Exploring the landscape of rheumatoid arthritis: piecing together risk factors and autoantibodies

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General introduction

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by joint inflammation.[1] The prevalence of RA differs per region, with a prevalence of 0.5-1% in European population. RA is up to three times more frequent in woman than in men, and the incidence rises with age.[2]

Presenting symptoms of RA are joint pain, stiffness and joint swelling typically of the small joints of the hand and feet. The disease has generally a gradual onset. As there is no single sign or symptom specific for RA, the diagnosis is made on a combination of symptoms and clinical findings. For research purposes classification criteria have been made to create homogeneous patient groups: the 1987 ACR criteria and the 2010 ACR/European EULAR criteria, with the latter being more sensitive and less specific.[3]

If patients are not treated for RA, the chronic inflammation leads to joint destruction causing disability. Besides joint involvement, extra-articular manifestations may occur in RA as well, such as secondary Sjogren syndrome, interstitial lung disease, vasculitis, pericarditis and pleuritis. In recent years considerable advances have been made in the treatment of RA emphasizing early treatment with disease-modifying anti-rheumatic drugs (DMARDS), therapy adjustments based on disease activity measures, and the arrival of targeted therapies.[4] This has led to major improvement in disease outcome: (1) more patients achieve clinical remission and drug-free clinical remission and (2) a decrease in joint damage and thereby less disability.

IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS

The pathophysiology of RA involves a complex interplay of genetic predisposition, environmental factors, immune system dysregulation, and tissue remodeling. Antigen-presenting cells (APCs) are critical in the initiation and perpetuation of the autoimmune response in RA. Dendritic cells, macrophages, and B cells serve as APCs that can capture, process, and present antigens to T cells, thereby triggering an immune response. It is hypothesized that environmental risk factors, such as smoking and specific pathogens, may initiate the formation of neoantigens or induce molecular mimicry. Molecular mimicry describes the cross-reactivity between foreign pathogens and self-antigens. These antigens will be presented APCs, which capture, process, and present the antigens on their surface in the context of major histocompatibility complex (MHC) molecules, specifically MHC class II. This presentation can activate CD4+ T cells, particularly in individuals with a genetic predisposition such as the HLA-DRB1 shared epitope, a sequence motif found in certain alleles of the HLA-DRB1 genes. Upon activation T cells can differentiate in T-helper (Th) subsets, including Th1, Th17, and T follicular helper cells, which al contribute to the disease process. Th17 cells are particularly important in

RA pathogenesis due to their production of IL-17, a cytokine that promotes inflammation by recruiting neutrophils and other immune cells to the site of inflammation.[5] IL-17 also stimulates fibroblast-like synoviocytes (FLS) and osteoclasts, leading to joint destruction.[6] Th1 cells produce pro-inflammatory cytokines such as interferon-gamma and tumor necrosis factor alpha (TNFalpha), which in turn further activates the inflammatory response and activates macrophages. T follicular helper cells also play a crucial role in RA by facilitating the differentiation of B cells and promoting the production of high-affinity antibodies.[7] The most well-known autoantibodies in RA include rheumatoid factor (RF) and a group of autoantibodies called anti-modified protein antibodies.[8] Moreover, these B cells also act as APCs, presenting antigens to CD4+ T cells and thus contributing to their activation. Moreover, B cells secrete cytokines like IL-6, further promoting inflammation and the autoimmune response.

The immune processes previously described lead to persistent inflammation of the synovium, leading the formation of an invasive pannus, a defining feature of RA.[9] Fibroblast-like synoviocytes (FLS) are central to this process, as they not only contribute to the inflammatory milieu but also drive joint destruction by secreting receptor activator of nuclear factor kappa-B ligand (RANKL).[10] RANKL promotes the induction of osteoclasts, the specialized cells responsible for bone resorption. In RA, osteoclast activity is enhanced, leading to the characteristic bone erosion and osteopenia seen in the disease.[11] Moreover, the persistent inflammation in the synovium results in cartilage degradation, driven by the production of matrix metalloproteinases (MMPs) and other catabolic enzymes. MMPs degrade the extracellular matrix of the cartilage, particularly type II collagen, further contributing to joint damage.[12] The interplay of these immune and tissue remodeling processes underlies the progressive joint destruction and functional impairment characteristic of RA.

