Circulating and Mucosal Antibodies to Citrullinated Antigens in Rheumatoid Arthritis

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Contents

Thesis Summary

Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint inflammation and subsequent destruction of cartilage and bone. The etiology is largely unknown, although genetic as well as environmental factors are involved. The manifestations and consequences of RA differ between individuals. This makes it important to find early markers for the disease course, in order to enable the most suitable treatment. IgG antibodies to cyclic citrullinated peptides (CCP) have high specificity for RA, but only around 60% of RA patients test positive for IgG anti-CCP.

The aim of this thesis was to evaluate the usefulness of serum IgA anti-CCP as a diagnostic maker compared to IgG anti-CCP, and to assess IgA versus IgG anti-CCP status in relation to smoking habits and genetic background. Another aim was to evaluate signs of mucosal immunization by analyzing salivary IgA anti-CCP.

IgA anti-CCP was present in a subgroup of RA patients with high levels of IgG anti-CCP and a slightly more severe disease course. Similar results were found regarding IgA class antibodies to modified citrullinated vimentin (MCV). IgG anti-MCV had slightly higher sensitivity for RA than IgG anti-CCP, thus identifying a group of IgG anti-CCP negative patients with an unfavourable disease course. However, the lower diagnostic specificity of IgG anti-MCV limits its usefulness.

Among 63 patients with established RA, salivary IgA anti-CCP was found in 22% and was associated with a more favourable outcome regarding erosive joint disease at follow-up. IgA anti-CCP in serum was strongly associated with smoking, and the earlier known interaction between smoking and shared epitope (SE) was here shown to be valid only for subjects positive for IgA anti-CCP in combination with IgG anti-CCP.

In conclusion, IgG anti-CCP is still the most useful serologic marker of RA, but IgA anti-CCP should be further investigated as a prognostic marker. The association between smoking and IgA anti-CCP strongly indicates a pathogenic role for smoking and IgA anti-CCP, supporting the possibility that RA may originate from chronic airway irritation. The less erosive disease in patients positive for salivary IgA anti-CCP indicates a protective role of secretory IgA anti-CCP.

Abbreviations

ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
BALT	bronchial-associated lymphoid tissue
CII	type II collagen
CCP	cyclic citrullinated peptide
CD	cluster of differentiation
CRP	C-reactive protein
DAS	disease activity score
DMARD	disease-modifying anti-rheumatic drug
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
Fab	Fraction antigen binding
Fc	Fraction crystallizable (Part of antibody binding to an Fc-receptor)
FcαR	Fcα receptor, receptor for Fc part of IgA
FcγR	Fcγ receptor, receptor for Fc part of IgG
GALT	gut-associated lymphoid tissue
HLA	human leukocyte antigen
iBALT	inducible bronchial-associated lymphoid tissue
Ig	immunoglobulin
JIA	juvenile idiopathic arthritis
MALT	mucosa-associated lymphoid tissue
MCV	mutated citrullinated vimentin
NALT	nasopharynx-associated lymphoid tissue
NSAID	non-steroid anti-inflammatory drug
OD	optical density
PAD	peptidyl arginine deiminase
PPAD	*Porphyromonas gingivalis* PAD
PPV	positive predictive value
pIgR	polymeric immunoglobulin-receptor
RA	rheumatoid arthritis
RF	rheumatoid factor
SE	shared epitope
SIgA	secretory immunoglobulin A
TNFi	tumor necrosis factor inhibitor
WHO	World Health Organization

List of Papers

I   **Svärd A**, Kastbom A, Reckner-Olsson Å, Skogh T. Presence and utility of IgA-class antibodies to cyclic citrullinated peptides in early rheumatoid arthritis: the Swedish TIRA project. *Arthritis Research and Therapy* 2008;10(4):R75.

II   **Svärd A**, Kastbom A, Söderlin MK, Reckner-Olsson Å, Skogh T. A Comparison Between IgG- and IgA-class Antibodies to Cyclic Citrullinated Peptides and to Modified Citrullinated Vimentin in Early Rheumatoid Arthritis and Very Early Arthritis. *Journal of Rheumatology* 2010;38:1265-72.

III   **Svärd A**, Kastbom A, Sommarin Y, Skogh T. Salivary IgA antibodies to cyclic citrullinated peptides (CCP) in rheumatoid arthritis. *Immunobiology* 2013;218(2):232-7.

IV   **Svärd A**, Skogh T, Alfredsson L, Ilar A, Klareskog L, Bengtsson C and Kastbom A. Associations to smoking and shared epitope differ between IgA and IgG class antibodies to cyclic citrullinated peptides in early rheumatoid arthritis**.** *Submitted.*

Preface

How is it possible that our immune system, which is usually so well-behaved, can suddenly turn on its own host?

Autoimmunity has to me always been surrounded by an air of mystery. This fascination has stayed with me from my first employment as a medical laboratory technologist within the field of immunologic research, via medical school to my present position as a clinical rheumatologist and doctoral student. Having the opportunity to meet patients with one of the most common autoimmune diseases – rheumatoid arthritis – and seeing the consequences of the disease as well as our increasing possibilities to slow down disease progress, has added to this interest.

Much research is constantly going on all over the world within this field and new knowledge is continuously generated. The picture today is quite different from what it was on the day I started out with this PhD project. However, even though rheumatoid arthritis is one of the most extensively studied autoimmune diseases, much is still unclear and the etiology remains a mystery.

Introduction

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease that primarily affects the joints. There are skeletal remains from North America indicating that the disease existed at least 3000 years ago, and it was given its name by Alfred Baring Garrod in 1859 [1].

Clinical picture

Patients developing RA typically present with several swollen and painful small joints of hands and feet, fatigue and morning stiffness. If not treated, the disease proceeds to affect more joints, and more constitutional symptoms like weight loss and fever may develop. The joint inflammation often leads to erosions of bone and eventually joint deformities and loss of function (figure 1). Involvement of other organs such as lungs, kidneys, skin, eyes, heart and blood vessels occurs in some patients. Patients with RA have an increased mortality compared to the general population, especially due to cardiovascular disease [2].

Figure 1. Hands of a woman with RA, with typical ulnar deviation in the metacarpo-phalangeal joints (a). Hands of a woman with a long history of erosive RA, with joint deformities (b). Published with approval of the patients.
Photographs by Per-Johan Hedin and Kajsa Öberg.

However, rheumatoid arthritis is a heterogeneous disease. Some patients have a mild, self-limiting arthritis and others a rapidly progressing, highly inflammatory disease with extensive joint damage.

Epidemiology

The prevalence of RA is 0.5-1% in most studied communities [3], and is one of the most common autoimmune diseases. There are populations with a considerably higher prevalence, such as American Indians [4] and those with a lower prevalence, such as Asian populations [5]. Women in the pre-menopausal age are affected 2-4 times as often as men, but this difference decreases with increasing age [3].

Diagnosis and classification

A clinical diagnosis of RA is often based on the presence of symmetrical swelling of small joints, autoantibodies and morning stiffness. More detailed classification criteria have been set up for scientific purposes in order to obtain well-defined study populations. The 1987 ACR (American College of Rheumatology) criteria [6] involve radiographic changes and rheumatoid nodules, which usually are late manifestations of the disease (table 1).

Table 1. 1987 ACR classification criteria for rheumatoid arthritis. (4/7 criteria are needed for a classification of RA. Criteria 1-4 must have been present for ≥6 weeks.)

Criterion	Definition
1. Morning stiffness	Morning stiffness at least one hour
2. Arthritis of three or more joint areas	(PIP, MCP, wrist, elbow, knee, ankle, and MTP joints)
3. Arthritis of hand joints	At least one swollen wrist, MCP or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas on both sides of the body
5. Rheumatoid nodules	Subcutaneous nodules
6. Serum rheumatoid factor	Abnormal levels of serum rheumatoid factor
7. Radiographic changes	Erosions in hands or wrists

PIP = proximal interphalangeal, MCP = metacarpo-phalangeal, MTP = metatarso-phalangeal

The newer 2010 ACR/EULAR (American College of Rheumatology/European League against Rheumatism) criteria [7] were elaborated to better reflect early disease, and emphasize autoantibody levels and erythrocyte sedimentation rate (ESR)/C-reactive protein (CRP), as markers of an immune activation and signs of ongoing inflammation (table 2). The 1987 ACR criteria are still used to enable comparisons between new and old studies, often in combination with the 2010 ACR/EULAR criteria.

Table 2. 2010 ACR/EULAR classification criteria for rheumatoid arthritis. (A score of ≥6 is needed for a definite classification of RA.)

A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

ACPA = anti-citrullinated protein antibody, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate

Treatment

As RA is a common disease, which can lead to considerable suffering for the individual as well as increased costs to society, effective treatment is of great importance. Modern treatment regimens aim at minimizing symptoms, preventing joint deformities, maintaining a good physical function and also preventing cardiovascular disease and mortality. Early initiated, effective treatment has improved the prognosis considerably [8-10].

Traditional Disease Modifying Anti-Rheumatic Drugs (DMARDs) include methotrexate, sulfasalazine, hydroxy-chloroquine and leflunomide, with methotrexate being the most frequently used. The introduction in the late 1990s of the so called biologic drugs has added valuable, but costly, treatment options, and are now being used by approximately 30% of RA patients in Sweden.

Glucocorticoids are often used as oral treatment and/or intra-articular injections in early disease and during episodes of increased inflammatory activity. Non-steroidal anti-inflammatory drugs (NSAIDs) are used for symptomatic relief of pain and stiffness when needed.

Monitoring of the disease activity is generally done by the 28-joint count disease activity score (DAS-28) [11], an algorithm based on the patient's global health assessment on a 100-mm visual analogue scale, number of swollen and

tender joints out of 28 defined joints, and ESR. In Sweden, functional disability is assessed using the Swedish version of the 'health assessment questionnaire' (HAQ) [12]. Regular X-rays of hands and feet are made to survey development of bone erosions.

Considering our increasing possibilities to potently treat patients with RA, the demands for early accurate diagnosis increase. Also, predictive information that may increase an individualized therapy becomes more important.

Etiology

The etiology of RA is not known. There is epidemiologic and experimental evidence for a number of genetic risk factors and environmental triggers, but much knowledge is still lacking.

A current model of initiation and development of RA include [13]:
1. A genetic predisposition.
2. An asymptomatic phase in which triggering environmental exposures (such as smoking) are encountered.
3. Mucosal inflammation (e. g. oral cavity or airways) with locally exaggerated generation of autoantigens (citrullinated proteins). Immune activation with autoantibodies and inflammation markers (cytokines) many years before onset of clinically manifest RA [14-16]. Although it has not been clearly shown what comes first, autoantibodies or inflammation, it seems reasonable that there is some degree of local inflammation which contributes to the formation of autoantibodies [17].
4. A 'pre-clinical' phase with arthralgia and possible subclinical arthritis.
5. Manifest arthritis.

The immune system

After encountering the physical barriers such as skin and mucosal membranes, micro-organisms are fought back by the *innate immune system*. This is a fast working defense system using 'pathogen' and 'danger' associated molecular pattern recognition by phagocytic cells, which activates NK cells and mast cells leading to release of cytokines and a wide range of other soluble mediators. This part of the immune system does not have an immunological memory.

We also have the constantly changing *adaptive immune system*, which generates tailor-made targeted responses and has a memory for what it has earlier encountered. The adaptive immune system has a sophisticated ability to dis-

tinguish between self and non-self, which means that it is designed to specifically eliminate foreign invaders and modified, harmful self-molecules, but to tolerate healthy self-molecules and tissues. The effector cells of the adaptive immune system are T-lymphocytes (T-cells) and B-lymphocytes (B-cells).

