarkers

Having the newly-developed peptide ELISA available, we aimed at validating the sensitivity and specificity of the most-promising antibody biomarkers. A first panel of four biomarkers – UH-RA.1, UH-RA.9, UH-RA.14, UH-RA.21 – was selected for further exploration based on prevalence in the original cohort and presence in early and/or seronegative RA. The first two candidates (UH-RA.1 and UH-RA.9) were obtained from screenings of patients with early disease, UH-RA.14 was identified from screening seronegative RA patients, and UH-RA.21 resulted from screenings on both pools. Previously, explorative screenings were performed by means of phage ELISA resulting in an antibody sensitivity and

associated specificity of 10% and 97% for UH-RA.1, 4% and 100% for UH-RA.9, 12% and 100% for UH-RA.14 and 29% and 95% for UH-RA.21.

In **Chapter 3**, we described the results from a validation screening in two large European cohorts. In a first cohort consisting of 292 RA patients and 187 controls, antibodies against UH-RA.1 and UH-RA.21 emerged as the most prevalent antibody responses and both were present in early and seronegative RA patients. Therefore, both antibodies were measured in a second cohort, the Early Arthritis Clinic (EAC) cohort from Leiden, including 600 early RA patients, 309 undifferentiated arthritis (UA) patients and 157 early rheumatic controls (RC) [272]. The results from these new cohorts are of utmost importance in the validation of novel candidate biomarkers. In **Chapter 4**, antibody measurements were performed in longitudinal samples, in order to reveal whether antibody reactivity is stable or fluctuating, and whether changes in reactivity or titers can be linked to disease activity or outcome.

Does testing for the novel autoantibodies improve diagnostics for seronegative RA patients?

In two independent cohorts, the UH cohort and the EAC cohort, the proportion of seronegative (i.e. RF-negative ACPA-negative) RA patients was 38%. We investigated whether this percentage could be decreased, in other words, if testing for the novel autoantibodies can aid in the diagnosis of RA by complementing the current markers RF and ACPA.

Within the UH cohort, 23% of these seronegative RA patients were identified by testing for autoantibodies against UH-RA.1 (6%) and UH-RA.21 (17%). The serological gap of 38% was therefore reduced to 29%. Also within the early subpopulation, a reduction of 9% was observed in the UH cohort, which included 39 early RA patients of which 10 were seronegative. These findings were confirmed in the EAC cohort containing 600 early RA patients of which 229 were seronegative: here, a 6% reduction was achieved. Thereby, the sensitivity of the antibody markers in the overall early RA population of the EAC cohort was 6% (UH-RA.1) and 14% (UH-RA.21), together yielding 18%.

The finding that the sensitivity of both UH-RA biomarkers in early and seronegative RA is similar in two independent cohorts is of high value in the

validation of their diagnostic potential. Further validation in additional cohorts is mandatory, but consistency so far is promising in the pursuit to improve diagnostics in seronegative RA patients. The detection of new biomarkers in one fifth of the seronegative RA population, does not close the serological gap, but its relevance should be interpreted within the current concept of RA in which RA is considered a highly heterogeneous population [71, 332]. The presence of the new biomarkers is not restricted to seronegative patients and therefore does not seem to imply subpopulations of RA as the presence of ACPA does [31, 33]. However, completing the diagnostics can, and in our belief will, be achieved by combined efforts: a so-called multiplex-biomarker approach [71, 332]. This was illustrated in **Chapter 3**, in which the serological status of anti-carbamylated protein antibodies (anti-CarP) [75] was known for patients from the EAC cohort. Up to 12% of the seronegative early RA patients were positive for anti-CarP, reducing the serological gap from 38% to 34%. Combining the novel UH-RA antibodies with anti-CarP, further reduced the serological gap from 38% to 27%. These results illustrate that combined antibody testing can further close the serological gap.

Is an early diagnosis within reach using the novel autoantibodies?

Early disease markers are of utmost importance when aiming at preventing joint damage and disability. Several studies demonstrated joint damage in early stages of RA [20-22]. Recent studies even suggest bone loss prior to symptom onset, especially in ACPA-positive patients [52, 53]. Therapy with disease-modifying anti-rheumatic drugs (DMARD) appears to be most effective when initiated early [23, 25, 26, 28, 333]. This lead to the concept of a 'window of opportunity' during early disease, marked by better treatment response and outcome [24, 27, 29].

The occurrence of antibodies against UH-RA.1 and UH-RA.21 in early RA has been addressed in **Chapter 3**. The UH cohort contained 39 patients that were diagnosed not more than one year ago. One third of these patients (33%) tested positive for antibodies against either UH-RA.1 (individual sensitivity of 10%) or UH-RA.21 (individual sensitivity of 23%). The presence of these antibodies was therefore further studied in 600 early RA patients from the Leiden EAC cohort. These patients had clinical arthritis of at least one joint for no longer than two

years at time of sampling. All patients were diagnosed with definite RA within one year of follow-up, 55% fulfilled the diagnostic criteria already at baseline. From the early RA patients within the EAC cohort, 19% tested positive for antibodies against UH-RA.1 (individual sensitivity of 7%) or UH-RA.21 (individual sensitivity of 13%). These results confirmed the peptides' ability to detect reactivity in early disease, an important property of potential biomarkers for RA diagnosis.

Are the novel autoantibodies predictive for the development towards RA?

As described above, antibodies against UH-RA.1 and UH-RA.21 can be detected in the early disease stages of RA. Reasonably, this raises the question whether the antibodies precede and even predict the development of RA. The identification of individuals who will develop RA, prior to disease onset, would allow early intervention thereby extensively improving patients' outcome. On the other hand, in patients likely to remit spontaneously or follow a mild disease course, overtreatment could be avoided. With the onset of clinical arthritis, many patients do not immediately fit within specific rheumatic disorders and are classified as having UA, a stage in which no definite diagnosis can yet be made [334]. Patients with UA might not yet fulfill enough criteria for the diagnosis of RA or present with overlapping symptoms fitting different rheumatic diseases. The majority of the patients with UA (40-50%) will undergo spontaneous remission, while one-third develops RA and the rest develops other conditions [272, 334]. In attempts to predict the progression from UA to RA, several factors have been linked with RA development: patient characteristics such as age, gender and a positive family history of RA, but also the localization of the symptoms, morning stiffness and inflammatory parameters (C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and the number of tender and swollen joints) [272, 335]. Finally, also the presence of autoantibodies was associated with progression to RA: RF and particularly ACPA were demonstrated to be highly predictive of the future development of RA, in both HC and UA patients [30, 247, 256, 336]. Also the recently discovered anti-CarP antibodies appear predictive for RA development [335, 337, 338].

Next to early RA patients and other rheumatic diseases, the EAC cohort included 309 UA patients that were screened for the presence of antibodies against UH-RA.1 and UH-RA.21 (**Chapter 3**). The UA patients from the EAC cohort had been followed up for at least one year (8 years on average), 31% of them developed RA within the first year of follow-up, 4% developed RA afterwards [272, 339]. Interestingly, the antibodies were detected in the undifferentiated stages of rheumatic disease: antibodies against UH-RA.1 and UH-RA.21 were detected in 60 UA patients, with individual sensitivities of 5% and 16%, respectively. However, the antibody-positive patients included both patients developing RA and not developing RA. Therefore, none of the two anti-UH-RA antibodies tested in UA patients was predictive for the progression from UA to RA.

It is however interesting that antibodies against UH-RA.1 and UH-RA.21 can precede RA development, whether they do so by many years – as is the case for RF and ACPA [30, 274, 300, 340] – is not known yet. If they do, healthy individuals testing positive might not be true false positives, but patients in their pre-RA stage. Reactivity to the UH-RA peptides was detected in 4% (UH-RA.1) and 8% (UH-RA.21) of the healthy control (HC) population, but whether this predicts the development of rheumatic disease has still to be elucidated. Furthermore, the sole presence of antibodies is not the only factor to be determined, also the isotype distribution and fine specificity might aid in distinguishing between health and disease. A Swedish study demonstrated that for both RF and ACPA, all isotypes occurred more frequently and in higher concentrations in unaffected first-degree relatives from multicase families than in unrelated HC, but with a different isotype distribution from patients with RA [341]. The isotype distribution of antibodies against UH-RA.1 and UH-RA.21 was found to be similar in RA patients, RC and HC (**Chapter 5**), but a higher number of antibody-positive controls should be tested to be conclusive, if possible complemented with samples from symptom-free first-degree relatives and individuals who subsequently develop RA (pre-RA patients).