In conclusion, the pathophysiology of rheumatoid arthritis involves a multifaceted interaction between immune cells, autoantibodies, and tissue remodeling processes. T cells and B cells drive the inflammatory response, while fibroblasts contribute to the formation of invasive pannus and cartilage damage. Genetic predisposition and environmental triggers further modulate the risk and severity of the disease.

In the following paragraphs, I will further focus on the role of autoantibodies and risk factors in the pathogenesis of RA. I will start with an introduction on autoantibodies in rheumatoid arthritis, followed by genetic and environmental risk factors and their association with various autoantibodies, and conclude with a pathophysiological model of rheumatoid arthritis.

AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

Different autoantibodies are known to occur in RA patients. These autoantibodies are useful as diagnostic markers and can give insight into the pathophysiology of RA. Rheumatoid factor (RF) is the first autoantibody which was found to be associated with RA. It is a marker of future RA development, the specificity of RF is however modest (85%).[13]

More recently, autoantibodies with a higher specificity for RA have been found, anti-modified protein antibodies (AMPA's). AMPA's are antibodies that recognize proteins that have undergone post-translational modification and currently three RA specific AMPA's are known: anti-citrullinated protein antibodies (ACPA), anti-carbamylated protein antibodies (anti-CarP) and anti-acetylated protein antibodies (AAPA).[8]

Anti-citrullinated protein antibodies (ACPA)

ACPA are autoantibodies that bind proteins and peptides containing citrulline. Citrulline is a post translationally modified amino acid, formed in the presence of high calcium concentrations by deimination of arginine by a group of enzymes called peptidyl arginine deiminases (PAD);[14] (figure 1A). Citrullination is a physiological process occurring intracellularly, often during apoptosis, and plays a role in the degradation of intracellular proteins. Citrullination reduces the net charge of proteins by removing a positive charge from each modified arginine residue, increasing hydrophobicity, inducing protein unfolding, and altering molecular interactions.[15] These structural changes often result in a loss, and occasionally a gain, of protein function.[15-17] Due to the significant and potentially irreversible impact on protein function, citrullination must be tightly regulated to prevent excessive modification and maintain proper cellular processes. In RA, citrullination is more prevalent and over 100 citrullinated proteins are identified in synovial fluid of patients with RA, both intra- and extracellular.[18, 19] PAD enzymes leading to citrullination, can be "hyperactivated" in neutrophils by signals leading to calcium influx such as bacterial toxins, membrane attack complexes and perforin.[20] Extra-articularly, it is hypothesized that bacterial toxins from sites such as the gingiva and gastrointestinal tract may contribute to the hyperactivation of PADs.[21] In contrast, intra-articular hypercitrullination is primarily driven by immune-mediated membranolytic pathways.[22] The extensive neutrophil cell death observed in RA results in the release of PAD enzymes, leading to enhanced extracellular PAD activity in affected tissues.

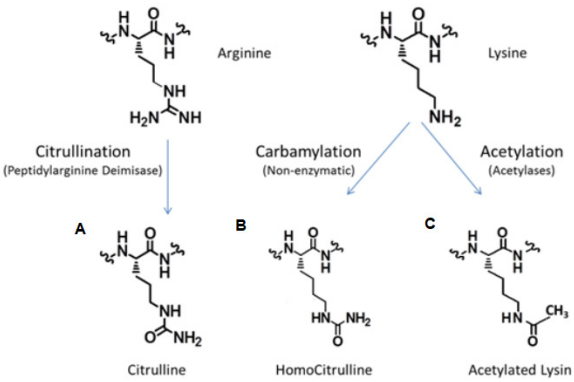

Figure 1. The formation of citrullinated proteins (A), homocitrullinated proteins (B) and Acetylated proteins (C)