T-cells

T-cells are developed in the bone marrow and matured in the thymus. This maturation process is essential for the cells to acquire the ability to distinguish self from non-self, and self-reactive cells are eliminated in the thymus during this process.

In order to alert the T-cells to invaders, antigen presenting cells (APCs) have to 'present' the invader to the T-cells (figure 2). APCs, such as dendritic cells, macrophages or B-cells bind, internalize and degrade proteins, and then present peptide fragments on their surface bound in a pocket of the MHC class II molecules and present them to the T-cell receptors. These MHC molecules have different shapes depending on genetic variants, which influence their ability to bind and present different antigens. This is of relevance as certain MHC class II genetic variants are strongly associated with RA.
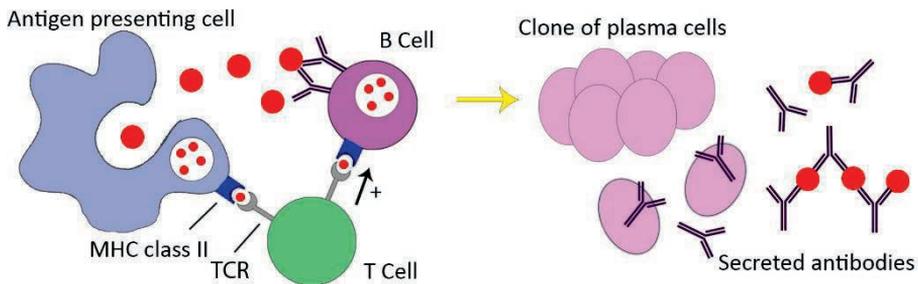

Figure 2. Antigen presenting cell (dendritic cell or B-cell) internalizing an antigen and displaying part of a peptide on its MHC class II molecule, thereby activating a T-cell via the T-cell receptor (TCR), which in turn activates a B-cell that develops into antibody-secreting plasma cells.

Upon activation, the T-cells induce production of cytokines such as tumor necrosis factor (TNF) and interleukin 2 (IL-2), which in turn activate other T-cells, B-cells, macrophages and NK-cells. T-cells are divided into CD (cluster of differentiation) 4+ and CD8+ T-cells. Upon activation, the CD8+ T-cells differentiate into cytotoxic T-cells and CD4+ T-cells differentiate into T helper cells; Th1, Th2 or Th17 cells. Th17 cells are of special interest at mu-

cosal sites as they are able to promote up-regulation of the polymeric immu-noglobulin-receptor (pIgR), which leads to increased secretion of IgA both in the gut [18] and in the bronchial epithelium of the lungs [19].
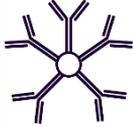
B-cells

A main function of B-cells is to develop into antibody-producing plasma cells. They also have the ability to present antigens to T-cells. B-cells are developed in the bone marrow, and are found in the blood and lymph circulation, in re-gional lymph nodes and in mucosal associated lymphoid tissue (MALT).

Immunoglobulins

As presented in table 3, antibodies occur in five different immunoglobulin (Ig) isotypes; IgM, IgG, IgA, IgD and IgE. Basically, they are Y-shaped proteins with two identical antigen-recognizing 'claws' called Fab (Fraction antigen binding) parts, and one Fc (Fraction crystallizable) part – a 'tail', with a dif-ferent structure for each isotype.

Table 3. Overview of the five major classes or isotypes of antibodies.

CLASS	MAIN STRUCTURE CIRCULATION	FUNCTION CIRCULATION	MAIN STRUCTURE MUCOSAE	FUNCTION MUCOSAE
IgM		First class of antibodies re-leased after primary antigen challenge. Attached (mono-meric) to surface of B-cells and free in circulation. Com-plement activation.		Mucosal immunity, es-pecially important in IgA deficiency.
IgG		4 subclasses. 80 % of serum antibodies. Opsonization. Classical complement activa-tion.		
IgA		2 subclasses (IgA_1 and IgA_2). Largely unknown functions.		2 subclasses (IgA_1 and IgA_2). Mucosal immun-ity. Non-inflammatory neutralization of toxins.
IgE		Protects against parasites. Involved in atopic allergic re-actions.		
IgD		Cell surface receptor of ma-ture B cells.		

All antibodies belonging to the same isotype have similar Fc parts, which determine the biologic action of the antibody. The 'claws' determine what structure the antibodies recognize, i.e. which specific bacterium/foreign antigen or, in autoimmune disease, which self-antigen.

Human circulating IgG, IgA, and IgE are mainly monomeric, whereas IgM is pentameric. IgD (monomeric) is only seen as antigen receptors on the surface of B-cells, often together with IgM.

IgG is the most abundant isotype in serum. One major role is to opsonize pathogens, i. e. binding to antigens and allowing FcγR-mediated elimination. Upon binding its target, IgG-class antibodies expose their Fc parts, leading to rapid "classical" complement activation. This in turn attracts phagocytes which can eliminate the target.

IgA is found in serum as well as in mucosal secretions. Circulating IgA has a rapid turn-over and constitutes 15-20% of the serum Ig concentration, whereas IgA clearly dominates in mucosal secretions. There are two subclasses of IgA; IgA1 and IgA2. The two subclasses are similar, but IgA1 contains an extra amino-acid sequence which makes it more sensitive to proteolytic cleavage. IgA1 dominates in serum and IgA2, thus being more resistant to enzymatic cleavage, dominates on mucosal surfaces in the gut. Parotid secretions on the other hand, consist of approximately 65% IgA1 and 35% IgA2 [20].

Serum IgA antibodies are mainly monomeric (>95%) and they are produced by B-cells in the bone marrow [21]. The functions of IgA antibodies in serum are largely unknown, although both pro-inflammatory and anti-inflammatory properties have been described. IgA up-regulates the pro-inflammatory IL-1β and down-regulates the anti-inflammatory cytokine IL-10 [22]. IgA also down-regulates many cell responses, such as release of the pro-inflammatory TNF and IL-6 [22-25]. In contrast to IgG-class antibodies, IgA-class antibodies are not complement activating via the classical pathway [26] and even prevent complement activation by IgG and IgM [27].

These pro- and anti-inflammatory effects of IgA have been found to depend on the dual action of the Fcα-receptor 1 (FcαR1) [28]. Circulating antibodies exert many of their effects by binding to Fc receptors, which form the link between antibodies and cellular reactions. Each Ig class binds to its own Fc-receptor and IgA mainly binds to the FcαR1, which is found on the surface of phagocytic cells: neutrophils, monocytes, eosinophils, macrophages, Kupffer cells and interstitial dendritic cells. Binding of single IgA molecules to the receptor induces an inhibitory effect whereas IgA bound in complexes leads to activation. This implicates that serum IgA under physiological conditions

may have an inhibitory role, whereas IgA bound to soluble antigens (in complexes) or pathogen surfaces during an infection would promote inflammation [29-31].

Deficiency of monomeric IgA is one of the most common immune deficiencies with a prevalence of around 0.2%. There is a clearly increased frequency of autoimmune diseases among IgA deficient individuals; for instance, occurring in 7- 36% in celiac disease [30], and systemic lupus erythematosus (SLE) [32]. This could imply that IgA plays a role in maintaining immune homeostasis, which is proposed by Jacob et al. [30] in a hypothesis where interaction of IgA with the FcαR1 leads to inhibition of activating pathways of the immune system. There is however no strong evidence for an association between IgA deficiency and RA, although a modest increase in RA prevalence has been reported among IgA deficient subjects [30, 33, 34]. Interestingly, it has been suggested that monomeric IgA may prove therapeutically useful in human inflammatory diseases [23].

Secretory IgA (SIgA) has an important role in mucosal immunity. It is usually dimeric, but to some extent polymeric and is produced locally by submucosal B-cells and connected by a joining chain. Di- and polymeric IgA bind to the pIgR located on the basolateral surfaces of mucosal epithelial cells. The whole complex is transcytosed and released at the apical surface of the epithelial cell in the form of SIgA, leaving the anchoring part of the receptor bound to the cell surface (figure 3). Thus, the extracellular part of the receptor remains bound to the polymeric IgA and becomes the secretory component (SC), a glycoprotein providing SIgA antibodies with resistance to enzymatic degradation [35].
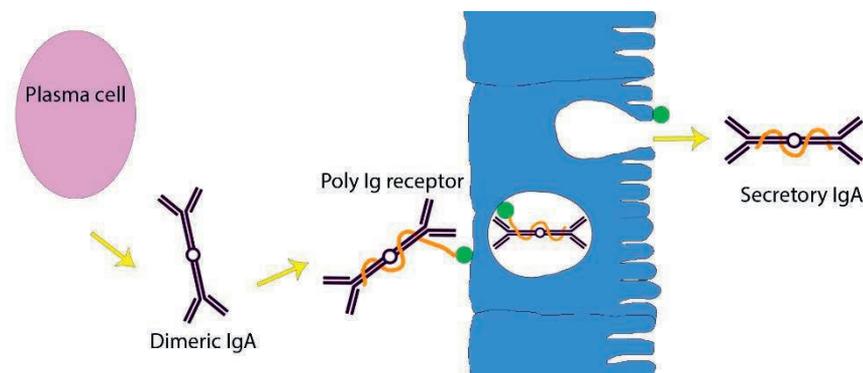

Figure 3. Formation of secretory IgA (SIgA). Dimeric IgA is produced by submucosal plasmacells, binds to the poly-Ig receptor (pIgR), is transcytosed through the epithelial cell and released as secretory IgA with a secretory component (SC).

The role of SIgA is to defend the body against microorganisms, without damage to the host barriers, while at the same time allowing commensal bacteria to reside in the mucosal environment. This role involves direct neutralization of toxic molecules, prevention of microorganism adherence to mucosal surfaces and intracellular neutralization of viruses during transepithelial transport [30, 36-38]. SIgA and IgG are the major immunoglobulins found in saliva. Most salivary IgG is derived from serum, via the gingival epithelium, whereas SIgA is derived from salivary glands. There are 3 major types of salivary glands, the submandibular, parotid and sublingual glands, which besides SIgA also secrete a large number of smaller proteins probably taking part in the innate immune system [39].

Autoimmunity in RA – What happens when it goes wrong?

Considering the complexity of the immune system and the sometimes minimal structural differences between pathogens and self-antigens, it is not surprising that it sometimes goes wrong. Autoimmune diseases are characterized by breakdown of self-tolerance, leading to immune reactions towards the own body. The total prevalence of all autoimmune diseases, depending on the definition, has been estimated to approach 4.5% [40].

A characteristic feature of autoimmune diseases is the presence of autoantibodies, i. e. antibodies directed to (or cross-reactive with) self-structures. Some of these autoantibodies have high diagnostic specificity, whereas most do not. In a few diseases, the autoantibodies are proven (or are highly likely) to have a pathogenic role, but in many diseases their function remain unknown.

Rheumatoid factor (RF)

Rheumatoid factor (RF), i.e. antibodies with affinity for the Fc-part of IgG, are autoantibodies present in about 60-70% of RA patients [41]. The presence of RF is related to significantly higher disease activity over time, more extra-articular manifestations, bone erosions and an increased risk of cardiovascular disease [42].

RF occurs among all immunoglobulin isotypes. Although IgM RF is the most frequently analysed, IgA RF has been considered of particular interest as a predictor of aggressive disease [43, 44], and high IgM, IgG and IgA-class RF have been reported in systemic rheumatoid vasculitis, where IgA RF has been

considered to add the highest diagnostic value [45]. IgA and IgM RF have also been shown to be associated with cigarette smoking [46].