Are the novel autoantibodies specific for RA?

The diagnostic potential of biomarkers does not only rely on the sensitivity for RA, i.e. the number of RA patients carrying the biomarker, it also depends on the specificity, i.e. the number of non-RA patients with a negative test. Important to note, the specificity is determined by the control patients considered. As described above, antibodies to the novel UH-RA peptides were detected in some HC included in the UH cohort. This resulted in a specificity of 96% for antibodies against UH-RA.1 and 92% for antibodies against UH-RA.21, associated with a sensitivity of 6% and 18%, respectively, in the general RA population (**Chapter 3**).

Biomarkers cannot only serve to distinguish between health and disease, but also between disorders which are very much alike. In the context of RA, biomarkers can aid in discriminating RA from other rheumatic diseases. Therefore, the prevalence of the candidate biomarkers was studied in different joint-related conditions (**Chapter 3**). The UH cohort contained 90 RC including patients with ankylosing spondylitis (SpA), psoriatic arthritis (PsA), Sjögren syndrome (SS) and osteoarthritis (OA). Antibody testing in these patients resulted in RA-specificities of 93% (UH-RA.1) and 88% (UH-RA.21). Similar specificities were observed in the EAC cohort, which included 157 RC in early stages of disease and with a broader range of diagnoses (SpA, PsA, SS and OA, but also reactive arthritis, (pseudo-)gout and several others). The specificity of antibodies against UH-RA.1 and UH-RA.21 in the EAC cohort was 96% and 88%, respectively. In both cohorts, reactivity in RC – and thus loss of RA-specificity – was mainly due to antibody-positive patients with SpA and PsA, interrelated inflammatory autoimmune diseases with common clinical features. As for RA, the exact causes of SpA and PsA are still unknown, and these three conditions are the most common inflammatory rheumatic diseases [342]. They all differ in clinical and laboratory hallmarks: SpA mainly affects joints in the spine and pelvis with variable involvement of peripheral joints and PsA primarily affects the distal interphalangeal joints of the hands and feet [342-344]. In RA, the metacarpophalangeal and proximal interphalangeal joints are principally affected [3]. While arthritis in RA occurs in the same joints on both sides of the body, this is not the case in SpA and PsA. Furthermore, SpA and PsA are considered as seronegative spondyloarthropathies as they are generally RF negative [343, 345, 346].

Although the humoral immune response is not likely to induce SpA, autoantibodies have been described, mainly targeting joint structures such as cartilage, bone and tendons [346, 347]. Moreover, while RA is associated with class II human leukocyte antigen (HLA) alleles, SpA and PsA are associated with class I HLA alleles [343, 344]. However, for all three conditions the role of Th17 cells has gained more interest, and TNF-α seems to be a crucial factor [348]. Most compelling is the shared feature of joint and bone destruction, in which osteoclasts play a major role [7]. It is not unlikely that RA, SpA and PsA share common pathways in inflammatory-mediated joint damage and the initiation and perpetuation of autoimmunity and chronic inflammation. These shared mechanisms may lead to overlapping autoantibody systems. The finding that anti-UH-RA.21 antibodies may be involved in bone resorption (**Chapter 6**) further supports this hypothesis.

Does antibody reactivity towards the novel peptides hold prognostic information?

Biomarkers are not only applied in diagnostics, but also in prognostics and theranostics, which means that they can provide information on the course of the disease and the response to therapy, respectively. As such, the current biomarkers for RA – RF and ACPA – are both predictive for the development towards RA [30, 247, 256, 336]. Moreover, ACPA-positive patients have more radiological damage [22, 252, 253] and are more likely to develop extra-articular symptoms [250, 251]. Similarly, RF positivity is correlated with more severe disease [3, 219]. Since seropositive patients have disease mechanisms at least partly different from seronegative RA, it is reasonably that they also respond differently to certain treatments. Indeed, whereas methotrexate is able to prevent progression from UA to RA in ACPA-positive patients, it has no such effect in ACPA-negative patients [28]. Similarly, treatment with rituximab, a B cell depleting agent, is more effective in ACPA-positive RA [255]. Therapy-decisions can be made based on ACPA-positivity but additional theranostic markers are more than welcome, especially for the seronegative RA subpopulation. Not only can novel biomarkers reveal novel therapeutic targets in ACPA-negative pathogenesis, they could also help to predict the most efficacious therapy in a personalized medicine approach, halting the trial-and-error approach which

burdens many patients. Besides predicting therapy response, a close monitoring of disease activity prior and during treatment initiation is recommended. Measurement of inflammatory markers (CRP and ESR) and the disease activity score based on 28 joints (DAS28) reflect ongoing disease status, but additional markers are desired to be one step ahead of upcoming flares.

We evaluated whether antibody reactivity can be linked to measures of disease activity and outcome, and whether antibody-positive patients present clinically different compared to antibody-negative patients. Our results pointed towards a better outcome linked to anti-UH-RA.1 antibodies as they were associated with sustained DMARD-free remission in the EAC cohort, defined as the absence of swollen joints for at least one year after cessation of DMARDs [272]. The presence of antibodies against UH-RA.21 on the other hand, was associated with a negative outcome since associations with inflammation and erosions were found in the UH cohort. Data from the EAC cohort did not confirm any link between UH-RA.21-specific antibodies and erosions, but demonstrated a higher number of swollen and tender joints in anti-UH-RA.21 antibody-positive patients, supporting the link between positivity for this antibody system and a worse prognosis. The association between the presence of anti-UH-RA.21 antibodies and bone erosion in RA patients, is supported by recent data from the *in vivo* model collagen-induced arthritis (CIA). Preliminary results show a role for antibodies against UH-RA.21 in increased bone resorption in CIA mice injected with the antibodies shortly after disease onset (**Chapter 6**).

The prognostic value of the novel autoantibodies was not only investigated in cross-sectional studies. We also assessed changes in serology in a longitudinal setting, and evaluated whether changes in antibody status or levels were related to clinical measures of disease activity and outcome (**Chapter 4**). Serology for anti-UH-RA.1 antibodies appeared stable in 96 RA patients during a follow-up of 17 months, with 6-months intervals. However, in the same study, levels of anti-UH-RA.21 antibodies showed an overall decrease in time. For 8 out of 35 patients initially testing positive for antibodies against UH-RA.21 – 7 with low-positive reactivity – antibody levels had dropped below cutoff by the end of follow-up (seroreversion). Changes in anti-UH-RA.21 antibody serology were not

associated with clinical parameters such as acute-phase response, disease activity, clinical joint assessment or the presence of erosions. We also explored a putative link with treatment strategy. The decline in anti-UH-RA.21 antibody levels was particularly present in patients continuing DMARD treatment, while not in patients treated with a biological at study entry or during follow-up. Furthermore, the results indicated that anti-UH-RA.21 antibody levels only decrease in patients responding to DMARD therapy. Further analyses will have to determine whether the decline in antibody levels is attributable to DMARD effects. Specific intervention cohorts might elucidate the effects of DMARDs and other therapies on antibody levels, and define whether the measurement of anti-UH-RA.21 antibodies might help in monitoring treatment response.

Is repeated testing for RF and ACPA in RA patients recommended?

The follow-up study in RA described in **Chapter 4** was the first time the novel autoantibodies against UH-RA.1 and UH-RA.21 were tested longitudinally. For RF and ACPA, several studies have already been published addressing the question of repeated testing. While both antibody systems predate the onset of RA by years [30, 300, 340], seroconversion (becoming seropositive) probably occurs mainly in the upcoming years of disease onset. In established RA, seroconversion and seroreversion (becoming seronegative) are barely observed [275, 278, 279, 284, 293].

The results of our longitudinal study demonstrated fairly constant RF levels during follow-up. Low rates of seroconversion and seroreversion were observed, and within seropositive patients RF levels showed minimal fluctuations. Repeated testing for RF in established RA patients, therefore seems pointless based on our findings.