Notably, ACPA-IgG is highly glycosylated, with complex-type variable domain glycans (VDG) found on more than 90% of the molecules, compared to conventional IgG, which exhibits only 12–14% VDG.[23, 24] Moreover, ACPA-IgG from synovial fluid of RA patients possess VDG glycosylation >100%, indicating that multiple glycans are attached to the variable domain region of an ACPA-IgG molecule.[23] Glycans are mono- or polysaccharides that are attached to proteins co- or post-translationally in the endoplasmatic reticulum. N-linked glycosylation is the most common type of glycosylation and these glycans are attached to a nitrogen atom of an asparagine residue (N). Most frequently N-glycans are built from monosaccharides, cyclic structures consisting of five carbons and one oxygen atom within the ring structure. N-linked glycosylation sites are selectively and abundantly introduced into the hypervariable regions of ACPA-IgG through somatic hypermutation (SHM), whereas these sites are absent in ACPA-IgM and germline-encoded sequences.[25, 26] Structural analysis has shown that ACPA-IgG variable domain glycans (VDGs) contain highly sialylated, galactosylated and bisected glycans.[23] Notably, recent studies indicate that the abundant presence of VDGs on ACPA-IgG in healthy First Nation Canadian individuals is a strong predictor of RA progression.[27] Since VDGs are selectively introduced, present on the majority of ACPA IgG molecules, and predictive of disease development, it is plausible that their expression confers a selective survival advantage to ACPA-expressing B cells, allowing them to persist long-term. In addition, glycosylation of the hypervariable regions may offer an additional mechanism for autoreactive B cells to modulate immune tolerance. This glycosylation may contribute to the pathogenicity of ACPA in RA.

ACPA is an important biomarker in RA since it is both a predictor for developing RA and is associated with more severe disease outcomes. In

healthy populations 2% is ACPA positive, in undifferentiated arthritis (UA) 20% are ACPA positive and in RA patients 60% are ACPA positive.[28] With regard to more severe disease outcome ACPA positive RA patients more often develop joint destruction and extra-articular manifestations of the disease and are less likely to achieve drug-free remission.[28]

Anti-carbamylated protein antibodies (anti-CarP)

Anti-CarP is an autoantibody that binds to homocitrullinated proteins. Homocitrullinated proteins are formed by carbamylation, a non-enzymatic modification of lysine residues. Thus, it is also a post-translationally modified amino acid, being one methylgroup longer than citrulline (Figure 1B).[29] This process occurs primarily under conditions of uremia and high inflammation. Homocitrulline is generated from a free NH2- groups of lysine residues upon binding with cyanate, which is in equilibrium with urea. In physiological circumstances, carbamylation through this process is low, however in renal failure this process increases.[29] In inflammation myeloperoxidase is released from neutrophils, which converts thiocyanate to cyanate which then can bind to a free NH2- group of lysine residue and forming homocitrulline.[30] Homocitrulline residues have, like citrulline, an effect on the net charge of the peptide, leading to impairment or loss of function.[31] Interestingly, carbamylation is increased in smokers by an increase in thiocyanate.[32] Homocitrulline containing peptides are present in the joints of both healthy individuals as well as in patients with RA.[33] In a mice model homocitrulline containing proteins activated T cells and in rabbits, homocitrulline containing proteins induced ACPA.[29, 34]

Although the similarity in the chemical structure of the targeted antigen of ACPA and anti-CarP, Anti-CarP and ACPA recognise different antigens and are only partly cross-reactive.[35, 36] Anti-CarP IgG are found in 45% of RA patients, and both in ACPA-positive and ACPA-negative RA patients, with 16% of ACPA-negative RA patients being positive for anti-CarP. With regard to disease outcome, anti-CarP IgG is associated with more radiographic progression in ACPA-negative RA patients.[35]

Anti-acetylated protein antibodies (AAPA)