A disadvantage with RF, however, is that the diagnostic specificity for RA is rather low. RF is frequently found in a number of other inflammatory conditions, including infectious diseases, and with the suggested cut-off limit of $\geq 95\%$ among healthy referents [6], close to 5% of the healthy population tests positive for RF.

Citrullination

The amino acid citrulline was first isolated from water melon (*lat. Citrullus vulgaris*) in 1914 [47]. Citrullination is the enzymatic conversion of an arginine residue to citrulline in a protein/peptide (figure 4). The reaction involves removal of an imine (double bonded nitrogen) and is catalyzed by the enzyme peptidylarginine deiminase (PAD).
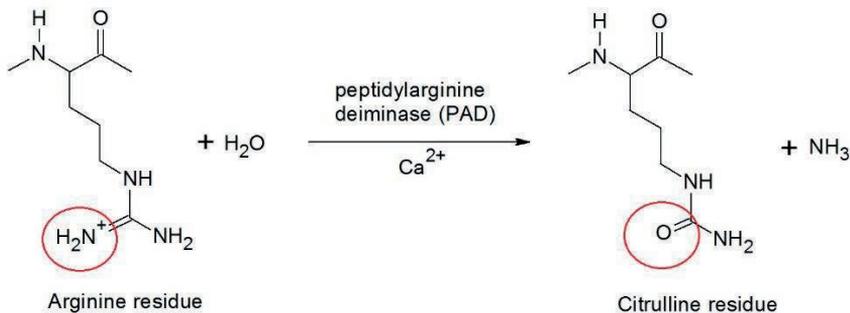
Figure 4. Conversion of an arginine residue to a citrulline residue occurs in an environment rich in calcium, through the action of the enzyme peptidyl arginine deiminase (PAD).

Citrullination is involved in many physiological processes in the body such as skin keratinization, maturation of hair follicles and insulation of nerve fibres (reviewed in [48]). Citrullination is also involved in pathological processes but although increased citrullination is found in the myelin sheaths in patients with multiple sclerosis (MS) and in lung cancer tissue, no antibodies to citrullinated proteins are found in these conditions [49].

Increased citrullination occurs under inflammatory conditions [50-52]. The PAD enzyme requires high calcium levels for activation, which in inflammation can occur inside the leaking cells. Citrullination can also occur outside

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the cells, where calcium levels are high, when the enzyme escapes from dying cells.

ACPA - Antibodies to citrullinated proteins and peptides

Although citrullination of proteins is common in inflammatory environments, the generation of specific antibodies directed to these proteins is almost exclusive to patients with RA, or individuals at increased risk of developing RA. Anti-citrullinated peptide/protein antibodies (ACPA) were first reported by Schellekens and colleagues in 1998 [53] and have had a large impact on routine serological testing [54, 55]. ACPAs often coincide with the presence of RF, but have higher diagnostic specificity for RA, and are better predictors of disease course and outcome [14, 41, 56-58]. The presence of circulating IgG ACPA may precede clinical onset of disease by several years [14], indicating a pathogenic role.

ACPA occurrence can be tested by several assays, most of which are primarily used for research purposes. In clinical practice the most widely used and most extensively evaluated analyses measure IgG-class antibodies to cyclic citrullinated peptides (anti-CCP). These have high diagnostic specificity for RA with a diagnostic sensitivity comparable to traditional RF tests, making them useful in clinical practice as diagnostic and prognostic tools [54, 55].

Antibodies to modified citrullinated vimentin (anti-MCV) can also be used as diagnostic and prognostic markers of RA. Anti-MCV has in a number of studies shown a higher sensitivity for RA than anti-CCP [59-61] which means that the anti-MCV test can identify more RA patients than the anti-CCP test. On the other hand, many studies show that anti-MCV has a lower specificity for RA [59, 61-65], meaning that, compared to anti-CCP, a larger number of subjects who do not and will not suffer from RA will test positive for anti-MCV.

ACPA is a large family of antibodies, including not only anti-CCP and anti-MCV, but antibodies to a wide range of different citrullinated proteins. The number of different ACPA specificities increases with time as pre-RA individuals get closer to developing clinical RA, a phenomenon called epitope spreading [66]. Subjects with different sets of antibodies have different risks of developing RA, and the influence from genes and smoking habits vary between these groups [67]. Besides epitope spreading, pre-RA patients develop increasing serum levels of ACPA and an increasing number of ACPA isotypes closer to the time of diagnosis [68].

Genetic risk factors

Twin studies indicate that heritability accounts for 66-68% of the risk of developing ACPA positive as well as ACPA negative RA [69]. The human leucocyte antigen (HLA)-DRB1 locus codes for an antigen-binding region of the major histocompatibility (MHC) complex. Some alleles code for a sequence of amino acids in position 70-74, which results in a similar three-dimensional configuration and consequently similar antigen-binding properties. These are collectively called shared epitope (SE) [70], and they have a strong connection to ACPA positive RA [71].

Other genes of importance include HLA-DRB*15, which appears to promote the production of high ACPA levels [72]. PTPN22 is a gene coding for protein tyrosine phosphatase N22, which is a negative regulator of T-cell reactivity [73]. Polymorphism of this gene increases risk of RA in SE carriers.

These and other known genetic factors explain about 50% of the genetic variance responsible for the variance in susceptibility to RA, whereas the remaining 50% awaits discovery [74].

Environmental triggers

Tobacco smoke has by western medical practitioners been regarded as a medicine appropriate to treat a number of conditions. Both ileus [75] and victims of near drowning [76] were treated with tobacco smoke enemas until the early 19th century, when nicotine was discovered to be poisonous. In the 1780s the Royal Humane Society installed resuscitation kits, including smoke enemas, at various points along the River Thames [76]. Tobacco smoking is now recognized as the single greatest cause of preventable death globally and is responsible for 5.4 million deaths every year [77], with lung cancer and cardiovascular disease being the principal causes of death.

Smoking has also been found to induce inflammation [78], and to associate with a number of autoimmune diseases [79-83]. However, components of cigarette smoke, e. g. nicotine, have also been shown to have immunosuppressive/anti-inflammatory effects [84, 85]. Long-term smoking reduces serum immunoglobulin levels, and smokers have been reported to have IgA, IgG and IgM levels up to 10-20% below normal [86, 87]. Cigarette smoking is associated with a reduced glandular focus score in lip biopsies among patients with Sjögren's syndrome [88], indicating an immunosuppressive effect. Epidemiological data indicate that smoking might decrease the incidence of certain chronic inflammatory diseases, like ulcerative colitis, sarcoidosis and Parkinson's disease [86]. Total levels of salivary IgA are lower in smokers than in

non-smokers [89, 90], which may hypothetically lead to an increased sensitivity to infections and inflammation, possibly promoting increased citrullination.

In the case of RA, cigarette smoking was estimated to be responsible for 20% of all RA cases and 35% of ACPA positive cases in the Swedish Epidemiologic Investigations in Rheumatoid Arthritis (EIRA) study [91]. As RA is a common disease, with a prevalence of 0.5 – 1 %, this means that the disease could have been avoided in about 15.000 persons, out of the total of 75.000 cases of RA in Sweden. Furthermore, in patients with established RA, there are indications that smoking is associated with more active disease and worse outcome [92], and also with a poorer response to treatment with methotrexate and TNF inhibitors (TNFi) [93, 94].

It is well established that interactions between smoking and SE increase the risk of developing ACPA positive RA [13, 95]. It has recently been shown in a population of pre-RA cases, that smoking is a risk factor not only for ACPA positive RA, but also for the development of autoantibodies in pre-RA subjects. This strengthens the hypothesis that smoking is of etiological importance in the very early stages of RA development [96]. Among pre-RA patients who are smokers, IgA anti-CCP antibodies appear significantly earlier than in non-smokers [97]. However, the mechanism by which cigarette smoking contributes to the development of RA is not known.

Although cigarette smoking is the most well-documented environmental risk factor for RA, epidemiologic research has presented convincing evidence for other factors as well. Silica dust exposure, occurring in occupations such as rock drilling and mining, is a risk factor for ACPA positive RA, and an interaction can be seen between silica and smoking [98]. Hormonal factors are implicated by the fact that women are 2-4 times more likely to develop RA than men [99], and that RA usually develops at times when sex hormone levels change, i.e. post-partum and around menopause [100]. The role of infections in the etiology of RA has been debated for decades. At present, the oral bacterium *Porphyromonas gingivalis (P. gingivalis)*, involved in periodontal inflammation, receives attention due to the known association between periodontitis and RA and the unique ability of *P. gingivalis* to citrullinate its own and human proteins [101]. Among dietary factors, omega 3 fatty acids have a well-documented anti-inflammatory effect [102] and fish and fish oil have shown a modest protective effect against developing RA [103-105]. Alcohol consumption is associated with a lower risk of developing RA [106, 107].

Where does RA start?

According to the prevailing hypothesis, the pathogenesis of ACPA-positive RA thus involves a local inflammation in the pre-clinical period. The most obvious place to start looking might be the joints, but MRI findings and synovial biopsies from autoantibody positive (IgM RF and/or ACPA) subjects without arthritis did not shown signs of inflammation [108].

Mucosal sites

Epidemiologic data indicate probable associations between mucosal immunity and the development of RA [109-113], and several mucosal sites have been suggested to be involved in the pathogenesis of RA.

The mucosal immune system is located in close association to mucosal surfaces, in organized mucosa-associated lymphoid tissue (MALT), which is more specifically subdivided into gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

The connections between the mucosal and systemic immune responses, and between different mucosal compartments, are not clear. It has been shown that enteric cholera vaccination leading to an intestinal SIgA response is poorly correlated with salivary IgA antibody production. However, there are clear differences between different salivary glands. In celiac disease, for instance, IgA antibodies to gliadin can be found in serum, intestinal secretions and in whole saliva, but not in parotid secretions. This indicates that B-cells from the gut-associated lymphoid tissue (GALT) migrate to submandibular and sublingual salivary glands but not to parotid salivary glands [114].

Oral immunization engaging GALT stimulates SIgA as well as serum IgA and IgG antibodies, whereas NALT stimulation only stimulates SIgA and not circulating immunoglobulins [115].

The gut

The human intestine accommodates 10 times as many bacteria as the total number of human cells in the body, and the relationship is of mutual benefit as the bacteria are provided with a favourable environment and the human body is provided with energy from otherwise indigestible polysaccharides [116]. These bacteria also influence our immune system. This is done partly through Th17 cells, as some species induce Th17 cells and other species induce T regulatory cells. These facts suggest that the gut microbiome may play a role in inducing autoimmunity [17].

Oral tolerance, the induction of systemic immunological tolerance after oral administration of an antigen, has been demonstrated in a number of animal disease models, and has also been suggested as a possible therapy in autoimmune disease [117]. It has long been known that mucosal immunisation may induce systemic tolerance against delayed type of hypersensitivity (DTH) reactions, whereas already established T-cell mediated inflammation is difficult to deviate by mucosal antigen challenge [26, 118].

Systemic immunization with type II collagen (CII) is a standard method to induce RA-like polyarthritis in mice [119], whereas mucosal CII immunization induces systemic tolerance [120]. During 1993 to 2001, a number of trials to treat RA patients with oral collagen type II were carried out [121]. In the majority of the studies some amelioration was registered, comparable to the effect of NSAIDs. A large placebo-controlled phase III study including 760 patients showed no difference between the groups [121], whereas in a Chinese study from 2009 with 454 patients the collagen type II group showed modest improvement [122].

