FACULTY OF MEDICINE AND HEALTH SCIENCES

Can metabolomics provide promising perspectives for future patients with Rheumatoid Arthritis?

Karen Vereecke
Student number: 01509416

Supervisor: Prof. Dr. Lennart Martens

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Medicine in Medicine

Academic year: 2019 – 2020

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PREFACE

Two years ago, writing a thesis was unknown territory for me. I was lucky to have the enthusiastic guidance of my supervisor, Prof. Dr. Lennart Martens. He directed me in the field of metabolomics, and gave me the space to develop a personal interest for its possibilities. He provided counsel and encouragement to tackle the complexities of metabolomics, Rheumatoid Arthritis, and academic writing. I am grateful for his effort.

I would also like to thank my friends and family for their support. Especially my parents for their comfort and curiosity during the writing process, my aunt, Danny Vereecke, for sharing her expertise with me, and Niels Notable for providing advice and a listening ear when necessary.

Thanks to these people I have achieved a satisfying result and learned a lot, while creating a thesis of personal interest.

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ABSTRACT (DUTCH)

Reumatoïde artritis (RA) is een invaliderende ziekte met een grote invloed op de levenskwaliteit, socio-economische status, morbiditeit en mortaliteit van patiënten. De ziekte wordt al lange tijd uitgebreid onderzocht met behulp van verschillende *omics*-analyses, maar veel aspecten van de pathogenese, diagnose, therapie, prognose en preventie zijn nog niet gekend. In deze thesis wordt daarom bekeken of *metabolomics* veelbelovende perspectieven kan bieden bij deze uitdagingen voor toekomstige patiënten met RA.

Gezien de grote omvang van de reeds beschikbare informatie over RA is het frappant om te zien dat exacte oorzaak(en), diagnostische methoden, behandelingsstrategieën voor remissie of, meer ambitieus, voor genezing en/of preventie grotendeels afwezig blijven. De verwachting is dan ook dat *omics*-analyses hieraan kunnen bijdragen met voornamelijk *exposomics*, *genomics*, *transcriptomics*, *epigenomics* en *proteomics*. *Metabolomics* is een recentere toevoeging, en alle *omics*-technologieën kunnen worden gecombineerd in de systeembiologie. Elke methode heeft specifieke uitdagingen en mogelijkheden, wat de combinatie ervan waardevol maakt. Dit is echter nog niet mogelijk, omdat de meeste *omics*-methoden zelf nog intensief bestudeerd en geoptimaliseerd worden, en systeembiologie nog in de kinderschoenen staat. Desalniettemin zijn de *omics*-vakgebieden veelbelovend voor de analyse van complexe ziekten zoals RA en de verwachtingen van met name *metabolomics* voor toekomstige RA-patiënten verdient gedetailleerde aandacht.

Terwijl relevante biomerkers voor RA ontdekt zijn in het exposoom, genoom, transcriptoom, epigenoom en proteoom, en zelfs in vroege systeembiologische analyses, blijft de bijdrage van *metabolomics* schaars. Deze thesis beschrijft daarom de bestaande vooruitgang dankzij *metabolomics* met identificatie van potentiële biomerkers voor klinische RA op basis van specifieke veranderingen in verschillende metabole *pathways*, en verzamelt de beperkte informatie die tot nu toe beschikbaar is over merkers bruikbaar voor preventie, preklinische RA en opvolging van therapie.

Op basis van de verzamelde informatie, met name met betrekking tot de potentiële waarde van *metabolomics* analyses voor toekomstige patiënten met RA, worden suggesties gegeven over hoe dit kan worden bereikt. Ook wordt rekening gehouden met de zwakke en sterke punten van de momenteel beschikbare informatie en de onderzoeksmogelijkheden die in deze thesis aan bod komen.

Tot slot wordt een samenvatting gegeven van de meest veelbelovende perspectieven van *metabolomics* en andere *omics*-analyses voor toekomstige RA-patiënten, samen met voorstellen voor toekomstig onderzoek.

ABSTRACT (ENGLISH)

Rheumatoid Arthritis (RA) is a debilitating disease that has a large impact on the quality of life, socio-economic status, morbidity, and mortality of patients. It has already been extensively researched using various omics analyses for a long time, but still many aspects on pathogenesis, diagnosis, therapy, prognosis, and prevention remain undiscovered. This thesis therefore reviews whether metabolomics can provide promising perspectives towards these goals for future patients with RA.