More recently, AAPA have been found: autoantibodies directed against acetylated proteins. Acetyl lysine is formed by acetylation, a reversible post-translational modification of lysine residues(Figure 1C).[37] Acetylation of lysine is induced on the ε-amino group of lysine side chains and is catalyzed by lysine acetyltransferases. This modification can be affected by inflammatory and metabolic states, and it is potentially reversible.[38] In bacteria lysine acetylation can also occur through a non-enzymatical process by acetyl-CoA.[39] Lysine acetylation is important in cell processes, especially in the regulation of

histones and nuclear transcription.[38] In RA patients increased acetylation of certain proteins was found, for example of α-enolase.[40] Interestingly, bacteria can not only acetylate self-proteins, but also host proteins, which may be a potential trigger to induce AAPA.[41]

AAPA are found in 40% of RA patients and are mainly found in ACPA-positive patients.[42] AAPA are highly cross-reactive to the other AMPA's, although also monoclonal AAPA IgM with no reactivity to the other PTMs do exist.[43, 44] The association of AAPA with risk factors and disease severity of RA has been scarcely studied so far.

Anti-modified protein antibodies (AMPA)

Collectively, ACPA, anti-CarP, and AAPA are autoantibodies that target post-translational modifications and these autoantibodies are highly specific for RA. Several other autoantibodies directed against post-translational modified proteins have been studied, however these autoantibodies are not as specific for RA and are also found in other inflammatory conditions.[45] Despite the chemical similarities between citrullination, carbamylation, and acetylation, the corresponding autoantibodies are partial cross-reactive, however monoclonals with only reactivity to citrulline, to homocitrulline or acetyl lysine exist, supporting the suggestion that ACPA, anti-CarP ant AAPA are different autoantibodies.[35, 36, 43, 44]

AUTOANTIBODY ISOTYPES

With this knowledge of different autoantibodies in RA, it is also crucial to explore the various antibody isotypes involved in the autoimmune response. Autoantibodies can exist in various isotypes, each serving distinct functions in the autoimmune response. This paragraph will first introduce the different antibody isotypes and subsequently elucidate the current understanding of AMPA isotypes.

Antibody isotypes, also known as immunoglobulin (Ig) classes, are variants of antibodies that perform distinct roles in the immune response. There are five main isotypes in humans: IgA, IgD, IgE, IgG, and IgM. Each isotype exhibits unique structural characteristics and functions. For instance, IgA is predominantly found in mucosal areas and secretions such as saliva and tears, playing a crucial role in mucosal immunity. IgD is present on the surface of B cells, and while its function is not fully understood, it is believed to be involved in initiating B cell activation. IgE is associated with allergic reactions and defense against parasitic infections, whereas IgG is the most abundant antibody in serum, critical for opsonization, neutralization of pathogens, and activation of complement. IgM, the first antibody produced in response to an infection, is particularly effective in forming antigen-antibody complexes due

to its pentameric structure, which allows for increased avidity and efficient activation of the immune response

Antibody diversity arises from VDJ recombination, a process in which variable (V), diversity (D), and joining (J) gene segments are rearranged to generate unique antigen-binding regions in the heavy and light chains of antibodies. Although each isotype originating from 1 progenitor B cell retains the same antigen-binding (VDJ) region, they differ in their constant (C) regions. The constant region determines the isotype and determines the antibody's effector functions. Following antigen stimulation, isotype switching allows B cells to transition from producing IgM to other isotypes (IgG, IgA, IgE, etc.), thereby enhancing the specificity and effectiveness of the immune response. The sequence of isotype production begins with IgM, followed by IgG, IgA, or IgE, depending on the signals received from cytokines and other immune mediators.

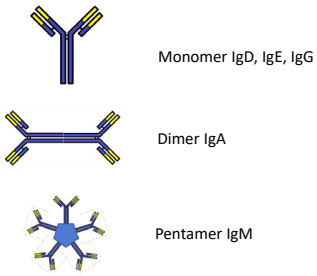

Figure 2. Structure of human antibody isotypes (IgA, IgD, IgE, IgG, IgM).