The oral cavity

In the first decades of the 20th century an association between oral health and arthritis was presumed, and dental extraction was recommended to treat a number of systemic inflammatory diseases [123]. Extraction even of healthy teeth was advocated with a preventive intention [124]. Modern epidemiologic research has confirmed that patients with periodontitis have a significantly higher prevalence of RA (4%) compared to the general population (0.5-1%) [125] and, similarly, patients with RA have a significantly higher prevalence of periodontitis (51%) compared to healthy controls (22%) [126].

This connection has recently attracted much attention and possible mechanisms behind this association have been hypothesised. The 'main suspect' is *Porphyromonas gingivalis (P.gingivalis),* a Gram-negative anaerobic bacterium commonly involved in periodontitis. This is the only prokaryote known to express a PAD, *P. gingivalis* PAD (PPAD), which enables it to citrullinate both its own and human peptides [127]. It has been proposed that oral citrullination of human and bacterial proteins by PPAD could break the host tolerance to citrullinated auto-antigens and trigger an antibody response against citrullinated proteins [128]. Once tolerance is broken, citrullination of host proteins by human PADs maintains the immune response through epitope spreading, resulting in a chronic inflammatory disease [48]. The findings that antibodies to both *P. gingivalis* [129] and PPAD [130] are more common in RA patients than in healthy controls support this hypothesis.

Studies reporting that the presence of periodontitis in RA patients is associated with circulating IgG anti-CCP prevalence [126], and an association between

ACPA and antibodies to *P. gingivalis* in a population of North American natives [131] further support this hypothesis.

The lungs

A connection between RA and lung involvement was first recognized in 1948 by Ellman, who reported 3 cases of RA with concurrent lung affection [132]. Since then, it has become established that several pulmonary manifestations can be associated with RA.

Recent studies indicate that pulmonary involvement may precede arthritis or other manifestations of RA [113]. Sensitive diagnostic methods such as high resolution computed tomography (HRCT) have revealed structural changes in the lungs in early disease. Metafrazi found lung abnormalities, including ground glass opacities, among 69% of 43 never-smoking patients with early RA, but not in control subjects [133]. Demourelle has reported structural lung changes among ACPA positive non-smoking subjects [134], indicating that other irritants than cigarette smoke may contribute to the initial inflammatory process preceding clinically manifest RA.

Smoking is associated with an increased proportion of BAL (broncho-alveolar lavage) cells expressing citrullinated peptides/proteins [13, 135]. Also, ACPA has been found in BAL from RA patients with structural lung changes [136], indicating that ACPA is involved in inflammatory processes in the lungs. Furthermore, ACPA and RF have been found in sputum samples of seronegative first-degree relatives of RA patients [113], indicating that the lungs are involved in early RA development.

BALT is not found under physiological conditions in human lungs, but at antigen stimulation, inducible BALT (iBALT) can develop in the peribronchial and interstitial tissues of the lung [137]. In patients with pulmonary complications of RA, the iBALT prevalence is higher than in other lung diseases and production of RF and ACPA has been documented in the pulmonary interstitium [112], further demonstrating an involvement of the lungs in RA development.

To conclude, there is a need for better diagnostic and prognostic markers for RA in order to enable the most suitable treatment, and there is a need for further exploration of the role of smoking and IgA anti-CCP in the development of RA, to increase our understanding of the association between the mucosal and systemic immune system.

Aims

The overall aim of this PhD project was to increase knowledge about the oc-currence, and the diagnostic and/or prognostic utility, of circulating and mu-cosal IgA class antibodies to citrullinated peptides, and about their relation to genetic and environmental background factors.

The specific aims were:

- to examine whether analysis of IgA class antibodies to cyclic citrulli-nated peptides in early RA adds diagnostic and/or prognostic infor-mation to IgG anti-CCP analysis.

- to evaluate analysis of IgA and IgG class anti-MCV and anti-CCP antibodies as diagnostic and prognostic markers in early arthritis.

- to determine if IgA anti-CCP antibodies occur in saliva of patients with established RA and, if so, to relate salivary IgA anti-CCP anti-bodies to disease activity at the time of diagnosis, and to erosive joint disease at follow-up.

- to explore relations between smoking habits/presence of SE and cir-culating IgA/IgG anti-CCP antibodies in patients with early RA.

Methods

Study subjects

The studies in this thesis are based on five different cohorts of patients.

(a). TIRA-1

320 patients with recent-onset RA (onset of joint swelling <12 months prior to inclusion) were enrolled in the Swedish TIRA project (Swedish acronym for 'early intervention in rheumatoid arthritis') during 1996-98, and were followed for 8 years. 97% of these fulfilled the 1987 ACR classification criteria. The remainder met the following criteria: morning stiffness ≥60 minutes, symmetrical arthritis, and arthritis of hands (wrists, metacarpophalangeal or proximal interphalangeal joints) or feet (metatarsophalangeal joints). A large database was created, containing information on life style and environmental factors, immunological markers, and longitudinal clinical data on disease activity, functional abilities, medication, etc.

(b). The Kronoberg Arthritis Incidence cohort

In Kronoberg county, 151 patients with acute arthritis were rapidly referred to a rheumatologist during one year (1999-2000). The 71 patients who had experienced symptoms <3 months were included in this very early arthritis cohort [138]. At the 2-year follow-up, the diagnoses as assessed by an experienced rheumatologist were RA (n=16), reactive arthritis (n=28), undifferentiated arthritis (n=10) and other arthritides (n=15), including 5 psoriatic arthropathy, 2 systemic lupus erythematosus, 2 sarcoid arthritis, 2 gluten enteropathy, 1 Lyme arthritis, 1 mixed connective tissue disease, 1 ankylosing spondylitis and 1 polymyalgia rheumatica. All patients diagnosed with RA in the Kronoberg cohort fulfilled the 1987 ACR criteria. Two patients with osteoarthritis were excluded, and among the remaining 69 patients 22% were after two years of follow-up diagnosed with RA.

(c). RA patients from the Rheumatology Clinic in Falun

63 patients with a clinical diagnosis of RA, and with a scheduled visit to the Rheumatology Clinic in Falun, were consecutively enrolled during 2009 and 2010; 43 women and 20 men (median age=64, range 30-84 years). Fulfilment of the 1987 ACR and/or 2010 Euro-American classification criteria was assessed from data registered in the medical records. Among the 49 patients where such data were available, 96% of the cases could be classified as having

RA; 80% according to the 1987 ACR classification criteria and another 16% according to the 2010 criteria. Two of the 49 patients (4%) did not fulfil any of the above mentioned criteria. For the remaining 14 patients included in the study, they had been diagnosed with RA before 1990, or the patients had moved to the Falun region with an RA diagnosis established elsewhere, and sufficient data was lacking to allow classification according to the RA classification criteria. With no exception, the clinical diagnosis of RA had been settled by an experienced rheumatologist. The majority of the cases (83%) were selected on the basis of a previous positive serum test for IgG anti-CCP.

(d). TIRA-2
During 2006-2009, 507 RA patients were recruited to a new early arthritis cohort with a design similar to TIRA-1. In this cohort, patients fulfilling the 1987 ACR classification criteria as well as patients positive for IgG anti-CCP and presenting ≥1 arthritis were included.

(e). EIRA-1
During 1996-2006, 2097 RA patients, aged 18-70 years, were reported from rheumatology units in Southern and Central Sweden. In total, 1998 (95%) participated in the study and 85% of these cases had less than one year of symptom duration at inclusion and fulfilled the 1987 ACR criteria. Controls were randomly selected from the study base, matched on sex, age and residential area. In total, 2770 controls were identified and of these 2252 (81%) participated in the study.

Study design

Study I - Longitudinal cohort study
Serum samples from 228 patients were obtained from the TIRA-1 cohort at the time of inclusion, and sera from 72 of these patients were also available at the 3-year follow-up. Disease activity and functional ability measures (ESR, CRP, DAS 28, the physicians assessment of disease activity, and the Swedish version of HAQ) were registered at inclusion and regular follow-ups during three years. Genotyping was performed by PCR amplification (GenoVision, Oslo, Norway), and shared epitope was defined as HLA-DRB1*01, *0401, *0404, *0405, *0408, *0409, *0410, *0413, *0416, *0419, *0421 or *10. Information on smoking habits were in both cohorts obtained from a questionnaire described in detail by Stolt [139]. Only cigarette smoking was included, and patients were classified as current smokers, former smokers or never smokers.

Study II – 2 longitudinal cohort studies

Serum samples from 215 patients with early RA from the TIRA-1 cohort were available for antibody analyses in this study. Antibody status was related to disease course, smoking habits and shared epitope status, similar to study I.

In the Kronoberg Arthritis Incidence cohort, serum samples from all 69 patients with very early arthritis, developing into a number of different diagnoses, were available. This cohort was included to enable assessment of the specificity for RA among the analyzed antibodies.

Study III - Cross-sectional study

Salivary samples were obtained from 63 consecutive patients with established RA. Data regarding disease activity parameters were retrospectively obtained from the patients' medical records. Radiographic assessments were based on written reports from an experienced radiologist, evaluating the presence or absence of bone erosions in hands or feet. 20 healthy subjects were recruited as controls.

Study IV - Case-control study (EIRA-1) and cross-sectional cohort study (TIRA-2)

Only subjects with complete data on antibody status, smoking habits and SE status were included in this study. Thus, 1663 out of 1998 RA patients in EIRA-1 were included and 199 out of 507 RA patients in TIRA-2. The relatively large dropout from TIRA-2 was mainly due to lacking information on smoking habits, as data on smoking habits was available only from the 199 of the TIRA-2 patients who were also included in EIRA-2.

Genotyping in EIRA-1 was conducted by Olerup SSP, Stockholm, Sweden and SE genes were defined as HLA-DRB1*01, *04 or *10. In TIRA-2 HLA-DRB1 was genotyped by Sanger sequencing at BGI Clinical Laboratories, Shenzhen, China. SE was defined as HLA-DRB1*01, *0104, *0405, *0408, *0409, *0410, *0413, *0416, *0419, *0421, or *10. Information on smoking habits were in both cohorts obtained from the same questionnaire as in study I and II. Only cigarette smoking was included, and patients were classified as current smokers, former smokers or never smokers.

Controls were randomly selected from the study base, matched on sex, age and residential area. In total, 1100 controls participated in this study.

The interaction between smoking and SE was calculated, and described as deviation from additivity, i.e. the effect exceeding the sum of the two risk factors added, as suggested by Rothman et al. [140] and as done by e.g. Padyukov et al. [141]. To quantify the interaction, the attributable proportion (AP) due to interaction (i.e. the proportion due to deviation from additivity) is expressed as a value between 0 and 1, and represents the proportion of RA incidence,

among persons exposed to both smoking and SE, that is attributable to the interaction.

Antibody analyses

All autoantibody analyses were performed using enzyme-linked immuno-sorbent assay (ELISA) technique. IgG anti-CCP was analyzed using the commercially available enzyme-immunoassay CCP2 (Euro-Diagnostica, Arnhem, The Netherlands), according to the manufacturer's instructions.

The IgA anti-CCP antibody assay for serum was developed based on the commercially available enzyme-immunoassay from Euro-Diagnostica (CCP2), replacing the detection antibody by an anti-human-IgA antibody. A positive IgA anti-CCP test was defined by the 99th percentile among healthy blood donors.