Given the large amount of information already available on RA, it is surprising to see that exact cause(s), diagnostic methods, treatment strategies for remission or, more ambitiously, for curative and/or preventive strategies remain largely absent. There is therefore an expectation that omics analyses could help advance these goals, with exposomics, genomics, transcriptomics, epigenomics, and proteomics as the main strategies. Metabolomics is a more recent addition, and all omics technologies can be combined in systems biology. Each omics method has its specific challenges and opportunities, which makes their combination more informative. However, it is not yet possible to properly combine the various techniques, because most of these omics methods are still being intensively studied and optimised themselves, and because systems biology remains in its infancy. Nevertheless, the omics fields do hold substantial promise for the analysis of complex diseases such as RA and the promise for future RA patients of metabolomics in particular deserves detailed attention.

Indeed, while relevant biomarkers for RA have been discovered in the exposome, genome, transcriptome, epigenome, and proteome, and even in early systems biology analyses, to our knowledge, the contributions of metabolomics to RA remain scarce. This thesis therefore describes the existing advances achieved in metabolomics by identifying potential biomarkers for clinical RA based on specific changes across a variety of metabolic pathways, and assembles the limited information available so far for markers useful in prevention, preclinical RA, and therapy monitoring.

Based on the information collected in this thesis, especially with regards to the potential value of metabolomics analyses on future patients with RA, suggestions are provided on how this value can be achieved. Weaknesses and strengths of the currently available information, as well as of the research scope possible in this thesis, are also considered.

Finally, a summary of the most promising perspectives of metabolomics and other omics analyses for future RA patients is provided, alongside possible proposals for future research.

1 INTRODUCTION

Rheumatoid arthritis (RA) is the most common form of inflammatory arthritis. It is a worldwide problem that affects 0.5-1% of the population (1, 2), including a member of my family. RA prevalence has been projected to increase by 22% between 2005 and 2025 (3). RA patients have a higher death rate when compared to the general population (1, 4) and, if left untreated, suffer from debilitation. Over the past years this debilitating state has evolved to a more chronic and controllable disease (1) because of therapeutic evolution (5). Nevertheless, patients with RA have an associated economic burden because of absenteeism, function loss at work, and direct medical costs, which has been calculated as a yearly expenditure of $7,941 for anti-citrullinated peptide antibodies (ACPA)-positive and $5,243 for ACPA-negative patients (6). Clearly, more can be done to aid patients and to address the negative consequences of RA at the personal and the economic level.

In this introduction, I first describe the current knowledge on RA and the types of analytical methods that are used in the diagnosis and study of RA. I then discuss the rapidly developing field of metabolomics and end by explaining why metabolomics can provide promising translational perspectives for future patients with RA.

1.1 RHEUMATOID ARTHRITIS

1.1.1 Definition and classification

Although a precise description is hard to pin down, a consensus definition of RA could be formulated as follows: RA is an autoimmune disease (7) that is characterized by chronic inflammation with joint swelling, joint tenderness, and destruction of synovial joints, and it is a systemic inflammation with changes in the immune system (7, 8). Eventually the disease leads to severe disability and premature mortality (1, 7, 9).

RA has been known since 1850, but classification criteria to define RA in a standardized way were developed only 50 years ago (10) (see section 1.1.4). Internationally, the 1987 American College of Rheumatology (ACR) criteria (11) were used until a working group of the ACR and European League Against Rheumatism (EULAR) developed the 2010 ACR/EULAR classification criteria for RA (7). Evaluation of these new classification criteria showed that these “are sensitive to detect cases of RA among various target populations, independent of how the latter is referenced” (12). However, the 2010 ACR/EULAR classification criteria are not diagnostic (7, 12), since these were created to select RA patients at earlier stages of the disease to facilitate their study and comparison between RA studies.

1.1.2 Pathogenesis

Although the detailed etiology of RA is unknown, the mechanisms of disease and associated disease pathways have been well-studied (13). At the same time, the literature on RA is quite complex and difficult to summarize, because different studies focus on different metabolites. I therefore structured the information around the three phases of the general disease pathway (the at-risk, the preclinical, and the clinical phase) and the role of cytokines in this pathway.