In RA, AMPAs are predominantly present as IgG antibodies, although IgA and IgM are also observed. Approximately 60% of RA patients are ACPA-IgG positive, while ACPA-IgM and ACPA-IgA are almost exclusively found in ACPA-IgG-positive patients.[28, 46] Among those positive for ACPA-IgG, 62% are also positive for ACPA-IgA and 61% for ACPA-IgM. The presence of anti-CarP is in general lower compared to ACPA, with 45% of RA patients testing positive for anti-CarP IgG, 24% for anti-CarP IgA, and 16% for anti-CarP IgM.[47] Notably, both anti-CarP-IgA and anti-CarP-IgM can be detected in patients who are either positive or negative for anti-CarP-IgG. Regarding AAPA, current measurements include only AAPA-IgG and AAPA-IgA, with AAPA-IgG found in 29-40% of RA patients and AAPA-IgA in 7.5%.[42, 48]

While the frequencies of the various AMPA isotypes have been studied, ACPA-IgG has been most extensively studied, particularly in relation to environmental and genetic risk factors, such as smoking and the shared epitope (SE) allele. The potential role of different isotypes in the pathogenesis of RA is worth exploring; for example, since smoking primarily affects the

mucosal surfaces of the lungs, investigating the association between smoking and IgA could provide valuable insights. The subsequent paragraphs will discuss genetic and environmental risk factors in relation to autoantibodies and isotypes in RA.

GENETIC RISK FACTORS FOR RHEUMATOID ARTHRITIS

The genetic contribution of RA is estimated to be between 50% and 60%,[49] with the most significant association being found in the human leukocyte antigen (HLA) region. In the HLA region, the strongest association is with alleles of the class II HLA-DRB1-gene, that encode a conserved amino acid sequence at positions 70 – 74.[50] This amino acid sequence is shared by the following HLA-DRBB1-alleles: HLA-DRB1*01:01, HLA-DRB1*01:02, HLA-DRB1*04:01, HLA-DRB1*04:04, HLA-DRB1*04:05, HLA-DRB1*04:08, HLA-DRB1*10:01, HLA-DRB1*14:02 and/or HLA-DRB1*14:04 and is associated with RA. This amino acid sequence is referred to as the shared epitope (SE). SE alleles influence the structure of the peptide-binding groove, suggesting their role in the binding and presentation of peptides to T cells, potentially including arthritogenic peptides.[51] Interestingly, the association of SE is primarily found in ACPA-IgG positive RA, and not with other ACPA-isotypes or autoantibodies such as RF and anti-CarP.[52, 53] Another prominent genetic risk factor are Protein tyrosine phosphatase non-receptor type 22 (PTPN22)-variants.[54] PTPN22 encodes proteins that play a key role in the adaptative immune systems by regulating both T and B cells. SNPs in PTNP22 are associated with multiple autoimmune disease.[55] Many other genetic risk factors have been described as risk factors for RA, involved in both adaptive as innate immunity.[56] Interestingly, the vast majority of genetic risk factors predispose to ACPA-IgG positive RA including SE and PTPN22.

ENVIRONMENTAL RISK FACTORS FOR RHEUMATOID ARTHRITIS

Smoking is the most extensively studied environmental risk factor and is associated with autoantibody-positive RA.[57-59] A gene-environment interaction between SE and smoking has been identified in ACPA-IgG positive patients supporting the intriguing hypothesis that autoimmunity in RA has a mucosal origin.[60] The mucosal origin hypothesis posits that the initial trigger in RA development occurs at mucosal sites such as the lungs, oral cavity, or gut, where autoantibodies are locally produced. These antibodies may subsequently cross-react with self-antigens in the joints, leading to disease onset. In line with the mucosal origin hypothesis other risk factors such as parotitis, air pollution and disbalance in the microbiome have been described as risk factors for RA.[61-63] Given that IgA is the mucosal isotype , it would be of interest to investigate the association of these risk factors with AMPA-IgA.