Patient sera were diluted 1:100 using the diluent provided with the kit. As secondary antibody, we used a horse radish peroxidase-conjugated polyclonal rabbit anti-human α-chain antibody (DakoCytomation, Glostrup, Denmark), which was diluted 1:2000 with the kit diluent. A 7-step serial dilution of a high-levelled IgA anti-CCP patient serum served as calibrator and the results were expressed as arbitrary units (AU/mL). Serum samples were analyzed in duplicate and the cut-off limit was set at 25 AU/mL based upon the 99th percentile of 80 blood donors (no differences were seen comparing female and male blood donors). The intra-assay coefficient of variation (CV) of the IgA anti-CCP assay was 13% based upon 6 sera analysed 13 times each, and the inter-assay CV (nine separate analyses) was 15%. (Study I-III).

In study IV, a more automatized method was set up to enable analysis of a larger number of samples. IgA anti-CCP antibodies were analysed on the Pha-Dia® 250 instrument by a fluoro-enzyme immune assay (EliA™, ThermoFisher AB, Uppsala, Sweden). A cut-off limit of ≥2 µg/mL was settled based upon >99th percentile among 101 blood donors.

Anti-MCV antibodies were analyzed with a commercial kit (Orgentec Diagnostika, Mainz, Germany). This test was modified for IgA-class antibody detection similar to the IgA anti-CCP analysis.

Salivary IgA anti-CCP antibodies were analyzed using a modification of an anti-CCP2 kit (CCPlus®, Euro-Diagnostica AB, Malmö, Sweden). After thawing, the saliva samples were centrifuged for 10 min at 15000 x g to remove non-soluble material. The remainder of each centrifuged sample was diluted 1:20 using the kit diluent. As secondary antibody we used polyclonal rabbit IgG anti-human α-chain antibodies conjugated with horse-radish peroxidase (HRP; DakoCytomation, Glostrup, Denmark) diluted 1:200 with the kit diluent. To evaluate the specificity of anti-CCP reactivity, plates coated with cyclic arginine peptide (CAP, Euro-Diagnostica) were used as control, following the same protocol.

All samples were thus analyzed on plates coated with cyclic arginine containing peptide (CAP) as a control, in addition to the CCP-plates, and the anti-CCP/anti-CAP ratio was calculated. An anti-CCP/anti-CAP ratio >1.5 was considered positive, which corresponded to the 99th percentile cut-off value among the 20 healthy subjects (one was positive with an anti-CCP/anti-CAP ratio of 1.62).

To further determine the specificity of the reaction, inhibition assays were performed in 9 saliva samples with CCP/CAP ratio > 1.5 and 8 samples with CCP/CAP ratio < 1.5. Centrifuged saliva samples (10 minutes at 15000 x g) were diluted with kit buffer with added soluble peptides (CCP and CAP respectively, Euro-Diagnostica) at final concentrations of 0-800 µg/mL. After 30 minutes of incubation at room temperature the samples were analyzed for IgA anti-CCP as described above.

The degree of inhibition was estimated by dividing the optical density (OD) value for the sample without soluble peptide with the OD value for the sample with 800 µg/mL. These values, indicating the degree of inhibition by CCP, thus reflect the specificity of the reaction. They were correlated to the anti-CCP/anti-CAP ratio, in order to see if the samples with a high CCP/CAP ratio, which we regard as positive, also show a high degree of inhibition indicating a specific reaction.

Summary of Results

Study I

From the TIRA-1 cohort, IgA anti-CCP was analysed in 320 patients with recent-onset RA, at inclusion and at 3 years follow-up. At inclusion, 29% of the patients tested positive for IgA anti-CCP compared to 64% for IgG anti-CCP. Out of the IgG anti-CCP positive patients, 45% tested positive also for IgA anti-CCP. All IgA anti-CCP positive patients were also positive for IgG anti-CCP. IgA anti-CCP positive patients had significantly higher levels of IgG anti-CCP, as seen in figure 5.

Figure 5. Median levels of IgG anti-CCP among RA patients negative for IgA anti-CCP compared to patients positive for IgA anti-CCP.

RA patients positive for IgA anti-CCP had significantly higher disease activity at the 3-year follow-up compared to IgA anti-CCP negative patients. Also after considering the IgG anti-CCP level, the disease activity tended to be higher in the IgA anti-CCP positive cases, although this difference reached statistical significance only concerning ESR (figure 6).
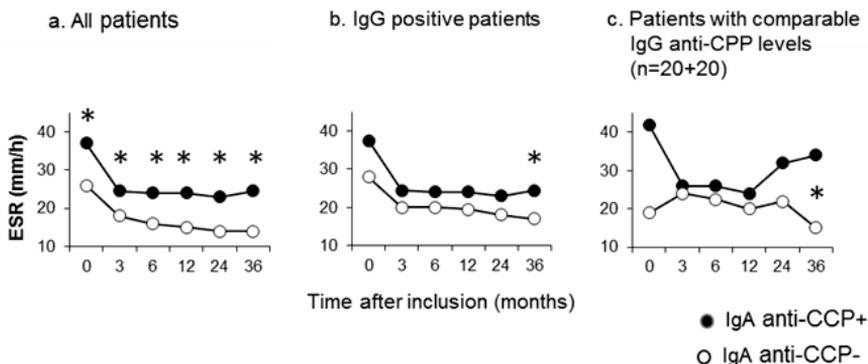
Figure 6. Course of the erythrocyte sedimentation rate (ESR) in IgA anti-CCP positive patients and IgA anti-CCP negative patients over 3 years. (a) All patients. (b) Only IgG anti-CCP positive patients. (c) 20 IgA positive patients and 20 IgA negative patients with pairwise comparable IgG levels. (*p<0.05).

Patients positive for IgA anti-CCP fulfilled a significantly larger number of ACR classification criteria than IgA anti-CCP negative patients. The proportion of RA patients prescribed DMARDs did not differ between IgA anti-CCP positive and IgG anti-CCP positive patients, whereas patients negative for both IgG and IgA anti-CCP were prescribed DMARDs to a significantly lower extent.

The proportion of IgA anti-CCP positive patients was larger among smokers than among never-smokers. Among current smokers, 43% were IgA anti-CCP positive (n=40), compared to 37% among previous smokers (n=38), and 25% among never smokers (n=150), (p=0.027). The corresponding difference regarding smoking and IgG anti-CCP status was not statistically significant.

Study II

In the TIRA-1 cohort, occurrence of IgG anti-MCV and IgG anti-CCP showed a 93% overlap, and serum levels were strongly correlated ($r = 0.87$). Patients testing positive for IgG anti-MCV had significantly higher disease activity over time, judged by ESR, CRP, DAS28 and the physicians assessment of disease activity at 6, 12, 24 and 36 months, than patients testing negative for IgG anti-MCV. No difference in HAQ was noticed.

Ten out of 78 patients negative for IgG anti-CCP tested positive for IgG anti-MCV, compared to 4 out of 72 IgG anti-MCV negative patients testing positive for IgG anti-CCP (table 4).

Table 4. IgG anti-MCV and IgG anti-CCP status among 215 early RA patients at the time of diagnosis.

	IgG anti-MCV+	IgG anti-MCV-	total
IgG anti-CCP+	133	4	137
IgG anti-CCP-	**10**	68	78
total	143	72	215

Among the IgG anti-CCP negative patients, IgG anti-MCV positive patients had a significantly higher disease activity than IgG anti-MCV negative patients, as exemplified by ESR in Figure 7.
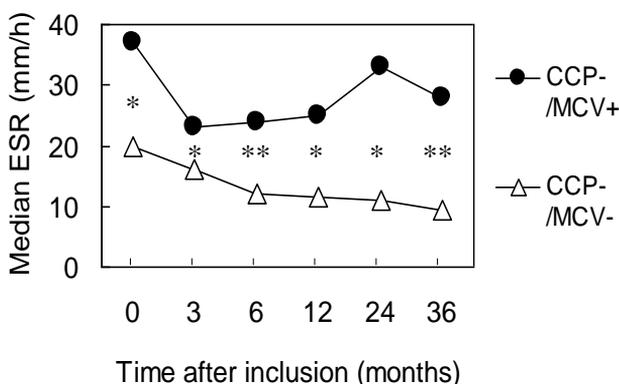

Figure 7. Median erythrocyte sedimentation rate (ESR) over three years after inclusion in IgG anti-CCP negative patients with positive (n=10) or negative (n=68) IgG anti-MCV tests. (* $p<0.05$, ** $p<0.01$).

A positive IgA anti-MCV test was found in 24% of RA patients, and they had significantly higher levels of IgG anti-MCV than IgA anti-MCV negative patients. IgA anti-MCV positive patients had significantly higher ESR and DAS 28 throughout the 3-year follow-up period, and these differences remained when comparing patients with similar IgG levels, although not reaching statistical significance.

Smoking habits and presence of SE was assessed in the three groups with different ACPA status: IgG-/IgA-, IgG+/IgA- and IgG+/IgA+. The lowest proportion of smokers was found in the IgG-/IgA- group, a slightly higher proportion in the IgG+/IgA- group, and the highest proportion of smokers in the IgG+/IgA+ group (figure 8). The pattern was similar for anti-MCV and anti-CCP, but statistical significance was found only for anti-MCV.
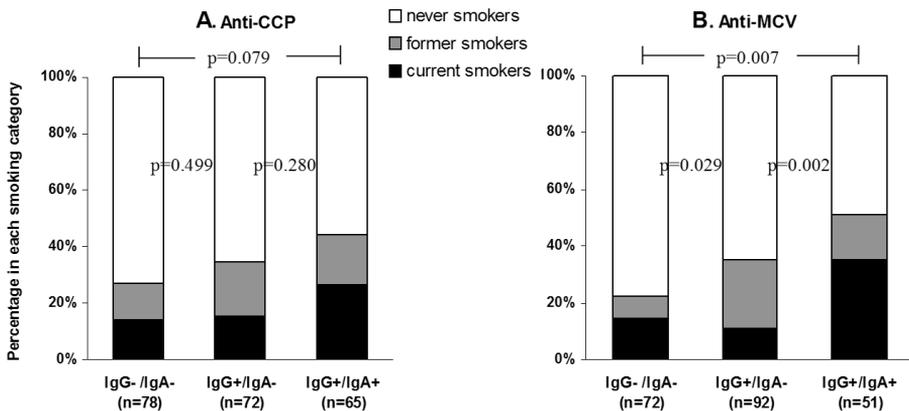

Figure 8. Smoking habits in groups with different ACPA status. A. Antibodies to cyclic citrullinated peptides (anti-CCP). B. Antibodies to modified citrullinated vimentin (anti-MCV). P-values refer to chi-square analysis.

The number of SE copies was significantly higher in IgG ACPA positive patients than in IgG ACPA negative patients, irrespective of IgA ACPA status, with a similar pattern for anti-CCP and anti-MCV.

In the Kronoberg Arthritis Incidence cohort, serum samples from all 69 patients included were analyzed. Sera from 2 patients that had earlier tested positive now tested negative in repeated tests, probably due to prolonged storing and repeated thawing, and these 2 patients were excluded. In the remaining 67 serum samples, all results were reproduced.

The sensitivity for early RA was 40% for both IgG anti-CCP and IgG anti-MCV, whereas the specificity for IgG anti-CCP was 98% compared to 92% for IgG anti-MCV (table 5).

Table 5. Performance profiles of IgG- and IgA ACPAs in relation to RA diagnosis in the very early arthritis cohort (n=67). PPV=positive predictive value.

ACPA type	sensitivity	specificity	PPV
IgG anti-CCP	40 %	98 %	86 %
IgA anti-CCP	13 %	100 %	100 %
IgG anti-MCV	40 %	92 %	60 %
IgA anti-MCV	13 %	98 %	50 %

IgA anti-CCP and IgA anti-MCV both had high specificity for early RA, 100% and 98% respectively, and both tests had a sensitivity of 13%.