For anti-CCP3 antibody levels on the other hand, seroconversion and increased reactivity was found in a considerable number of RA patients. Maybe these findings were due to the use of a more sensitive peptide ELISA (CCP3) [244-246]. Studies using CCP2 report lower seroconversion and seroreversion rates [275, 278, 279, 284, 293]. The higher seroconversion rates in our study, might suggest a possible benefit of repeated testing in seronegative patients and patients with low-positive antibody levels. However, as we are among the first to apply the new third-generation CCP test in a longitudinal setting, further studies including

repeated measures for anti-CCP3 antibody reactivity are needed to validate our results. Furthermore, repeated testing did not seem to provide additional information over baseline testing, and fluctuations in antibody levels could not be linked to clinical measures of disease activity or outcome – questioning the potential benefit of repeated testing for anti-CCP3 antibody reactivity in seropositive patients.
Intriguingly, overall anti-CCP3 antibody levels increased in three-quarter of the RA patients, while the remaining patients showed a decrease. The nature of this difference remains unclear and might be found in treatment response. Therefore, data on therapy strategy and response will be gathered. These future analyses will provide information on whether changes in anti-CCP3 serology reflect responses to therapy, and repeated testing for anti-CCP3 antibodies might be helpful in monitoring treatment efficacy.

PART 3: The biological relevance of the novel candidate biomarkers

Investigating the potential of the novel autoantibodies as biomarker for diagnosis and prognosis was one of the main pillars in this thesis, and a direct approach in improving the diagnostic work-up. However, as biomarkers reflect certain pathogenic processes, further characterization of the antibody systems might lead to the elucidation of pathways involved in RA. The antibodies are related to a secondary effect of specific disease mechanisms or they are actively involved in RA pathogenesis. In the latter case, new therapeutic targets might be identified by characterizing the antibodies' properties and binding partners.

As pathogenic properties of antibodies are partially determined by their corresponding Ig class, the isotype profile of the novel antibodies against UH-RA.1 and UH-RA.21 in RA patients and controls was studied in **Chapter 5**. We found that both antibodies exist in the IgG, IgM and IgA type, and that patients can carry multiple isotypes. We also noticed that the two antibody systems are characterized by a different distribution of immunoglobulin (Ig) classes and subclasses.

In a second part of the characterization of the novel autoantibodies, we addressed the role of the antibodies in the arthritic disease process of RA in

order to evaluate their biological relevance. The finding that the antibodies against UH-RA.1 and UH-RA.21 are linked to a good and bad prognosis, respectively, raised interest in the role these antibodies play in arthritis. Therefore, in **Chapter 6** we described the antibodies' effect on disease incidence and severity in animal models for RA. Below, we review and discuss our main findings so far on the putative biological properties of the autoantibodies.

Which isotype repertoire do the novel autoantibodies possess and how does it connect to their potential biological properties?

As already mentioned, anti-UH-RA.1 and –UH-RA.21 antibodies exist in the IgG, IgM and IgA type. Surprisingly, anti-UH-RA.1 antibodies were primarily of the IgM isotype, which was encountered twice as often as IgG and IgA. Subtyping of IgG revealed IgG3 as the dominant IgG subclass with barely other subclasses detected. On the contrary, for anti-UH-RA.21 antibodies the IgG and IgA type were more common than IgM. Additionally, the IgG subclass distribution for anti-UH-RA.21 antibodies was more diverse with the highest prevalence for IgG2. For both antibody systems, the isotype profile in antibody-positive controls was not substantially different from RA patients except for some differences in IgG subclasses. Anti-UH-RA.1-IgG1 and –IgG2 were RA-specific compared to RC and HC, but with low overall prevalence. For UH-RA.21, antibody-positive HC only carried IgG2 while RA patients and RC also carried IgG1 and IgG3. Finally, the Ig (sub)class profile was similar in seronegative and seropositive RA patients, as well as in early and established disease.

The autoantibodies against UH-RA.1 and UH-RA.21 have a diverse isotype repertoire, reflecting putative biological properties. Antibodies of the IgM type are the first secreted Ig and its presence typically indicates recent antigen exposure [210, 302]. The presence of IgM in the anti-UH-RA.1 antibody system, and to a lower extend also in the anti-UH-RA.21 antibody system, therefore suggests a continuous antigenic stimulation and an ongoing immune response. It is not fully understood how IgM production is sustained in the presence of IgG against the same antigen. Switching to IgG not only leads to antibodies with higher affinity, it also prolongs the half-life of the antibody [210]. For UH-RA.21-specific antibodies, IgG was more common than IgM while for anti-UH-RA.1 antibodies

the opposite was observed. Also for RF, IgM is the primary isotype from early to late disease despite the presence of IgG and IgA [8].

IgA guards the mucosal barriers, often the first encounter for inhaled or ingested pathogens [304, 349]. IgA can be found in mucosa from the lungs and gut but also in secretions such as saliva. The presence of IgA within an antibody system therefore points towards a link with environmental exposure such as smoking, a major risk factor for RA. In line with this theory, anti-UH-RA.1 antibodies of the IgA type were only observed in smokers and not in non-smokers (**Chapter 5**). Similar findings have been reported for RF and ACPA. Pre-RA patients who were smokers, were significantly more often IgA-RF positive [305]. Furthermore, IgA-ACPA appeared earlier in smokers than in non-smokers [228, 299, 300]. Not only smoking is a risk factor for RA, also respiratory and gut microorganisms have been linked to the disease and may form a source of novel antibody targets [39]. Within the gastrointestinal tract, a role for *Porphyromonas gingivalis* has been described in inducing periodontitis – a risk factor for RA [102] – and promoting ACPA production [350]. A recent study further studied the oral and gut microbiomes and found them to be perturbed in RA but partly normalized after treatment [351]. From this point of view, it should be interesting to determine the antibody response to the UH-RA.1 peptide target, in which the arginine residue is converted to a citrulline residue (GLQEFGT**R**EK**R**QEITTE), similar as we did for UH-RA.21 in **Chapter 2**.

IgG is the most prevalent isotype, but nonetheless its intricate ways of modulating the immune response is far from fully understood. Effector functions are determined by the capability to activate complement, and by the interaction with the Fragment crystalline receptor (FcR), which exists in different types, with different cellular distribution and principal functions [352]. Generally, type I FcR and type II FcR primarily mediate pro- and anti-inflammatory actions, respectively [308]. The glycan core structure of IgG and modifications therein such as galactosylation and sialylation tip the balance from pro- to anti-inflammatory [309-312]. As the four IgG subclasses differ in their constant region, involved in FcR binding, they all differ in antigen binding, half-life and effector mechanisms such as complement activation and the formation of immune complexes [315]. Whereas IgG1 and IgG3 are potent pro-inflammatory subclasses, IgG2 and IgG4 typically induce more subtle responses [315]. In order to comprehend the dominant role

played by IgG3 in the UH-RA.1 antibody response – yet linked with a better outcome – other antibody properties and modifications should definitely be addressed. Nevertheless, an IgG3-dominated antibody response is uncommon [315]. The pro-inflammatory feature of IgG1 and IgG3 is however likely to explain the presence of these subclasses only in RA and RC, and not in HC, within the UH-RA.21-specific antibody system. Within these anti-UH-RA.21 antibodies, IgG2 is the most prevalent IgG subclass, perhaps suggesting a microbial trigger for the development of the antibodies. IgG2 is generally produced in response to bacterial infections (polysaccharide antigens) [315, 353]. Molecular mimicry, in which antibodies cross-react from the bacterial-derived epitope to *self*-targets, is an intriguing phenomenon involved in autoimmunity [354]. Interestingly, IgG2 binds preferentially to only two specific IgG-FcR (FcγR): FcγRIIA (also known as cluster of differentiation (CD)32A; H131 polymorphic variant in particular) and FcRn (neonatal FcR), while IgG1 and IgG3 bind all FcγR. FcγRIIA is the most abundantly expressed activating FcR, expressed on all myeloid cells such as mast cells, basophils, neutrophils and eosinophils, but not on lymphocytes [316]. FcγRIIA has been shown to play a pivotal role in the activation of inflammation. As FcRIIA is not expressed in mice, transgenic mice were created expressing the human FcγRIIA gene [355]. These transgenic mice developed spontaneous autoimmunity with RA-like features such as erosive pannus formation [356]. Furthermore, they had a significantly increased susceptibility to CIA, CAIA and pristane-induced arthritis, even CIA-resistant mice became susceptible to CIA after the transgenic expression of FcγRIIA [357, 358]. All together, FcγRIIA appears to be involved in inflammation and autoimmunity. Therefore, the absence of the FcγRIIA in mice might have significant consequences for the *in vivo* study of anti-UH-RA.21 antibodies, marked by a high IgG2 participation. Next to FcγRIIA, also FcRn is bound by IgG2 – and all other IgG subclasses – in acidic conditions. FcRn prolongs the life span of IgG [318-320] and has the ability to transport IgG within and across cells and may therefore contribute to the transfer from autoantibodies from one joint to another [206, 317, 321, 322]. So far, we can only speculate about the isotype-specific contributions of the autoantibodies. Further characterization of biological properties and post-translational modifications of anti-UH-RA.1 and -UH-RA.21 antibodies and their binding to different FcR, will further clarify the significance of the isotypes in the pathophysiology of RA.