At-risk phase – Because RA is a disease initiated by genetics as well as random (or as-yet unknown) events, the pattern of inherited genes can put a person at risk of developing RA. A positive family history increases the risk of RA with a factor three to five, unless the person with RA is seronegative. In the latter case, the risk is lower (14). The most important markers are the human leukocyte antigen (HLA) major histocompatibility genes. Especially HLA-DRB1, but also single nucleotide polymorphisms (SNPs) in PTNP22, CTLA4, and STAT4 (13), all involved in T-cell activation, are associated with a higher risk of developing seropositive RA. Additionally, T-cell and cytokine cell signaling genes increase susceptibility (15). Less is known about the genetic susceptibility of seronegative RA, but genetic factors on HLA-DR1 and HLA-B have been discovered (16). On the other hand, changes in genome, transcriptome, epigenome, proteome, and metabolome, together called the exposome, can be caused by several environmental factors and trigger RA (see section 1.2) (14, 15).

Preclinical phase of systemic autoimmunity – Although most studies on RA focus on inflamed joints and peripheral blood cells (17), the induction of RA does not necessarily start in the joints (13). Ramwhadhdoebe *et al.* (17) were the first to describe preclinical changes in lymph nodes of preclinical and clinical RA patients. The induction of RA is characterized by repeated stimulation of the innate immune system at mucosal surfaces (15) by one or more environmental factors (13) (section 3.1.1). This activates the innate inflammatory cascade and upregulates pro-inflammatory genes in susceptible persons through epigenetic changes. Smoking, for example, causes the upregulation of peptidyl arginine deiminase (PPAD) in alveolar macrophages, which converts arginine to citrulline (15). Citrullinated peptides become autoantigens that are presented by dendritic cells to the adaptive immune system, which can trigger autoantibody formation, rendering the immune response persistent (13, 15). Similarly, carbamylation of peptides can also trigger autoantibody formation (13). In early disease, citrulline-specific T helper (Th) 1 cells are increased in the circulation, but their contribution to autoimmunity is uncertain (14). Up to ten years before disease development in the synovium, autoantibodies – including rheumatoid factor (RF), ACPAs and others – can be present in blood (13-15, 17). ACPAs activate macrophages and osteoclasts and potentiate the effect of RF. RF activates macrophages and induces cytokine production more directly (14). The concentration and epitope diversity of ACPAs and the serum concentration of cytokines both

increase in the preclinical phase and in particular right before onset of the clinical phase (14). However, despite the fact that ACPAs and RF are not always present in RA patients, these are nevertheless used as biomarkers of the persistent immune response (13, 15, 17). At this preclinical phase regulatory T (Treg) cells are decreased in lymph nodes (17).

Clinical phase – The next step in disease development is the formation of soluble immune complexes in the circulation. When reaching the microvasculature, these complexes bind to mast cells, neutrophils and monocytes, causing vascular permeability and allowing leucocytes to infiltrate joints and sometimes other organs (13, 15). These leukocytes are innate immune cells such as monocytes, dendritic cells, mast cells and innate lymphoid cells (ILCs), and adaptive immune cells such as Th1 and Th17 cells, B-cells, plasmablasts and plasma cells (14). The increased vascular permeability also facilitates the interaction of ACPAs to citrullinated peptides in the synovium and the cartilage (15).

The affected joints at this stage are in a persistent inflammatory state with cell-cell interactions and local production of pro-inflammatory cytokines, chemokines, antibodies, lipid mediators and metalloproteinases. In contrast to the decreased levels of Treg cells in the lymph nodes of RA-risk patients (17), Treg cells were elevated in affected joints and synovial fluid (13), but this did not limit the inflammation in the clinical phase of RA. Activation of neo-angiogenesis also occurs in this stage as a reaction to the low oxygen tension in the inflammatory microenvironment. This angiogenesis, together with the inflammation, promotes the proliferation of fibroblast-like synoviocytes (FLSs) that form tumor-like tissue that invades cartilage and bone, the pannus (10, 13). At the same time, osteoclasts are activated indirectly by inflammatory cytokines and directly by infiltrated ACPAs (13, 15), and cause damage to chondrocytes, collagen and proteoglycans. There is also an increased generation of osteoclasts because T-cells, B-cells and fibroblasts express receptor activator of nuclear kappa-B ligand (RANKL) that binds to RANK, expressed by pre-osteoclasts (14). Degraded collagen and proteoglycans form new neo-epitopes with joint-specific antigens to which dendritic cells react. These dendritic cells migrate to lymph nodes where these activate the adaptive immune system. This in turn causes T-cells to become activated and to lose self-tolerance (15). This process thus increases autoimmunity, as B-cells and T-cells react to self-antigens, which activates more cytokines. The resulting spiraling immune dysregulation establishes a chronic inflammatory and destructive state in the synovium. Cytokines that accumulate in the synovium also leak into circulation, where these cause systemic symptoms, such as fatigue and fever.