Study III

When performing the initial ELISA for IgA anti-CCP in salivary samples, optical density (OD) values were equally high among healthy controls as among RA patients (mean OD 1.17 vs 1.16, p=0.96). In order to evaluate the specificity of the antibodies, an ELISA with cyclic arginine peptide (CAP) was performed, and the anti-CCP/anti-CAP ratio was calculated. A positive test was defined as an anti-CCP (OD)/anti-CAP (OD) ratio >1.5. Using this cut-off limit, salivary IgA anti-CCP was found in 14 of 63 (22%) RA patients and in 1 (5%) of the healthy controls.

In the inhibition assays performed to further evaluate the specificity of the reaction, salivary IgA anti-CCP reactivity was inhibited by pre-incubation with soluble CCP but not soluble CAP, and this inhibition was seen only in patients with a high anti-CCP/anti-CAP ratio (figure 9). The degree of inhibition correlated strongly with the anti-CCP/anti-CAP ratio.
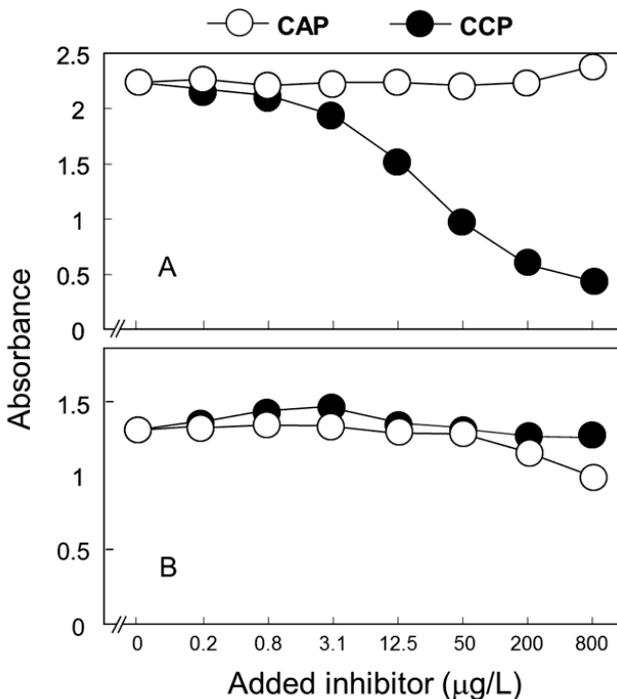

Figure 9. Examples of two inhibition experiments with pre-incubation with soluble cyclic citrullinated peptide (CCP) and cyclic arginine peptide (CAP) at a concentration of 0 - 800 µg/mL. The pre-incubation was followed by IgA anti-CCP ELISA, and the absorbance (OD value) is indicated on the Y-axis. A. In salivary samples with a high anti-CCP/anti-CAP ratio (>1.5) a dose-dependent inhibition is seen. B. In samples with a low anti-CCP/anti-CAP ratio (≤1.5) no inhibition was seen.
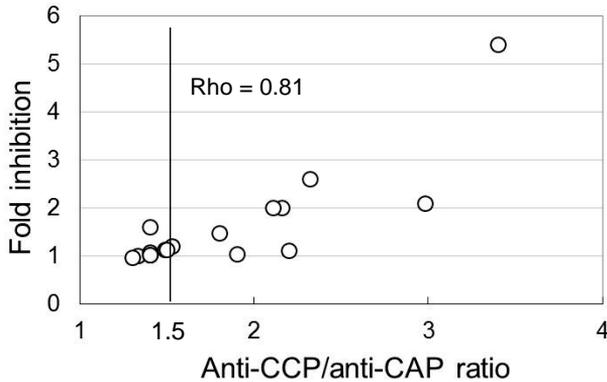
Figure 10. Illustration showing how pre-incubation with soluble cyclic citrullinated peptide (CCP) at a concentration of 800 μg/mL inhibits salivary IgA anti-CCP reactivity depending on the level of salivary antibodies. A positive IgA anti-CCP test is defined as a ratio >1.5 achieved by dividing the results of an anti-CCP enzyme immunoassay (EIA) with the results obtained with an EIA using the control peptide 'cyclic arginine peptide' (CAP) as antigen.

In RA patients positive for salivary IgA anti-CCP, the presence of joint erosions within 6 years of RA diagnosis was significantly lower (p=0.042), and at the time of diagnosis there was a trend towards lower median erythrocyte sedimentation rate (p=0.071) and C-reactive protein (p=0.085) (figure 10).
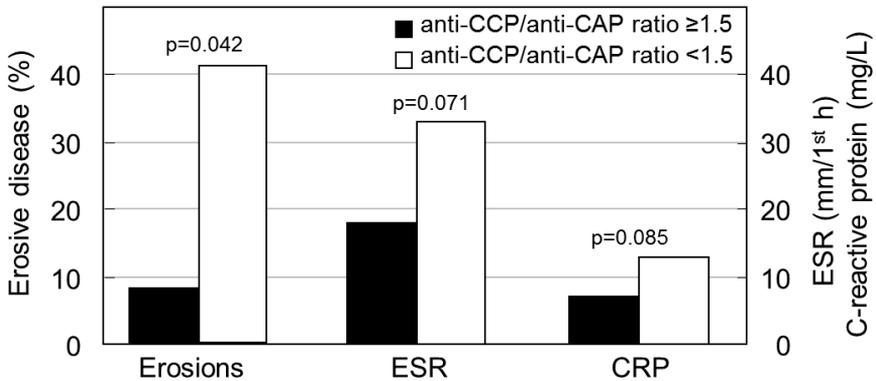

Figure 10. Disease activity reflected by median levels of erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) at diagnosis of rheumatoid arthritis, and presence of radiographic erosions in hands and feet within 6 years, in relation to occurrence of salivary IgA antibodies against cyclic citrullinated peptides (CCP). Antibodies against cyclic arginine peptide (CAP) served as control and a positive anti-CCP test is defined as an anti-CCP/anti-CAP ratio >1.5.

Study IV

Anti-CCP occurrence was similar in the two cohorts, TIRA-2 and EIRA-1. In the EIRA-1 material (table 6), IgA anti-CCP alone was detected in a minority of cases (n=54; 3%), whereas in TIRA-2 no patients positive for IgA anti-CCP alone were observed.

Table 6. The EIRA-1 cohort. Number of RA patients in the four groups with different anti-CCP status.

		IgA anti-CCP	
		positive	negative
IgG anti-CCP	positive	720	324
	negative	**54**	565

Antibody levels varied markedly between the groups. IgG anti-CCP levels were higher among IgA anti-CCP positive patients than among IgA anti-CCP negative patients. Similarly, IgA anti-CCP levels were higher among IgG anti-CCP positive patients than among IgG anti-CCP negative patients (figure 11).
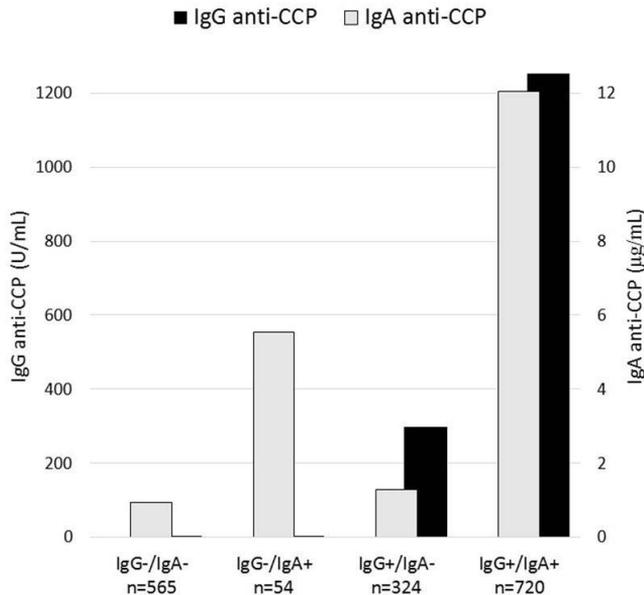

Figure 11. Antibody levels (mean values) in the four groups with different antibody status; IgG-/IgA-, IgG-/IgA+, IgG+/IgA- and IgG+/IgA+.

Smoking was significantly overrepresented among IgA anti-CCP positive patients. Presence of shared epitope (SE) genes was overrepresented among IgG anti-CCP positive patients with or without IgA class anti-CCP, but not among RA patients with IgA anti-CCP antibodies alone (figures 12 and 13).
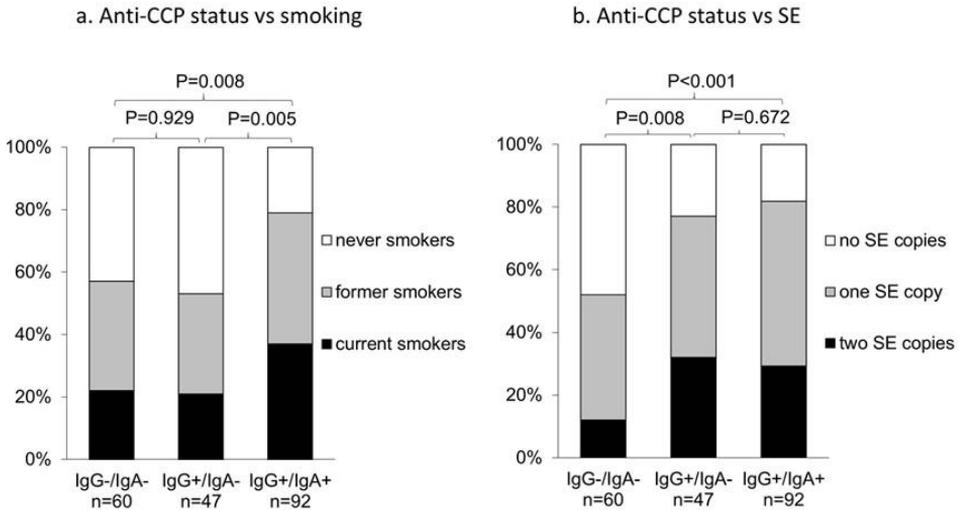

Figure 12. Distribution of (a) smoking habits and (b) SE status in relation to anti-CCP status in the TIRA-2 cohort (n=199). P-values are from Chi square testing, taking all three variants of smoking and SE status into account.
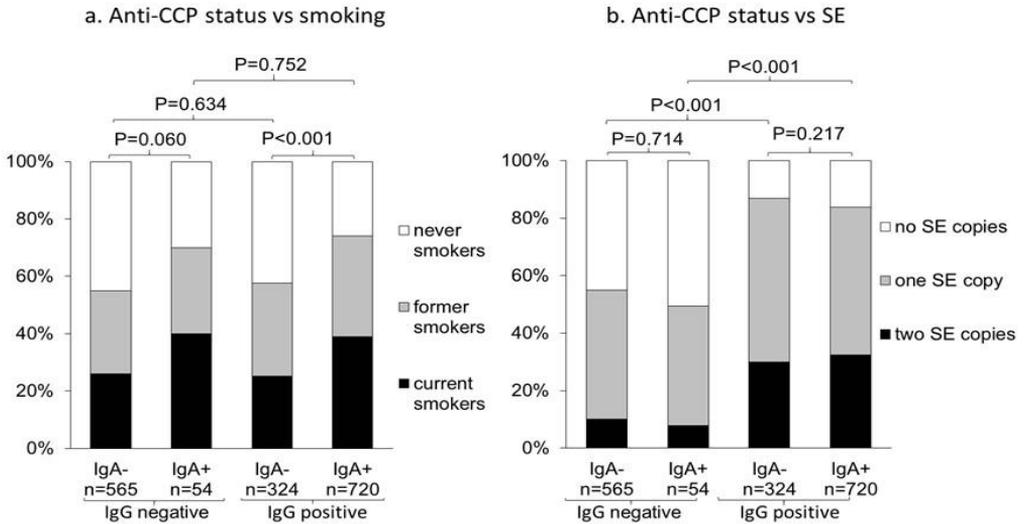

Figure 13. Distribution of (a) smoking habits and (b) SE status in relation to anti-CCP status in the EIRA-1 cohort (n=1663). P-values are from Chi square testing, taking all three variants of smoking and SE status into account.