What are the implications of the isotype profile for diagnostic and prognostic testing?

Exploration and validation screenings have been performed primarily focusing on IgG reactivity. After the discovery of other isotypes within the antibody systems, we investigated whether diagnostic or prognostic testing could be improved based on measuring particular isotypes (**Chapter 5**). The most prominent finding in isotyping the novel autoantibodies, was the dominance of IgM within the anti-UH-RA.1 antibody system. Since IgM was twice as prevalent as IgG or IgA, the sensitivity of testing for anti-UH-RA.1 antibodies could be doubled when targeting IgM instead of IgG: 18% vs 9%, respectively, with an associated specificity of 90%. Testing for combinations of isotypes could not reach beyond this sensitivity. It seems highly recommended to perform future screenings with the main focus on IgM rather than IgG.
When looking into the anti-UH-RA.21 antibody system, associated with an RA-specificity of 90%, IgG remained the most prevalent isotype (23%), closely followed by IgA (20%). Combined isotype testing (IgG+IgA) increased the sensitivity up to 27%. Whether IgA should be included in future screenings based on these results, will perhaps be a matter of practical and economical perspective. Testing for two isotypes separately will double the number of assays and will require more of the valuable test material. This should be weighted against limited additional information: an increase in overall sensitivity of 4% and no compelling prognostic value. To overcome the disadvantages, the test assay could be optimized for measuring both isotypes at once, as has been done for the third-generation anti-CCP test (QUANTA Lite CCP3.1 IgG/IgA ELISA, INOVA Diagnostics Inc, San Diego, USA) [246].

For both antibody systems we studied, the isotype profiles appear quite similar in antibody-positive RA patients and controls. None of the Ig classes of the M, A or G type were preferentially detected in either RA or control conditions. Nevertheless, we found differences in IgG subclasses. As mentioned before, anti-UH-RA.1-IgG antibodies were all from the IgG3 type in antibody-positive controls, while IgG1 and IgG2 were also detected in some of the RA patients and are therefore considered RA-specific. The low prevalence of these subclasses seems however negligible when it comes to improving RA diagnostics. Anti-UH-

RA.21-IgG distribution (IgG1, -2 and -3) was equal in RA patients and RC, while in HC it was restricted to IgG2. But here again, the relatively low share of IgG1 and IgG3 compared to the predominance of IgG2 in the UH-RA.21 antibody system, suggests no substantial improvement of diagnostic testing when including IgG-specific subclasses.

The principal population for which novel biomarkers are needed, are early and seronegative RA patients. We therefore studied these subpopulations in more detail and found the full isotype usage for both anti-UH-RA.1 and anti-UH-RA.21 antibodies within all serological subgroups – either positive or negative for RF and/or ACPA. The sensitivity of individual and combined antibody testing in seronegative RA is therefore equal to that within the overall RA population. The same was true for patients in early disease. The implications of isotype-specific testing are thus similar in early and seronegative RA as in the general RA population.

Do the novel autoantibodies play a role in the arthritic disease mechanisms?

Decoding the role autoantibodies play in underlying disease provides insight into the pathogenesis. Moreover, it can reveal new pathways with potential therapeutic targets. The effect of antibodies against UH-RA.1 and UH-RA.21 was investigated in two mouse models for RA (**Chapter 6**). Within the CIA model, the effect of the antibodies on disease severity was investigated by analyzing clinical arthritis scores and, for UH-RA.21, also bone resorption, after passive transfer of polyclonal antibodies. Antibodies against UH-RA.1 had no effect on the disease scores in CIA mice. For antibodies against UH-RA.21, irregular results were obtained. A first pilot experiment suggested a disease-exacerbating effect of the antibodies in CIA, but subsequent repeats of this experiment were not able to confirm these results. Whether the inconsistency is due to fortuitous results in the pilot experiment or to variations in the antibody properties (human vs rabbit, several different donors), is not clear. Surprisingly, despite any effect on clinical disease scores, 3D micro-CT images of affected knees suggested higher bone resorption in CIA mice treated with anti-UH-RA.21 antibodies (**Chapter 6**). Although quantitative analyses are still ongoing and require

further expansion, these preliminary findings are in agreement with the link between antibody positivity and erosions in RA patients (**Chapter 3 and 4**).

By means of the second model, collagen antibody-induced arthritis (CAIA), the arthritogenic capacity of anti-UH-RA.21 antibodies was evaluated (**Chapter 6**). There was no evidence for arthritogenicity as the antibodies were not arthritogenic on their own and they did not enhance the arthritogenic effect of collagen II-specific antibodies.

7.2 Concluding remarks and future perspectives

Based on the results gathered in this thesis, we try to answer two central questions – aimed at the autoantibodies' clinical application on the one hand, and at their biological relevance on the other – in this concluding paragraph. Also some suggestions for further research are put forward.

What can the novel autoantibodies mean for the management of RA and the socio-economic burden associated with the disease?

RA is the most common autoimmune disease affecting up to 0.5-1% of the Western population [3, 5, 6]. The high prevalence of RA, its chronic and disabling character, and the associated comorbidities lead to high costs for both patient and society [5, 359]. Moreover, the disease is characterized by a long diagnostic delay, aggravating the prognosis [23, 109, 256, 360]. Despite decennia of intensive research, the underlying cause and pathogenesis is still not fully understood. It is therefore of utmost importance to find biomarkers that can complement current diagnostics as one third of the RA patients is seronegative for RF and ACPA [220, 256]. Furthermore, novel biomarkers can provide prognostic information and predict the adequate therapy in a personalized-medicine approach. They may also provide insight into the underlying disease mechanisms and reveal putative targets for new treatments.

RA has always been a heterogeneous disorder, and revision of the diagnostic classification criteria in order to allow earlier diagnosis, has further increased this heterogeneity [8]. The discovery of ACPA has divided RA patients in two subsets, either ACPA-positive or ACPA-negative [31-33, 229, 258]. The relatively milder disease course of patients without RF or ACPA (seronegative patients) [22, 252, 253], initially lowered the priority to find biomarkers for this subgroup [33]. However, although to lower extent than in seropositive patients, radiological damage has also been demonstrated in seronegative patients and this subpopulation should no longer be neglected [33, 260]. The extensive quest for novel biomarkers for RA seems to result in antibody systems partially closing the serological gap, such as the recently discovered anti-CarP antibodies [75]. Also our novel autoantibodies, directed against UH-RA.1 and UH-RA.21, reduced the serological gap from 38% to 29%. We believe this is an important step in the closure of the serological

gap. Regarding the high heterogeneity of RA, it is beyond our expectation to find one biomarker identifying all (seronegative) RA patients. Instead, a multiplex biomarker approach seems more feasible. We illustrated how combined efforts – testing for both anti-CarP antibodies and our novel candidate markers – increase the diagnostic window. Additional markers can further complete the biomarker panel so in the end all patients can be identified, preferentially early in their disease. The finding that our antibodies are present in early stages of the disease is thereby highly relevant.

In order to find such additional markers, we have yet other autoantibody systems in the running. Besides antibodies against UH-RA.1 and UH-RA.21, also other candidate biomarkers were identified by SAS technology, such as antibodies against UH-RA.11, UH-RA.15 and UH-RA.16 [267]. In contrast to the peptide sequences UH-RA.1 and UH-RA.21 for which the corresponding *in vivo* targets are still unknown, the antibody targets UH-RA.11, UH-RA.15 and UH-RA.16 were identified as major histocompatibility (MHC) class IA (HLA-A), minichromosome maintenance complex component 2 (MCM2) and 40S ribosomal protein S6 (RPS6), respectively. For all three proteins, higher expression levels were found in RA synovial tissue compared to control synovial tissue [267]. To our knowledge, these three proteins are no established antigens in RA. Based on exploratory screenings using phage ELISA, Somers K *et al* already reported that antibodies against UH-RA.11, UH-RA.15 and UH-RA.16 were preferentially detected in ACPA-negative RA [267]. Therefore, these candidate biomarkers still have the potential to further complement the combined antibody testing approach. Currently, efforts are being made to produce these targets as recombinant proteins and apply them on a protein ELISA format, for subsequent validation in large patient populations.