Cytokines – Due to the success and failure of different therapies, the role of cytokines and cytokine networks in RA is now better understood (14) and identified them as possible targets in RA diagnosis, prognosis, therapy, and prevention (13, 15) (see section 1.1.6.). The main role of cytokines in inflammation cascades is to induce eventual synovial inflammation,

bone and cartilage destruction by collagenase and metalloproteinases, or both (10). T-cell cytokines secreted by different types of T-cells are: interferon (IFN) γ for Th1 cells; IL-4, IL-5, and IL-13 for Th2 cells; IL-17 and IL-22 for Th17 cells; and IL-10 for Treg cells. In contrast, activated macrophages and fibroblasts secrete IL-1, TNFα, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-CSF, transforming growth factor (TGF) β, and several chemokines. It should be noted that this distinction is not very strict, because several cytokines are produced by different types of cells (18).

Venuturupalli states that, although IL-17 (a TH17-derived cytokine) is elevated, most Th1 (IFNγ) and Th2 (IL-4, IL-5, IL-13) cytokines are “conspicuously absent or present at very low levels in RA synovium (15)”. This contrasts to macrophage and fibroblast cytokines, including TNFα, IL-6 and GM-CSF (14), which are more abundant in synovial fluid and tissue, and have a more central role in the pathogenesis of RA. In addition, Kim and Moudgil (18) stress the importance of an imbalance of the Th1/Th2 cell ratio and of the Th17/Treg cell ratio. Ramwadhdoebe *et al.* (17) showed that whereas the amount of CD4+ T-cells in lymph nodes of preclinical and clinical RA patients are equal to healthy controls, their balance and function is altered.

Pro-inflammatory cytokines. IL-6 normally regulates the acute-phase response of the innate immune response (19), which in RA is unregulated in the rheumatoid joint (15). It also increases survival and proliferation of immune cells (19). T-cells differentiate into TH17 cells, and B-cells mature to cause antibody production. This facilitates the transition from acute to chronic inflammation. The diffuse impact causes systemic features such as fatigue, cognitive dysfunction, fever, anemia, systemic osteoporosis and altered pituitary adrenal axis function (15, 19). It has been shown that anti-IL-6 therapies are effective (this in contrast to IL-1, see section 1.1.6).

IL-1 and TNFα stimulate cytokine production, adhesion cell profile expression and production, and metalloproteinase production. TNFα is the reason for the variety in different patients with RA (15), and is a key inflammatory pathway in RA (10). It stimulates prostaglandin E2 and collagenase, induces bone resorption, inhibits bone formation, and stimulates resorption of proteoglycans in rheumatoid joints and in the circulation (15). It also leads to an overproduction of many cytokines, like IL-6 (10), attracts neutrophils, stimulates proliferation and pannus formation of FLSs, and has systemic effects (18). IL-1 has multiple biological effects, including prostaglandin and collagenase synthesis, fibroblast stimulation, and B- and T-cell chemotaxis, and is one of the most important pro-inflammatory cytokines according to Venuturupalli (15). However, other studies state that the most important cytokines are GM-CSF, IL-6 and TNFα (14) and that IL-1 is a cytokine that is either less observable in RA or specific to one or more disease subsets (10). It is also worth noting that anti-IL-1 therapies are not very effective.

IL-17 induces the production of metalloproteinases through the activation of osteoclasts and triggers neo-angiogenesis (15, 18). It also amplifies the productivity and activity of other pro-inflammatory cytokines and chemokines, and of macrophages, neutrophils, and other cells in the synovium (15, 18). It is also noted that, IL-1 and TNFα have a synergetic effect on IL-17 (15).

Although IFNγ is extensively studied and is known to have several effects on inflammation in normal situations (18), this cytokine is not elevated in the synovium to an amount that would cause symptoms. This means that the IFNγ-like effects are likely due to other factors (15).