To assess the interaction between smoking and shared epitope we used the EIRA-1 cohort, and controls matched for age, sex and residential area.

Table 7 shows that among never-smokers, the odds ratio for developing IgG+/IgA+ RA for SE positive subjects compared to SE negative subjects was 4.0 (95% CI 2.7–6.1). Among SE negative subjects, the odds ratio for ever-smokers compared to never-smokers was 1.7 (95% CI 1.1–2.7). When combining the two risk factors, smoking and SE, it was shown that ever-smokers with SE had an odds ratio of 9.7 (95% CI 6.6–14.4) compared to never-smokers without SE. Thus, an interaction between smoking and SE genes was observed in association with IgG+/IgA+ RA, which was also reflected by the attributable proportion (AP) due to interaction (0.5, 95% CI 0.4–0.6). No interaction was observed between smoking and SE in association with the IgG-/IgA+ or IgG+/IgA- subgroup of disease.

Table 7. Odds Ratios for disease risk in subjects with different anti-CCP antibody status (IgG-/IgA-, IgG-/IgA+, IgG+/IgA- or IgG+/IgA+), in relation to smoking habits and shared epitope (SE) in the EIRA-1 cohort.

	No SE alleles		1 or 2 SE alleles		AP due to interaction
	Cases / controls	OR (95 % CI)	Cases / controls	OR (95 % CI)	
All					
Never smoker	178/228	Ref	416/244	**2.2 (1.7–2.8)**	**AP: 0.3 (0.2–0.5)**
Ever smoker	267/316	1.1 (0.9–1.5)	802/312	**3.5 (2.7–4.4)**	
IgG–/IgA– RA					
Never smoker	114/228	Ref	138/244	1.1 (0.8–1.5)	AP: 0.1 (-0.3–0.5)
Ever smoker	143/316	0.9 (0.7–1.3)	170/312	1.1 (0.8–1.5)	
IgG–/IgA+ RA					
Never smoker	8/228	Ref	9/244	1.0 (0.4–2.7)	AP: 0.1 (-0.8–0.9)
Ever smoker	19/316	1.7 (0.7–3.9)	18/312	1.8 (0.7–4.2)	
IgG+/IgA– RA					
Never smoker	21/228	Ref	116/244	**5.4 (3.3–9.0)**	AP: 0.2 (-0.1–0.5)
Ever smoker	22/316	0.8 (0.4–1.6)	165/312	**6.6 (4.0–10.8)**	
IgG+/IgA+ RA					
Never smoker	35/228	Ref	153/244	**4.0 (2.7–6.1)**	**AP: 0.5 (0.4–0.6)**
Ever smoker	83/316	**1.7 (1.1–2.7)**	449/312	**9.7 (6.6–14.4)**	

All estimates adjusted for age group, geographical area and sex. AP = attributable proportion

Discussion

Prognostic and diagnostic markers

Since RA is a disease that can take on various forms, it is an important task to find early markers for disease progression and response to therapy.

The discovery of antibodies against citrullinated peptides as specific markers for RA [54], and the subsequent development of anti-CCP tests for routine use [55], has had a major impact on the care of patients with early RA. The IgG anti-CCP positive RA patients form a relatively homogeneous subgroup with a more aggressive disease course and outcome compared to the IgG anti-CCP-negative patients [41, 57, 142]. In parallel with the introduction of IgG anti-CCP testing in clinical routine, the new biologic drugs were introduced. This has had a dramatic effect on everyday life for many RA patients [143]. The economic cost for biologic drugs is 30-40 times higher than for traditional DMARDs [144], and although inpatient care and indirect costs to society have decreased, the total cost of RA in Sweden, including inpatient care, outpatient care, drugs, sick leave and disability pension, has increased by approximately one-third between 1990 and 2010, mainly due to increased use of biologic drugs [145].

Even though IgG anti-CCP is a valuable diagnostic tool due to its high specificity for RA, there is still a need for additional markers. The 2010 classification criteria have made it possible to recognize anti-CCP/RF-positive RA earlier. However, antibody-negative patients with RA according to ACR-87, may in fact be missed using the 2010 criteria. For instance, an antibody-negative patient with morning stiffness, longstanding symmetric polyarthritis in >3 joint areas including up to 10 joint-swellings, hand-engagement and elevated CRP and/or ESR levels would be classified as RA by ACR-87, but not by the 2010 criteria. These patients may very well develop erosive disease and they would benefit from an earlier diagnosis. This underlines the need to search for further novel diagnostic and prognostic tools in early developing RA.

As ACPA isotypes other than IgG had not previously been investigated to any great extent, and in the light of IgA RF being associated with more extra-articular manifestations [45] and a more therapy-resistant disease [146], we developed a method to analyse IgA anti-CCP and subsequently also IgA anti-

MCV, hoping to find additional useful diagnostic and prognostic markers for RA.

Presence of IgA ACPA in serum

Since IgA anti-CCP and IgA anti-MCV performed similarly in these studies, they are here collectively referred to as IgA ACPA when not explicitly specified.

In the TIRA-1 cohort, IgA anti-CCP was detected in 47% of IgG anti-CCP positive patients, compared to 66% in TIRA-2 and 69% in EIRA-1. These findings are concordant with those of Verpoort (62%) [68] and Kokkonen (60%) [97]. Dos Anjos and colleagues later found as much as 83% of IgG anti-CCP positive patients to be positive also for IgA anti-CCP, using another variant of test; the CCP3 test [147]. This discrepancy in proportion of IgA anti-CCP positive patients might be due to different cut-off levels. We have consequently used a strict cut-off level, defined by the 99th percentile of a healthy blood donor population.

IgA anti-MCV antibodies largely overlapped with IgA anti-CCP antibodies, and were found among 36% of IgG anti-MCV positive patients. In a smaller study with 82 RA patients by Sghiri et al. IgA anti-MCV was found in 51% of all patients [148], which is a higher proportion than in our study.

Anzilotti et al. have analysed antibodies to a citrullinated viral peptide in sera from RA patients, and found IgA ACPA mainly in a subpopulation of IgG ACPA positive patients, but also a small proportion of IgG negative/IgA positive patients [149], while we found IgA ACPA only among IgG ACPA positive RA patients in TIRA-1 and in TIRA-2. However, in study IV, using the large EIRA cohort, we found a small proportion (3.2%) of IgG negative/IgA positive patients, despite using a strict cut-off level in the IgA ACPA analysis.

Prognostic markers

IgA anti-CCP, as well as IgA anti-MCV, was found to be associated with a slightly less favourable disease course at the 3-years follow-up.

IgA ACPA positive patients had higher levels of IgG ACPA than IgA ACPA negative patients, and one could argue that the high IgG ACPA levels may explain the higher disease activity seen in IgA ACPA positive patients. An association between IgG anti-CCP level and disease activity has been found by some [150], whereas others have not seen such an association [151]. Here, we selected patients with comparable IgG ACPA levels, to avoid this possible confounder. A small difference could still be seen, reaching statistical significance for ESR at 3-years follow-up for IgA anti-CCP but not for IgA anti-

MCV, which indicates that IgA anti-CCP might add extra prognostic value to IgG anti-CCP. In the study by Sghiri et al. [148] no correlation between disease course and IgA anti-MCV was seen, which is in line with our findings regarding IgA anti-MCV.

Another, indirect, way to compare disease course, is to look at the prescription of DMARDs. It has been shown that prescription of DMARDs differ significantly between patients positive for IgG anti-CCP and patients negative for IgG anti-CCP, with the prescribing physicians being unaware of the anti-CCP results [41]. We compared the prescription of DMARDs among patients positive for both IgG and IgA anti-CCP to the prescription among patients positive for IgG anti-CCP alone. As no difference could be seen between the groups this suggests that any difference in disease activity between IgA anti-CCP positive and IgA anti-CCP negative patients are of minor clinical importance.

Thus, although statistically significant, the differences in disease activity between IgA positive and IgA negative RA patients are to our judgement of minor clinical significance and we do not, at present, see a place for IgA anti-CCP testing in everyday clinical practice. However, the possible additional prognostic value of IgA anti-CCP need to be further studied in a larger cohort. Further investigation of the relation between IgA anti-CCP, disease activity and smoking habits would also be of great interest.

The IgG anti-MCV test identifies a small group of IgG anti-CCP negative RA patients with a disease activity as high as the IgG anti-CCP positive patients. This indicates that IgG anti-MCV (and perhaps other ACPA tests) might be useful as a prognostic marker among IgG anti-CCP negative patients. However, as the slightly lower diagnostic specificity of IgG anti-MCV makes it less useful as a diagnostic screening tool, e. g. in a primary care setting, it might rather serve as an additional prognostic tool, but not as a substitute for IgG anti-CCP.

Diagnostic markers

To evaluate the diagnostic utility of IgA anti-CCP and IgG/IgA anti-MCV compared to IgG anti-CCP, we used the Kronoberg Arthritis Incidence cohort, including patients with very early arthritis evolving into a variety of rheumatologic diagnoses. We found IgG anti-MCV to have equal sensitivity but lower specificity compared to IgG anti-CCP. A possible objection could be the limited size (67 patients) of the cohort. However, our results are in line with the majority of earlier studies on IgG anti-MCV reporting a higher sensitivity and a slightly lower specificity [59, 63, 152] or a higher sensitivity and equal specificity [153] for early RA, compared to IgG anti-CCP.

Different ACPA specificities have different genetic association and identify slightly different subgroups of RA patients [66, 154-156], but no specificity has yet proved superior to IgG anti-CCP when it comes to clinical usefulness as a prognostic or diagnostic marker.

Salivary IgA anti-CCP

Methodological issues

Sampling
Detection of antibodies from salivary samples involves a number of methodological difficulties which have to be considered. The sampling method is not as standardized as for serum. Whole saliva or selective sampling from e.g. the parotid glands can both be used. Unstimulated or stimulated samples can be used. If using unstimulated whole saliva, there are the alternatives of passive drooling or using inert swabs.

Unstimulated saliva, using the method of 'passive drooling' has been shown to yield three times higher IgA levels than stimulated saliva [157], reflecting a dilution effect from the increased secretion rate which is difficult to control. Further, leakage of serum proteins into the oral cavity can increase from chewing [114], and as chewing on paraffin is the common way of collecting stimulated salivary samples, we have preferred to use unstimulated saliva collected by passive drooling. In study III, the presence of salivary IgA did not differ significantly between patients with and patients without IgA anti-CCP in serum, supporting the notion that salivary IgA is essentially derived from salivary gland plasma cells, and that possible leakage from the circulation is of minor importance.

Quick freezing of salivary samples is important to maintain the antibody reactivity, as prolonged storage of 4-8 hours in refrigerator before freezing leads to 30% lower IgA levels [158]. Storage for six months at -80°C has however been shown not to affect the antibody levels [159]. In our study the test tubes for saliva sampling were placed on ice, and the samples were frozen to -80°C within 1-2 hours. No overnight storage in refrigerator or repeated thawing was allowed as this, in a small series of experiments, was found to markedly lower the antibody reactivity.