However, this has only been explored for smoking in relation to ACPA-IgA, where an association between smoking and ACPA-IgA has been identified.[48, 64]

Dietary risk factors for RA are another interesting field of research, with possible protective effects of low to moderate alcohol consumption and healthy eating behaviours including omega-3 fatty acids.[65, 66] High dietary salt consumption has been found as risk factor for especially autoantibody positive RA.[67, 68] However, little research has been performed in the pathological role of sodium in RA. Yet further research in the field of RA is of interest since a pro-inflammatory role of sodium has been described. Bacterial skin infection leads to a remarkable sodium accumulation in the skin, and the hypertonic microenvironment led to increased macrophage activation.[69] Furthermore, sodium can induce the differentiation of T helper 17 (Th17) cells, which plays a role in autoimmune diseases in general and arthritis in particular.[70]

In addition to smoking and dietary factors, numerous other environmental risk factors for rheumatoid arthritis (RA) have been identified, including obesity, specific infections, and hormonal differences.[71] These factors may interact with genetic predispositions, thereby influencing the development and progression of the disease. A pathophysiological model illustrating these interactions will be described in the following paragraph.

PATHOPHYSIOLOGICAL MODEL OF RHEUMATOID ARTHRITIS

The first and second hit model in rheumatoid arthritis (RA) describes a two-step process in the development of the disease, integrating genetic susceptibility, environmental triggers, and subsequent immune system dysregulation (Figure 3). The “first hit” involves a preclinical phase where individuals with a genetic predisposition, such as those carrying SE alleles encounter environmental risk factors. These risk factors can lead to the post-translational modification of self-proteins, such as citrullination. This modification creates neoantigens that are recognized by the immune system as foreign, leading to the production of AMPA. During this phase, these autoantibodies are generated, but there are no clinical symptoms of arthritis yet.

The “second hit” occurs when these autoantibodies, now circulating in the bloodstream, encounter a triggering event that leads to the clinical manifestation of RA. This could involve further environmental insults, infections, or physical trauma to the joints. The presence of autoantibodies facilitates the formation of immune complexes within the synovial fluid, activating complement pathways and leading to an influx of inflammatory cells into the synovial membrane. This inflammatory cascade results in synovitis, characterized by joint swelling, pain, and eventual destruction of cartilage and bone. The second hit represents the transition from a preclinical state to overt rheumatoid arthritis, driven by the interaction of autoantibodies with joint tissues and the subsequent

inflammatory response.

The mucosal origin hypothesis integrates seamlessly with the first and second hit model for RA by providing a potential explanation for the initial trigger (the first hit) in genetically susceptible individuals. According to this hypothesis, environmental exposures at mucosal sites such as the lungs, oral cavity, or gastrointestinal tract lead to the post-translational modification of proteins, such as citrullination, at these local sites. These modified proteins act as neoantigens that are recognized by the immune system, resulting in the local production of autoantibodies.

AIM

Although a pathophysiological model for RA has been formed, many questions remain regarding the pathophysiology of RA. In recent years new autoantibodies such as anti-CarP and AAPA have described as well as features of RA autoantibodies that were until recently unknown, such as the high glycosylation of ACPA antibodies. It is unknown if risk factors and clinical outcomes of AMPA differ from each other. Moreover, most studies so far have investigated AMPA-IgG, whereas AMPA-IgA and AMPA-IgM have been scarcely studied. Yet, these isotypes are of interest for several aspects of the pathophysiology of RA. IgM is the first antibody formed, and therefore it is interesting to study AMPA-IgM since it might lead to the starting point of autoantibody formation in RA. Additionally, further investigation of AMPA-IgA could contribute to understanding the mucosal origin hypothesis. Understanding the isotype profiles of autoantibodies in RA patients can provide insights into the mechanisms of disease onset and the influence of environmental factors on autoimmunity. Therefor this thesis aims to investigate risk factors and clinical outcome of AMPA-isotypes in RA.

The described research was performed with the ultimate goal to clarify the pathophysiology of RA, as insights in disease pathophysiology can lead to new treatment opportunities and ultimately a cure for RA.