Anti-inflammatory cytokines. There is a natural negative feedback loop in the inflammation cascade of RA (18). In general, TNFα and IFNγ are primarily responsible for this self-regulation and control of inflammation. IFNγ inhibits many of the effects of TNFα. In RA patients, direct injection of IFNγ in the joints can even achieve some benefits without significant side effects. TNFα decreases Treg cell activity. The mechanism by which TNFα and IFNγ both play a pro- and anti-inflammatory role is not entirely understood, making it dangerous to focus therapy on either of these cytokines. Anti-TNFα, for example, is a treatment for RA, but some patients show aggravation instead of improvement with this treatment.

IL-10 is normally activated when effector T-cell differentiation starts and regulates tolerance and the immune response (17). In at-risk and preclinical RA, IL-10 producing T-cells are decreased in lymph nodes, which might eventually lead to an overactive immune system. However, in clinical RA IL-10 producing T-cells are increased in synovial tissue and fluid.

1.1.3 Extra-articular manifestations and comorbidities

In this section, I discuss the extra-articular manifestations caused by the chronic systemic inflammation (20) or the treatment (14, 21) of RA and the impact of RA on coexisting diseases, which I define as comorbidities. Even though detection and prevention of extra-articular manifestations and comorbidities are recommended by the EULAR (5), these remain often underdiagnosed and undertreated (20). Indeed, despite the fact that the amount of patients with severe disease has decreased because of modern therapies (14, 21), recognition of extra-articular manifestations and comorbidity remains a problem because some therapies cause manifestations themselves (14, 21) or their efficacy and safety is influenced by coexisting comorbidities (21). Nevertheless, extra-articular manifestations of RA, most of which are diseases of the circulatory and respiratory system, cancer (4), and infections (21), are the main cause of the lower survival rate of patients with RA (5, 22). Typical extra-articular manifestations include rheumatoid nodules, pulmonary involvement, vasculitis, secondary amyloidosis, lymphoma, cardiovascular disease, myocardial infarction (MI), angina, pulmonary

tuberculosis (tbc), asthma, thyroid disease, depression, hepatitis B virus (HBV), cerebrovascular events, serious infections, and malignancy (21).

Early predictors of cardiovascular disease in RA are vasculitis and rheumatoid lung disease, which are severe extra-articular manifestations, and a persistent elevated inflammation measured in blood. Additionally, there is a rapid progression of atherosclerosis at five months after diagnosis as a consequence of the direct impact of chronic inflammation on the vasculature and the indirect impact of physical inactivity, together with non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (GCs) which are typical initial, symptomatic treatments (21). Although the Korean National Health and Nutrition Examination Survey shows that most RA patients do not smoke (20), Turesson claims that, because RA is a risk factor for both cardiovascular disease and RA, smoking contributes to this extra-articular manifestation (21). Other studies explain that cardiovascular disease is caused by increased lipoproteins in RA (23, 24), but Turesson claims that hyperlipidemia does not consistently predict cardiovascular development in RA patients (21). The reason for this contrast is the “lipid paradox” described in some studies (25) (see section 3.2.2).

Lung disease is either a consequence of therapy or can be caused by RA itself (26), and results in morbidity and mortality in RA patients. Interstitial lung disease (ILD) is most common (26, 27). Conventional disease modifying anti-rheumatic drugs (DMARDs) (methotrexate (MTX) and leflunomide) and biologic antigens (TNFα-inhibitor or rituximab) can trigger or aggravate ILD (27), but this affects only a minority of patients (26). In most patients, treatment reduces the risk of lung disease. Risk factors for lung disease are: smoking, male gender, HLA-DRB1, RF and ACPA (26). The lung is affected because ACPAs bind to residues of self-proteins, and in the same way also other tissues can be affected. Why the lung is affected more frequently is unknown. However, it is known that smoking is a factor that induces citrullination and that the same citrullinated peptides are found in the lung as well as in synovial tissue (14). The association of asthma with RA (20) has recently been disputed (28), although a correlation was shown between chronic obstructive pulmonary disease (COPD) and RA independent of lifestyle confounders and mediators after diagnosis (28).