Finally, ultimate implications of the novel autoantibodies for the population, are situated on the longer term with regard to novel insights gained by the characterization of the biological relevance of the autoantibodies and their targets.

Is there evidence for an active role of the novel autoantibodies in rheumatic disease mechanisms?

Antibodies are related to a secondary effect of specific disease mechanisms or they are actively involved in RA pathogenesis. In the latter case, new therapeutic targets might be identified by characterizing the antibodies' properties and binding partners. Strongest evidence is gathered for the biological relevance of anti-UH-RA.21 antibodies in rheumatic disease. The presence of antibodies against UH-RA.21 appears to be accompanied by a worse prognosis. Large-scale screening of RA patients demonstrated associations with a higher number of tender and swollen joint counts, inflammation and bone erosion. The latter was supported by studying the antibodies' effects *in vivo*. Although no decisive proof is available yet regarding the disease-exacerbating or arthritogenic effects in mouse models for RA, preliminary bone analyses point towards increased bone resorption in mice who were injected with anti-UH-RA.21 antibodies. A putative role in bone damaging mechanisms could also explain why antibodies against UH-RA.21 are not exclusively found in RA, but also in SpA and PsA, two other rheumatic conditions marked by extensive bone and joint destruction [7].
Elucidating the role of anti-UH-RA.21 antibodies in inflammation-mediated bone loss will be a central ambition in future research on our novel antibody biomarkers. Quantification of the bone resorption data from the *in vivo* study is currently ongoing. While these preliminary data require further validation studies, immunohistochemical evaluation of affected knees will support the micro-CT results.

Another fundamental research goal is the identification of the corresponding *in vivo* targets of antibodies against UH-RA.1 and UH-RA.21. Both peptides constitute a mimotope, an epitope mimicking an *in vivo* antigen, and the identity of the corresponding antigen is unknown [265, 268]. Identifying these targets will provide an adequate context in which the results from this thesis should be interpreted. Additionally, it will open a broad range of opportunities to study and decipher the pathophysiological role of the antibody responses.

To conclude, in this thesis novel autoantibody systems were evaluated as candidate biomarkers for early and seronegative RA. After the development of noncommercial peptide ELISA tests, two large independent cohorts were cross-sectionally and longitudinally screened for the presence of autoantibodies against UH-RA.1 and UH-RA.21. Results from these screenings suggested a promising contribution in the diagnosis of seronegative patients, reducing the serological gap left by RF and ACPA. Moreover, the antibodies were already detected early in disease. While antibodies against UH-RA.1 were shown to be associated with remission, anti-UH-RA.21 antibodies seemed related to a worse outcome by associations with clinical and inflammatory parameters, and bone erosions. The latter is supported by preliminary observations *in vivo* in which the injection of anti-UH-RA.21 antibodies in a mouse model for arthritis seemed to increase bone resorption.

8

NEDERLANDSE SAMENVATTING

Reumatoïde artritis (RA) is een ziekte gekarakteriseerd door een chronische ontsteking van synoviale gewrichten die naar schatting 0.5-1% van de bevolking treft, voornamelijk vrouwen [4-6]. RA wordt gekenmerkt door gewrichtspijn en zwelling, maar ook vasculitis, cardiovasculaire problemen, anemie, lymfadenopathie en osteoporose reduceren de levensverwachting van RA-patiënten [10-13]. Uiteindelijk kan de ziekte leiden tot onomkeerbare destructie en vervorming van het gewricht, die gepaard gaat met invaliditeit. RA is een auto-immuunziekte, wat wil zeggen dat het immuunsysteem lichaamseigen weefsel aanvalt [4]. De verschillende immuuncellen die hierbij betrokken zijn infiltreren en accumuleren in de betrokken gewrichten [3, 4]. Ze zorgen onder meer voor de productie van cytokines en antilichamen (humorale immuunrespons). In RA spelen zogenaamde autoantilichamen een belangrijke rol, dit zijn antilichamen die zich richten tegen autoantigenen (lichaamseigen eiwitten). Deze afwijkende humorale respons is een belangrijke focus in het huidige onderzoek naar RA. Onder meer de ontdekking van reumafactor (RF) en antilichamen tegen gecitrullineerde peptiden en proteïnen (ACPA) betekenden een grote doorbraak. RF en ACPA zijn de enige twee klinisch gebruikte biomerkers om RA te diagnosticeren [8, 361]. RF is een antilichaam gericht tegen het Fc (*Fragment crystalline*)-gedeelte van immunoglobuline G (IgG) [216]. Ongeveer 70-80% van de RA-patiënten is seropositief voor RF [220]. Helaas wordt ook een verhoging van RF teruggevonden in andere chronische ontstekingsziekten waardoor de specificiteit van RF voor RA maar een matige 85% bedraagt [220]. Hoewel gecitrullineerde eiwitten aangetoond zijn in synoviaal weefsel bij patiënten met verschillende reumatische aandoeningen, blijkt de ontwikkeling van antilichamen hiertegen (ACPA) wel heel specifiek te zijn voor RA [222, 362]. Citrullinatie is een post-translationele modificatie waarbij een arginine aminozuur wordt omgezet in een citrulline aminozuur door peptidyl arginine deiminase (PAD) enzymen [222, 223, 225, 227]. Op basis van de aanwezigheid van ACPA wordt een onderverdeling gemaakt tussen ACPA-positieve en ACPA-negatieve RA-patiënten, waarbij beide subgroepen verondersteld worden een verschillende etiologie en pathogenese te kennen [31-33, 229, 258, 259]. In ACPA-positieve patiënten speelt citrullinatie vermoedelijk een belangrijke rol in de pathogenese van RA, terwijl in ACPA-negatieve patiënten echter nog veel onduidelijker is welke onderliggende mechanismen betrokken zijn.

Helaas volstaat het gebruik van RF en ACPA als diagnostische merkers voor RA niet. De sensitiviteit van deze tests is aanzienlijk lager in de beginfase van de ziekte [222, 256, 257] waardoor de diagnose vaak lang op zich laat wachten. Nochtans treedt er reeds in het vroege stadium van de ziekte irreversibele schade op [20-22, 52, 53] en blijken de bestaande behandelingen het meest effectief in deze vroege fase [23-29, 333]. Het belang van een vroege diagnose en de tekortkoming van bestaande diagnostische merkers om in deze vroege fases RA te detecteren, geven aan hoe essentieel het is om verder onderzoek te voeren naar ziektemerkers voor vroege RA-patiënten. Bovendien blijft een derde van alle RA-patiënten seronegatief voor RF en ACPA [220, 256], voor deze 'seronegatieve' RA-patiënten zijn nieuwe ziektemerkers nodig om een accurate en tijdige diagnose te kunnen stellen.

Daarnaast blijkt niet enkel de diagnose een uitdaging in RA-patiënten, maar ook therapie. De conventionele behandelingen gaan gepaard met nevenwerkingen en niet alle RA-patiënten reageren op de verschillende therapeutica. De respons op bestaande behandelingen is bovendien verschillend tussen seropositieve en seronegatieve RA-patiënten. Wanneer de diagnose van RA gesteld is, wordt de behandeling meestal gestart met de toediening van methotrexaat, een *disease-modifying anti-rheumatic drug* (DMARD), dat de chronische ontsteking onderdrukt en zo de progressie van de ziekte afremt. Patiënten die seronegatief zijn voor ACPA blijken minder te reageren op behandeling met methotrexaat dan ACPA-positieve patiënten [28]. Het bestuderen van ziektemerkers in RA-patiënten met het oog op het ophelderen van de onderliggende pathogenese, is dus ook van belang om effectieve therapieën te kunnen ontwikkelen voor alle RA-patiënten, maar vooral voor de seronegatieve subpopulatie. Voorheen werden met behulp van serologische antigen selectie, op basis van cDNA faag display, nieuwe kandidaat-autoantilichaammerkers voor RA geïdentificeerd uit plasmapools van vroege en seronegatieve RA-patiënten [266, 267]. Van deze merkers waren er 11 specifiek voor RA (enkel aanwezig in RA-patiënten) en 3 geassocieerd met de ziekte (ook aanwezig in enkele reumatische controles maar niet in gezonde controles) [267].