OUTLINE

This thesis follows the pathophysiological model for rheumatoid arthritis: first genetic predisposition is discussed, secondly we focused on environmental risk factors for RA, then on autoantibodies and lastly on clinical outcome in relation to autoantibodies.

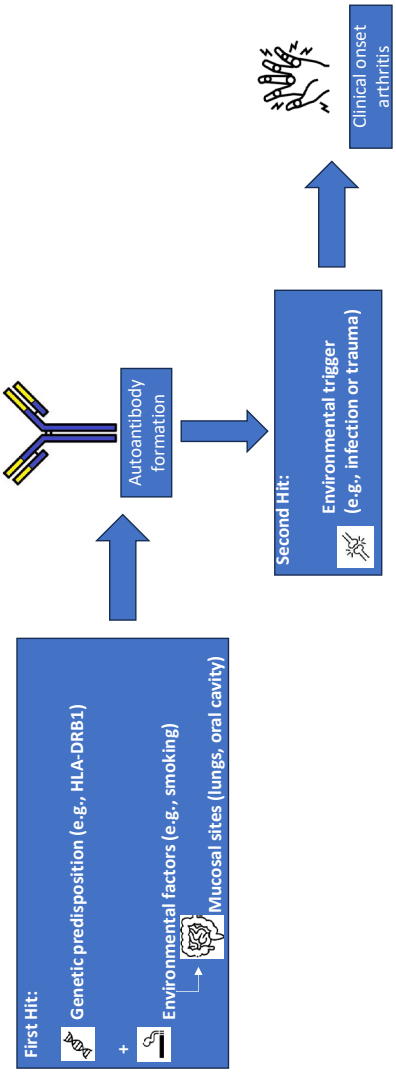

Figure 3. Pathophysiological model of rheumatoid arthritis

PATHOPHYSIOLOGY OF AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

GENETIC PREDISPOSITION

In the first two chapters, we focused on genetics of rheumatic diseases. In **Chapter 2**, rheumatic diseases were placed in a immunological landscape based on genetics and the presence of autoantibodies. The validity of our model was assessed by the congruence with medication use. In **Chapter 3**, we studied the association between SE and ACPA glycosylation. We hypothesized that SE was not associated with ACPA, but specifically with the presence of variable domain glycosylated ACPA-IgG.

ENVIRONMENTAL RISK FACTORS

In **Chapter 4** the association of smoking with the presence of RF, ACPA and anti-CarP was studied. In addition to the association of each separate autoantibody, the relationship between smoking and the number of autoantibodies was discussed. In **Chapter 5**, sodium was studied as both a risk factor and an inflammatory factor in autoimmune arthritis. Sodium MRI (Na-MRI) was used to investigate sodium concentration in inflamed joints (to assess the inflammatory component of sodium in arthritis) and in the skin and muscle (to evaluate sodium storage differences in RA and healthy controls).

AUTOANTIBODY ISOTYPES

In the last two chapters of this part we focused on autoantibody isotypes. In **Chapter 6** we studied the development of AMPA in RA by investigating the isotype that firstly developpes: IgM. To investigate this, AMPA-IgM and AMPA-IgG where measured in different disease stages of RA: healthy individuals, ACPA-positive healthy individuals, patients with arthritis with other diagnosis than RA and in RA. In **Chapter 7** the association of smoking and genetic risk factors was studied with AMPA isotypes. We hypothesized that smoking is associated with AMPA IgA, since smoking exerts it's influence on the mucosa of the lungs.

CLINICAL OUTCOME IN RELATION TO AUTOANTIBODIES

In **Chapter 8** we studied if AAPA are associated with baseline characteristics in RA patients, and if AAPA were associated with worse disease outcome measured as sustained DMARD free remission and radiological progression. In **Chapter 9** we investigated if the presence of several autoantibodies was associated with the onset of auto-immune related adverse events in the treatment with anti-PD1 antibodies.

All these findings are summarized and discussed in the Discussion.

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