Saliva is a viscous fluid, which increases the risk of analysing an unrepresentative portion of the sample if not properly homogenised and centrifuged. Centrifugation may, on the other hand, also affect the sample, and a loss of 12% of total antibody content has been demonstrated [159].

Despite these various disadvantages, unstimulated whole saliva is commonly used, and regarded as a 'representative' external fluid [114]. Some of these disadvantages can, as discussed, be overcome by careful handling while others cannot, and the results will therefore have to be interpreted with caution.

Unspecific reactivity

As the initial ELISAs for IgA anti-CCP in salivary samples yielded equally high optical density (OD) values among healthy controls as among RA patients, we suspected an unspecific (non-citrulline dependent) reaction. To pursue this idea, we acquired microtitre plates coated with control antigen, i.e. cyclic arginine peptides, from Euro-Diagnostica. All samples were analysed for both IgA anti-CCP and IgA CAP reaction. We then calculated the anti-CCP/anti-CAP ratio as a way to identify the samples containing specific IgA anti-CCP antibodies.

This kind of unspecific binding is not a problem when analysing serum samples from RA patients. Verpoort and colleagues found that only 2% of IgA anti-CCP positive sera also reacted with the control peptide [68]. In patients with autoimmune hepatitis and systemic lupus erythematosus however, serum samples have been shown to react with unspecific binding to the arginine-containing control peptide [160].

We also performed an inhibition assay with soluble antigen in order to further ensure the specificity of our salivary IgA anti-CCP assay. The results, showing a dose-dependent inhibition by soluble peptide and a correlation between the degree of inhibition and the anti-CCP/anti-CAP ratio, support that we have indeed demonstrated the occurrence of specific IgA anti-CCP antibodies in saliva.

Salivary IgA anti-CCP occurred in 22% of the 63 RA patients included. This number needs to be interpreted with caution as the majority (83%) of patients were selected on the basis of a positive serum IgG anti-CCP test. As none of the IgG anti-CCP negative patients tested positive for salivary IgA anti-CCP, this selection clearly constitutes a bias.

Salivary IgA anti-CCP and disease course

Contrasting to circulating IgG and IgA anti-CCP, we found that RA patients positive for IgA anti-CCP in salivary samples tended to have a less severe outcome of RA. This suggests that a mucosal antibody response to citrullinated peptides might be involved in the pathogenic process in RA. Hypothetically, this includes an anti-inflammatory and protective role of salivary IgA-

class autoantibodies against citrullinated antigens presented at mucosal surfaces.

The limited number of patients included in study III constitutes a limitation, along with the methodological difficulties associated with analyses of saliva. However, the clear-cut findings of the inhibition assays confirm CCP specificity, and hence the novel finding of ACPA production in this mucosal compartment.

Larger studies should be performed to confirm and expand these results. A prospective study with patients at risk of developing RA would be an ideal setting for further evaluation of the significance of salivary ACPAs, as the possible presence of salivary IgA ACPA before development of clinical symptoms would be very interesting to investigate. However, such a study would require significant effort, and a more feasible step forward, now that we have demonstrated the possibility to analyse IgA anti-CCP in salivary samples in this pilot study, might be evaluation of a larger number of patients with established RA. Evaluation of smoking habits in relation to salivary IgA ACPA would also be of great interest.

In order to further investigate these matters, 200 RA patients and 100 controls have just been recruited to a new cohort, SARA (Secretory IgA ACPA in RA). Salivary samples, blood samples, information on disease activity parameters as well as data on smoking habits and oral health have been assembled.

Smoking and IgA anti-CCP in serum

Besides the wish to find early prognostic markers, a second reason to investigate IgA anti-CCP is curiosity about how RA starts and develops. Can information about these processes help us find means of interfering, and thereby moderating or even stopping the disease process? And is IgA anti-CCP part of this process?

In study I and II, we noticed an association between smoking and IgA anti-CCP, which was not seen between smoking and IgG anti-CCP. The numbers were however not large enough to draw any firm conclusions, although the findings are in line with data from Verpoort [82]. Further, Kokkonen and colleagues showed that smoking pre-RA patients developed circulating IgA-CCP earlier than their non-smoking counterparts [97].

In order to confirm our findings, in study IV we chose to evaluate the association between IgA anti-CCP and smoking in two more cohorts: TIRA-2 and the large EIRA-1 cohort with matched control subjects. Cigarette smoking was in both cohorts found to correlate strongly with the presence of IgA anti-

CCP, whereas there was no correlation between smoking and IgG anti-CCP alone.

In most earlier studies, only IgG anti-CCP has been evaluated, and as the large majority of IgA anti-CCP positive patients are also IgG anti-CCP positive, this has led to an assumed association between smoking and IgG anti-CCP positive RA. Klareskog and colleagues found a strong interaction between smoking and IgG anti-CCP in 2006 [13]. This interaction has now, in paper IV of this thesis, been demonstrated to be valid only among subjects positive for IgA anti-CCP in addition to IgG anti-CCP. This implicates a pathogenic connection between smoking, IgA anti-CCP and the development of RA.

A considerable strength of this study is that we have confirmed our findings in two separate cohorts.

These findings are further supported by a recent study by Willis et al. [113], where IgA anti-CCP was evaluated in sputum and serum from both patients at risk of developing RA and patients with established RA. Interestingly, patients at risk had a high ratio of sputum IgA anti-CCP to serum IgA anti-CCP, whereas this ratio was low in patients with established RA. Whether this pulmonary IgA anti-CCP response in patients at risk for RA reflects a protective or a pro-inflammatory mechanism remains to be clarified.

Conclusions

- IgA anti-CCP is present in a subgroup of RA patients positive for IgG anti-CCP with signs of a slightly more severe disease, but does at present not add to the diagnostic and prognostic properties of IgG anti-CCP.

- IgG anti-CCP has a higher diagnostic specificity and equal sensitivity for RA compared to IgG anti-MCV in very early arthritis. In early RA, IgG anti-MCV identifies a subgroup of IgG anti-CCP negative patients with unfavorable disease course.

- IgA anti-CCP occur in saliva of patients with established RA and is associated with a less erosive joint disease at follow-up.

- IgA anti-CCP is strongly associated with smoking. The well-known interaction between smoking and SE concerning the risk of developing RA, that was earlier assumed to apply for all IgG positive subjects, has here been demonstrated to be valid only in the subgroup positive for both IgA and IgG anti-CCP.

Clinical Implications

- This work supports the continued use of IgG anti-CCP as the preferred diagnostic and prognostic marker for RA.

- Among IgG anti-CCP negative patients, 13% had a positive IgG anti-MCV test, and their disease course was as severe as that of IgG anti-CCP positive patients. This accentuates the need for careful clinical diagnosis and follow-up, irrespective of antibody status.

- The clear association between smoking and IgA anti-CCP that was seen supports a pathogenic role for smoking, and further emphasizes the importance to encourage all people to refrain from smoking.

Future Directions

- Further evaluation of the prognostic value of IgA anti-CCP in a larger population, preferably with a subgroup of IgA+/IgG- patients, and with access to radiographic data.

- Further development of the method for analyzing IgA anti-CCP in saliva, and evaluation of salivary IgA anti-CCP in a larger number of RA patients, with association to genetic variants, smoking habits, oral health and disease course.

- Examination of the similarities/differences between the mucosal immune system and the systemic immune system, by examining fine ACPA specificities in salivary and serum IgA anti-CCP.

- In vitro experiments to evaluate the pro- and anti-inflammatory effects of IgA compared to IgG anti-CCP.

- Large, prospective studies involving pre-RA patients, evaluating the temporal aspects of exposure to smoking, mucosal antibodies at different sites, circulating antibodies, onset of symptoms and disease course.

Acknowledgements

Sammanfattning på svenska

Reumatoid artrit (RA), ledgångsreumatism, är en autoimmun sjukdom som drabbar 0.5–1% av befolkningen. Sjukdomen ger ledinflammationer med svullna, ömma leder och efterhand skador på brosk och ben. Även andra organ som lungor, njurar och blodkärl kan drabbas, och patienter med RA har en ökad risk att dö av hjärt-kärl-sjukdomar.

Vissa individer har en mild variant, och klarar sig bra med en förhållandevis mild behandling, medan andra har en mycket aggressiv variant med snabb nedbrytning av brosk och ben och har behov av en intensivare behandling för att inte utveckla bestående skador.

De senaste åren har vi fått kunskap om vikten av tidigt insatt behandling, och vi har även fått tillgång till ett antal nya läkemedel med god effekt hos många patienter, men som även medför höga kostnader för samhället. Mot bakgrund av detta är det viktigt att vi så tidigt som möjligt kan identifiera patienter med RA och helst även förutsäga hur sjukdomen kommer att utveckla sig hos en enskild individ och vilka mediciner som kommer att fungera bäst.

Orsakerna till RA är ofullständigt kända, men man vet att både gener och miljöfaktorer spelar in. Rökning ökar risken för att utveckla RA, men man känner inte till mekanismerna bakom detta.

Flera år innan sjukdomen märks i lederna, aktiveras immunsystemet och angriper vävnader i den egna kroppen. Det bildas ofta antikroppar mot citrullinerade proteiner (ACPA) som är specifika för RA, och som är kopplade till en aggressivare sjukdomsbild. Den typ av ACPA som tidigare undersökts är främst IgG anti-CCP.

Syftet med denna avhandling har varit att undersöka ett antal antikroppar (IgA anti-CCP, IgA anti-MCV samt IgG anti-MCV), dels för att se hur användbara de är för att ställa diagnos och förutsäga sjukdomsutveckling, och dels för att få en ökad förståelse för de immunologiska processer som föregår utvecklingen av RA. På längre sikt skulle detta kunna leda till metoder för att bromsa, och i bästa fall förhindra, utvecklingen av RA.

Analys av serumprover från patienter med tidig RA visade att bland de patienter som har IgG anti-CCP, så har ungefär hälften även IgA anti-CCP. Dessa patienter har en något högre sjukdomsaktivitet när man följer dem under tre år, än de som saknar IgA anti-CCP. Fynden behöver bekräftas i större studier innan man eventuellt kan rekommendera att IgA anti-CCP används i klinisk vardag. Man såg även att rökare oftare har IgA anti-CCP i serum än icke-rökare.

Vid jämförelse mellan IgG anti-CCP och IgG anti-MCV, visade det sig att IgG anti-MCV-testet har förmågan att upptäcka ett antal patienter med aggressiv RA som IgG anti-CCP-testet (som vanligen används) missar. Nackdelen med IgG anti-MCV är att de även finns hos ett antal individer som inte har eller kommer att få RA, vilket minskar användbarheten.

I det tredje delarbetet, av de fyra som ingår i denna avhandling, analyserades IgA anti-CCP i saliv från patienter med RA. De patienter som hade IgA anti-CCP i saliven hade en mildare sjukdomsvariant än övriga, med mindre skelettskador på röntgenundersökningar. Detta kan tala för att IgA anti-CCP i saliv har en dämpande effekt på immunsystemet. Möjligen är det så att dessa antikroppar kan binda och oskadliggöra citrullinerade proteiner som finns på slemhinnor, så att en reumatisk inflammation dämpas.

Slutligen har kopplingen mellan IgA anti-CCP och rökning undersökts hos patienter med tidig RA. Den tidigare kända kopplingen mellan rökning och RA visade sig gälla endast för de patienter som har IgA anti-CCP i kombination med IgG anti-CCP, och detta talar för att rökning och IgA anti-CCP är inblandade i de processer som leder fram till RA, och stöder teorin att slemhinnor, sannolikt i lungorna, är inblandade i utvecklingen av ledgångsreumatism.

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