Het doel van deze thesis was om deze kandidaat biomerkers voor RA verder te bestuderen. Enerzijds trachtten we hun aanwezigheid in vroege en seronegatieve RA-patiënten te bevestigen en onderzochten we in hoeverre ze kunnen bijdragen aan een verbeterde diagnostiek. Daarnaast gingen we ook na of ze prognostische informatie kunnen verschaffen. En ten slotte wilden we weten of de ziektemerkers ons ook iets kunnen leren over onderliggende ziektemechanismen. Daarvoor richten we ons op de verdere karakterisatie van de autoantilichaamresponsen, onder meer door hun effecten te bestuderen in diermodellen voor RA.

DEEL 1: Ontwikkeling van een nieuwe testmethode

Voorheen werd de aanwezigheid van de nieuwe autoantilichamen in het bloed gedetecteerd met behulp van een faag *enzyme-linked immunosorbent assay* (ELISA), gebaseerd op de fagen die de autoantilichaam-targets tot expressie brengen [267]. Jammer genoeg is het gebruik van fagen zeer arbeidsintensief en vereist het specifieke veiligheidsvoorzieningen. Faag ELISAs zijn dus niet toepasbaar in klinische labo's. Bovendien werden de faag ELISAs gekenmerkt door relatief hoge achtergrondreactiviteit, hoge inter- en intra-assay variabiliteit en dus een lage reproducibiliteit. De eerste doelstelling in deze thesis was daarom het omzetten van de oorspronkelijk faag ELISAs naar peptide ELISAs, waarbij enkel het peptide-target dat door de fagen tot expressie werd gebracht, werd behouden in de ELISA. Zoals beschreven in **hoofdstuk 2**, werd met behulp van competitie-assays de specifieke herkenning van synthetische peptides door de nieuwe antilichaamresponsen bevestigd. Vervolgens werden peptide ELISAs met succes verder geoptimaliseerd. Bovendien kan op basis van één standaard protocol, de antilichaamreactiviteit tegen verscheidene autoantigenen gemeten worden. Dit vergemakkelijkt het gelijktijdig testen voor meerdere merkers en is van belang met het oog op het combineren van merkers in een biomerkerpanel.

DEEL 2: Het diagnostische en prognostische potentieel van de nieuwe autoantilichaammerkers

Met behulp van de nieuw ontwikkelde peptide ELISAs, trachtten we vervolgens de diagnostische en prognostische waarde van de autoantilichaammerkers te valideren. In **hoofdstuk 3** werd de sensitiviteit (het vermogen om RA-patiënten

te identificeren) en de specificiteit (het vermogen om RA-patiënten te onderscheiden van gezonde individuen of andere aandoeningen) bepaald in twee grote Europese cohorten: een cohort van Universiteit Hasselt (UH, n=479) en het *Early Arthritis Clinic* cohort uit Leiden (EAC, n=1066). Binnen deze patiëntpopulaties werd ook nagegaan of de antilichaammerkers prognostische waarde bevatten. Hun aanwezigheid en levels werden gecorreleerd met klinische parameters voor inflammatie, ziekte-activiteit en de aanwezigheid van erosies. In **hoofdstuk 4** werden vervolgens de nieuwe antilichaammerkers, evenals de huidige diagnostische merkers RF en ACPA, gemeten in een longitudinale studie waarin RA-patiënten gedurende gemiddeld 17 maanden werden opgevolgd met intervallen van 6 maanden. Het doel van deze studie was om te bepalen of de antilichaamreactiviteiten stabiel blijven of fluctueren, en of veranderingen in reactiviteit of antilichaamtiters gelinkt kunnen worden aan ziekte-activiteit of kenmerken van het ziekteverloop.

In welke mate kunnen de nieuwe antilichaammerkers bijdragen aan een vroege diagnose en aan een verbeterde diagnostiek voor seronegatieve RA-patiënten?

In **hoofdstuk 3** hebben we beschreven hoe de screening van een eerste panel van vier merkers – UH-RA.1, UH-RA.9, UH-RA.14 en UH-RA.21 – aantoonde dat twee merkers, de antilichaamreactiviteit tegen UH-RA.1 en UH-RA.21, de hoogste prevalentie vertoonden in het UH-cohort, bestaande uit 292 RA-patiënten, 90 reumatische controles en 97 gezonde controles. De sensitiviteit van antilichamen tegen UH-RA.1 en UH-RA.21 bedroeg gezamenlijk 24% met een bijhorende specificiteit van 87%. De belangrijkste bevinding echter, was de aanwezigheid van de antilichamen in vroege en seronegatieve RA-patiënten. Antilichaamreactiviteit voor beide merkers werd teruggevonden in een derde van de vroege patiënten (diagnose minder dan 1 jaar oud). Om deze resultaten te valideren, werd een tweede cohort gescreend: het EAC cohort uit Leiden. Dit cohort omvatte 600 vroege RA-patiënten (ziekteduur van maximaal 2 jaar), 157 reumatische controles met eveneens een vroege ziekteduur, en 309 patiënten met ongedifferentieerde artritis (UA). Ook in dit cohort kon de aanwezigheid van antilichamen tegen UH-RA.1 en UH-RA.21 aangetoond worden in 19% van de vroege RA-patiënten. Gezien het belang van een vroege interventie om de

progressie van RA te remmen, is de vroege detectie van biomerkers zeer relevant. De resultaten van de UA patiënten hebben bovendien bewezen dat de merkers ook te detecteren zijn in patiënten die (nog) niet voldoen aan de diagnostische criteria voor RA: 19% van de UA patiënten testte reeds positief voor anti-UH-RA.1 of -UH-RA.21 antilichamen. Omdat deze antilichaam-positieve groep zowel patiënten bevatte die verder progresseerden tot RA, als patiënten waarbij later geen diagnose van RA werd vastgesteld, bleken de antilichaammerkers niet in staat te voorspellen of UA al dan niet verder progresseert tot RA.

Naast een vroege biomerker, waren we ook op zoek naar een merker voor de RF-negatieve, ACPA-negatieve RA-patiënten. In beide cohorten behoorde 38% van de RA-patipënten tot deze 'seronegatieve' subpopulatie. Maar liefst 23% van de seronegatieve RA-patiënten testte positief op antilichamen tegen UH-RA.1 of UH-RA.21 (UH cohort). De serologische gap van 38% kon dus teruggebracht worden tot 29%. Ook binnen de vroege seronegatieve subpopulatie werd een reductie van 9% aangetoond. Deze resultaten werden tevens bevestigd in het EAC cohort, met een sensitiviteit van 18% in vroege seronegatieve RA (reductie van 6%). Hoewel een reductie van de serologische *gap* van 6-9% beperkt lijkt, is het belangrijk deze resultaten te kaderen in het heterogene karakter van RA. RA kenmerkt zich in zovele klinische gradaties, met grote verschillen in ziekteverloop en therapierespons, waarbij vermoedelijk vele verschillende ziektemechanismen betrokken zijn – allemaal met één gezamenlijk eindpunt: een volhardende chronische ontsteking van de synoviale gewrichten waarbij kraakbeen en bot onherstelbare schade oplopen [31, 33, 71, 332]. Het is weinig waarschijnlijk dat slechts één merker gebruikt kan worden voor diagnosestelling van al deze RA-patiënten. Een complete diagnostiek is naar onze mening dan ook pas binnen bereik wanner verschillende biomerkers gecombineerd worden in een zogenaamde *multiplex*-biomerker aanpak. Deze strategie werd verder geïllustreerd met een ander recent-ontdekte antilichaammerker (antilichamen tegen gecarbamyleerde eiwitten of anti-CarP [75]), welke de serologische kloof in het EAC cohort kon reduceren van 38% tot 34%, maar in combinatie met onze merkers verder tot 27% (**hoofdstuk 3**).

Worden de nieuwe autoantilichamen enkel teruggevonden in RA?

Zoals hierboven reeds vermeld, zijn de nieuwe antilichaammerkers niet 100% specifiek voor RA. Dit is voornamelijk te wijten aan reactiviteit in patiënten met ankyloserende spondylitis (ziekte van Bechterew) en psoriatische artritis. Samen met RA vormen deze aandoeningen de drie meest voorkomende inflammatoire reumatische ziektes [342]. Net zoals RA is ook voor deze aandoeningen de onderliggende oorzaak nog niet opgehelderd, en alle drie onderscheiden ze zich op vlak van klinische manifestaties, de rol van het humorale immuunsysteem, en met betrekking tot genetische en omgevingsfactoren [342-346]. Er zijn echter ook gelijkenissen tussen RA, Bechterew en psoriatische artritis, waaronder opvallend genoeg de schade aan gewrichten en bot, waarbij de rol van osteoclasten (botafbrekende cellen) centraal staat [7]. Het is daarom goed mogelijk dat deze drie vormen van ontstekingsreuma, ziektemechanismen gemeenschappelijk hebben die leiden tot overlappende antilichaamresponsen. De bevinding dat antilichamen tegen UH-RA.21 gelinkt werden aan boterosies in zowel de RA-patiënten uit het UH cohort (**hoofdstuk 3**), als in de diermodellen beschreven in **hoofdstuk 6**, ondersteunen deze hypothese.

Wat is het prognostische potentieel van de nieuwe antilichaammerkers?

Biomerkers vinden niet enkel hun toepassing in diagnostiek, maar ook in prognose en theranose, wat wil zeggen dat ze informatie kunnen verschaffen over respectievelijk het ziekteverloop en de respons op bepaalde behandelingen. Zo zijn bijvoorbeeld ook de huidige diagnostische merkers voor RA – RF en ACPA – voorspellend voor de ontwikkeling van RA [30, 247, 256, 336], radiologische schade en een hogere kans op extra-articulaire symptomen [3, 22, 219, 250-253]. Omdat in seropositieve patiënten in zekere mate verschillende onderliggende mechanismen betrokken zijn, is het ook aannemelijk dat seropositieve en seronegatieve patiënten verschillend zullen reageren op bepaalde behandelingen. Inderdaad, methotrexaat kan de progressie van UA naar RA afremmen in ACPA-positieve patiënten, maar niet in ACPA-negatieve patiënten [28]. Ook behandeling met rituximab, gericht tegen B-cellen, blijkt meer effect te hebben in ACPA-positieve RA [255]. Behandelingsstrategieën kunnen dus beïnvloed worden door ACPA-positiviteit, maar bijkomende theranostische merkers zijn meer dan welkom, met name voor de seronegatieve RA subpopulatie. Niet alleen

kunnen nieuwe merkers nieuwe doelwitten voor behandeling in ACPA-negatieve pathogenese onthullen, ze kunnen ook nuttig zijn in het voorspellen van de meest geschikte therapie in zogenaamde *gepersonaliseerde geneeskunde*. Dit in tegenstelling tot de huidige *trial-and-error* aanpak ten koste van vele patiënten. Naast het voorspellen van het effect van een behandeling, wordt de ziekte-activiteit van dichtbij opgevolgd en ook daarin kunnen biomerkers hun nut bewijzen.

In **hoofdstuk 3** gingen we na of antilichaamreactiviteit tegen de nieuwe kandidaatmerkers gelinkt is aan klinische parameters, en of antilichaampositieve patiënten klinisch verschillend zijn van antilichaamnegatieve patiënten. We zagen daarbij dat de aanwezigheid van antilichamen tegen UH-RA.1 gerelateerd is aan een beter vooruitzicht: in het EAC cohort waren anti-UH-RA.1 antilichamen geassocieerd met blijvende remissie na het stopzetten van de behandeling met DMARDs [272]. De aanwezigheid van antilichamen tegen UH-RA.21 bleek dan weer geassocieerd met een slechtere prognose: binnen het UH-cohort waren de antilichamen geassocieerd met ontstekingsparameters en de aanwezigheid van erosies. Binnen het EAC-cohort werd geen link tussen anti-UH-RA.21 antilichamen en erosies vastgesteld, maar een ernstiger klinisch beeld (groter aantal gezwollen en pijnlijke gewrichten) in antilichaampositieve patiënten bevestigde wel de link met een ernstiger ziekteverloop. Het verband tussen antilichamen tegen UH-RA.21 en botschade in RA-patiënten, wordt ook ondersteund door recente data uit de diermodellen voor RA (**hoofdstuk 6**). In muizen met collageen-geïnduceerde artritis (CIA), wijzen preliminaire resultaten op verhoogde botresorptie in muizen die geïnjecteerd werden met antilichamen tegen UH-RA.21.

De prognostische waarde van de nieuwe autoantilichaammerkers werd niet enkel onderzocht in cross-sectionele studies. Ook in een longitudinale set-up werden veranderingen in serologie bestudeerd en hun mogelijke link met klinische parameters (**hoofdstuk 4**). Niet enkel de antilichaamrespons tegen UH-RA.1 en UH-RA.21 werd herhaaldelijk gemeten, ook de huidige diagnostische merkers RF en ACPA. Er werden amper serologische veranderingen waargenomen voor de antilichaamrespons tegen UH-RA.1 en RF. Voor anti-UH-

RA.21 antilichamen werd algemeen een daling in antilichaamtiters waargenomen, met name in patiënten die in behandeling waren met conventionele DMARDs in tegenstelling tot patiënten die behandeld werden met een biological. Voor ACPA (gemeten met een nieuwe derde-generatie anti-CCP test [244, 245, 363]) werd dan weer een toenemende reactiviteit teruggevonden, net zoals voor UH-RA.21 enkel in patiënten onder DMARD behandeling. Om na te gaan of deze veranderingen te wijten zijn aan effecten van de therapie is verder onderzoek nodig. Tot dusver konden serologische veranderingen niet gelinkt worden aan klinische parameters of prognostische informatie. De verhoogde anti-CCP3 reactiviteit leidde in een aantal gevallen wel tot seroconversie (evolutie van een negatieve naar een positieve test), wat erop wijst dat het nuttig zou kunnen zijn om seronegatieve patiënten herhaaldelijk opnieuw te testen.

DEEL 3: Biologische relevantie van de nieuwe autoantilichaammerkers

Een belangrijk aandeel in deze thesis bestond uit het valideren van het diagnostische en prognostische potentieel van de kandidaat autoantilichaammerkers. Biomerkers weerspiegelen echter pathogene processen en kunnen dus ook inzichten verschaffen in de onderliggende ziektemechanismen. De antilichamen kunnen geproduceerd worden als 'neveneffect' van een ziekteproces, of ze kunnen een actieve rol spelen in de pathogenese. In dit laatste geval kan karakterisatie van de antilichamen en hun targets ook leiden tot nieuwe doelwitten voor therapie.

De pathogeniciteit van antilichamen wordt door verschillende factoren bepaald, waaronder bijvoorbeeld het isotype profiel. Isotypes zijn verschillende vormen van antilichamen die verschillen in localisatie en in de omstandigheden waaronder ze geproduceerd worden. Zo wordt het IgM type als eerste geproduceerd, en wordt IgG pas aangemaakt bij een tweede blootstelling aan datzelfde antigen of bij grotere hoeveelheden antigen [210, 302]. IgA is dan weer voornamelijk terug te vinden in mucosale weefsels en secreties [304, 349]. Het veranderen van isotype noemen we *isotype switching* of *class switching* [210]. De specificiteit voor een bepaald antigen wordt daarbij behouden maar de effector mechanismen veranderen.

In **hoofdstuk 5** hebben we aangetoond dat antilichamen tegen UH-RA.1 en UH-RA.21, voorkomen onder de isotypes IgG, IgM en IgA, en dat patiënten meerdere isotypes kunnen meedragen. Het isoype profiel bleek verschillend voor beide antilichaamresponsen. Binnen de antilichamen tegen UH-RA.1, kwam IgM dubbel zo vaak voor als IgG of IgA. Deze bevinding heeft belangrijke implicaties voor de diagnostiek. Het testen van antilichaamreactiviteit tegen de nieuwe merkers gebeurde op basis van de IgG respons. We toonden echter aan dat testen voor UH-RA.1-specifiek IgM de sensitiviteit verdubbelde ten opzichte van IgG (18% vs 9% bij een specificiteit van 90%). Het is dus raadzaam om de IgM respons op te nemen in toekomstige screenings voor anti-UH-RA.1 antilichamen. Een interessant gegeven was ook de aanwezigheid van deze antilichamen van het IgA type, en dit enkel in rokers en niet in niet-rokers. IgA is aanwezig in mucosale barrières en staat daarbij in contact met pathogenen die worden opgenomen via de luchtwegen of het maagdarmstelsel [304, 349]. De aanwezigheid van IgA in de anti-UH-RA.1 antilichaamrespons, verwijst dus mogelijk naar een link met blootstelling aan omgevingsfactoren. Ook voor RF en ACPA, werd het IgA isotype reeds gelinkt aan roken [228, 299, 300, 305].

Binnen de antilichaamrespons tegen UH-RA.21, bleek IgM minder prevalent dan IgG en IgA. Ook de IgG-subtypes bleken verschillend van anti-UH-RA.1 antilichamen: terwijl voor UH-RA.1 voornamelijk IgG3 domineerde gevolgd door IgG1, bestaat anti-UH-RA.21 IgG vooral uit IgG2. IgG2 wordt voornamelijk geproduceerd als reactie op bacteriële infecties [315, 353] en de frequente aanwezigheid van dit isotype wijst mogelijk op een microbiale trigger voor de ontwikkeling van anti-UH-RA.21 antilichamen, waarbij *molecular mimicry* verantwoordelijk is voor een kruisreactie tussen bacteriële antigenen en lichaamseigen eiwitten [354]. Algemeen beschouwd zijn vooral IgG1 en IgG3 sterk pro-inflammatoir [315]. De isotype profielen weerspiegelen dus niet meteen het beeld van de 'goede' (UH-RA.1) en 'slechte' (UH-RA.21) antilichaamresponsen zoals naar voren kwam uit voorgaande bevindingen, maar de eigenlijke rol van antilichamen in immunologische processen is echter nog van vele andere factoren afhankelijk. Zo bepalen modificaties van de glycaan structuur van IgG onder meer de balans tussen pro- en anti-inflammatoir [309-312]. Verdere karakterisatie van de antilichaameigenschappen is vereist om hun werkingsmechanisme te achterhalen.

In **hoofdstuk 6** tenslotte, werd de rol van de nieuwe autoantilichamen in de onderliggende pathogenese bestudeerd aan de hand van diermodellen voor RA. In het eerste muismodel, CIA, veroorzaakt immunizatie met collageen type II in compleet Freund's adjuvant (CFA) een chronische progressieve vorm van artritis [139, 141]. Net zoals in RA worden de synoviale gewrichten aangetast met bijhorende schade aan kraakbeen en bot [142-144]. In dit model werd het effect van anti-UH-RA.1 en anti-UH-RA.21 antilichamen op de ernst van het ziekteverloop bestudeerd. Klinische ziektescores, en voor UH-RA.21 ook botresorptie, werd geëvalueerd na passieve transfer van polyklonale antilichamen in de CIA muizen. Antilichamen tegen UH-RA.1 bleken geen effect te hebben op CIA ziektescores. De resultaten voor antilichamen tegen UH-RA.21 waren echter inconsistent. Een eerst pilootexperiment wees op een ziekteverergerend effect van de antilichamen in CIA, maar deze konden niet bevestigd worden in vervolgexperimenten. Ondanks het uitblijven van een effect op de ziektescores, werd in het laatste vervolgexperiment verrassend genoeg wel een effect waargenomen op subklinisch niveau: op 3D-microcomputed tomography (micro-CT) analyses van aangetaste kniegewrichten was een verhoogde botresorptie zichtbaar in muizen die geïnjecteerd waren met antilichamen tegen UH-RA.21, in tegenstelling tot muizen die controle antilichamen toegediend kregen. Hoewel deze resultaten nog met enige voorzichtigheid dienen geïnterpreteerd te worden in afwachting van quantitatieve analyses, sluiten onze bevindingen wel aan met de associatie tussen anti-UH-RA.21 antilichamen en de aanwezigheid van erosies in RA-patiënten aangetoond in **hoofdstuk 3 en 4**.

In een tweede muismodel voor RA, collageen-antilichaam-geïnduceerde artritis (CAIA), onderzochten we de artritogene aard van anti-UH-RA.21 antilichamen. De resultaten uit dit model toonden aan dat de antilichamen op zich niet in staat waren om artritis te veroorzaken. Bovendien konden ze het artritogene effect van anti-collageen type II antilichamen ook niet versterken.

Conclusie

Tot slot, in deze thesis werden nieuwe autoantilichaamresponsen geëvalueerd als kandidaat biomerkers voor vroege en seronegatieve RA. Na de ontwikkeling van peptide ELISA tests, werden twee grote onafhankelijke Europese cohorten cross-sectioneel gescreend voor de aanwezigheid van antilichamen tegen UH-RA.1 en UH-RA.21. Binnen één van beide cohorten werden de antilichaamresponsen bovendien ook in een longitudinale setting gemeten. De bevindingen van deze validatiescreenings hebben aangetoond dat de kandidaat biomerkers de diagnose van seronegatieve patiënten kunnen verbeteren. Belangrijk hierbij is dat de merkers reeds in vroege ziekte aanwezig bleken te zijn. Terwijl voor antilichamen tegen UH-RA.1 een associatie met remissie werd teruggevonden, bleken antilichamen tegen UH-RA.21 dan weer gerelateerd met een slechtere prognose, op basis van klinische en inflammatoire parameters, en de aanwezigheid van boterosies. Dit laatste bleek ook uit preliminaire observaties in een muismodel voor RA, waarin injectie met antilichamen tegen UH-RA.21 boterosie leek te verergeren.

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Curriculum Vitae

Liesbeth De Winter werd geboren op 9 juni 1987 te Edegem. In 2005 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in de afstudeerrichting Moderne Talen – Wetenschappen aan het Maris Stella Instituut te Oostmalle. Vervolgens startte ze haar opleiding Biomedische Wetenschappen aan de Universiteit Hasselt waar ze haar diploma Bachelor in de Biomedische Wetenschappen behaalde in 2008. Aansluitend behaalde ze haar diploma Master in de Biomedische Wetenschappen met onderscheiding. Haar masterstage, getiteld "Validation of kidney injury molecule 1 (kim-1) as an early, low-dose biomarker of metal-induced kidney damage in a population sample" voerde ze uit aan de Universiteit Hasselt in de onderzoeksgroep Fysiologie (Biomedisch Onderzoeksinstituut, Diepenbeek) onder leiding van dr. ir. Quirine Swennen en Prof. dr. Emmy Van Kerkhove, en in de onderzoeksgroep Milieubiologie (Centrum voor Milieukunde, Diepenbeek) onder leiding van Prof. dr. Tim Nawrot. Tijdens haar masteropleiding behaalde ze tevens de certificaten Proefdierkunde I (FELASA B) en Radioprotectie (UHasselt en SCK-CEN). In 2010 startte ze haar doctoraat in de Immunologie/Biochemie groep onder het promotorschap van Prof. dr. Veerle Somers en het co-promotorschap van Prof. dr. Piet Geusens en Prof. dr. Piet Stinissen. Haar doctoraatsonderzoek was gericht op nieuwe kandidaat biomarkers voor reumatoïde artritis (RA). Ze schreef een IWT beurs en nam actief deel aan het schrijven van een FWO en SBO project. Daarnaast, was ze lid van het onderwijsteam in de opleidingen Biomedische Wetenschappen en Geneeskunde, begeleidde stagestudenten uit de bachelor en master opleiding, en volgde zelf de cursussen Proefdierkunde II (FELASA C) (UHasselt), *Project Management* (True Colours), Presentatie en interview skills (Vocational Training and Testing), Bioveiligheid (UHasselt en Perseus), *Good scientific conduct* and *Lab book taking* (UHasselt), *Scientific writing and oral presentations* (VIB), *Career Management* (Braingain), Patent Databanken (VIB), Parametrische en niet-parametrische statistische methoden voor de levenswetenschappen (UHasselt CENSTAT), en Eiwit purificatie (GE Healthcare). Ze was betrokken bij de organisatie van het "International Life Sciences Master Student Research Conference" in 2012 te Diepenbeek, en nam zelf deel aan verscheidene nationale en internationale symposia waar ze haar onderzoeksresultaten

presenteerde aan wetenschappers van over de hele wereld. Haar resultaten gepresenteerd op het EULAR congres in 2013 te Madrid, Spanje, werden geselecteerd voor presentatie aan de pers, en voor het bijwonen van dat congres ontving ze een EULAR travel grant. Tijdens haar doctoraat droeg ze tenslotte bij aan de uitbouw van een onderzoeksovereenkomst met het Amerikaanse INOVA Diagnostics.

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"It always seems impossible until it's done."

-Nelson Mandela