Infections have caused morbidity and mortality in RA patients long before DMARDs were used (21). Predictors of a higher susceptibility are markers of disease severity (see section 3) and the presence of other comorbidities. The use of GCs is also a major risk factor, which is why these should be used for short terms or only when necessary (5).

In the context of malignancies and RA, the prevalence of lymphoma is increased. Predictors of this risk are severe disease, positive RF, and persistent high disease activity. This can be explained by the chronic activation of B- and T-cells, initiating lymphoproliferative disorders. Moreover, lung cancer is more prevalent in RA patients. On the other hand, there is a reduced risk of colorectal cancer possibly due to the extensive treatment with NSAIDs. Also

breast, ovary, endometrial, and prostate cancer are reduced, likely because of hormone exposures that predispose to RA development (see section 3.2) (21).

Despite their reported positive effects, DMARDs can also negatively contribute to extra-articular manifestations. NSAIDs cause an increased risk of cardiovascular disease, TNFα-inhibitors and other DMARDs can increase the risk of serious infections. There is no overall increased risk for cancer, but azathioprine and MTX are associated with a higher level of lymphoma, and DMARDs in general do increase the risk for melanoma.

1.1.4 Diagnosis

There is a crucial transition to chronic synovitis in the early phases of RA that makes the disease non-resolving. Therefore, diagnosis of at-risk and preclinical patients is important to start early, preventive therapy (see section 1.1.7) (14).

In the absence of a gold standard (7, 11, 14), the current method to diagnose clinical RA is based on the diagnostic criteria formulated by the ACR in 1987 (11). Of these criteria, the first four have to be present for at least six weeks, and if these four or more are present, the diagnosis of RA can be made.

1) "morning stiffness in and around joints lasting at least 1 hour before maximal improvement;
2) soft tissue swelling (arthritis) of three or more joint areas observed by a physician;
3) swelling (arthritis) of the proximal interphalangeal (IP), metacarpophalangeal (MCP), or wrist joints;
4) symmetric swelling (arthritis);
5) rheumatoid nodules;
6) the presence of rheumatoid factor; and
7) radiographic erosions and/or peri-articular osteopenia in hand and/or wrist joints."(11)

In 2010, the ACR/EULAR came up with a new set of criteria to classify RA patients for population studies (7). The difference between classification and diagnosis is that a diagnose aims to be correct on an individual level, whereas a classification maximizes a study population for study purposes (14). The classification criteria do not contain late disease presentations, because these were compiled to recognize early RA and start treatment to avoid complications, for example erosions. The criteria can only be used if two conditions are present: (1) there is evidence of clinical active synovitis in at least one joint (excluding the distal interphalangeal (DIP) joint, first metatarsophalangeal (MTP) joint, and first carpometacarpal (CMC) joint); (2) no other diagnose explains the synovitis better (*e.g.* Systemic Lupus Erythematosus (SLE), Psoriatic Arthritis (PsA), Gout). If these two conditions are met, the following four criteria for RA are assessed and given a score:

1) Joint involvement going from 1 large joint (0), 2-10 large joints (1), 1-3 small joints with or without large joints (2), 4-10 small joint with or without large joints (3), to finally more than 10 joints with at least 1 small joint (5).
2) Serology of RF and ACPA is done and both are negative (0), one or both are low-positive (1), one or both are high-positive (3).

3) Acute-phase reactants (CRP and ESR) are either both normal (0), or at least one is abnormal (1).
4) The duration of the symptoms is less (0) or more (1) than six weeks (7).

If the patient scores six or more out of ten, the disease is confirmed and treatment can be started. If the score is less than six, the disease cannot be classified as clinical RA, but the patient can still develop RA. In that case, it is suggested to reassess the patient.

1.1.5 Therapy

Here I describe a short history of treatment strategies for RA, the currently available therapies and management recommendations, the pathways tackled by therapies, and the current recommendations to monitor treatment.

Historically, a pyramidal model was taken to treat RA. The initial symptomatic treatment typically consisted of salicylates, like NSAIDs and analgesics, associated with bed rest, splinting, physical therapy, heat therapy, and occupational therapy (1, 29-31). Later in the disease course, DMARDs were introduced as additional therapy consisting of gold salts, MTX, and penicillamine (1, 29). Changes in this treatment model were necessary due to the side-effects of NSAIDs (17, 32) and the debilitating evolution of the disease, both with significant morbidity and mortality (30, 31). DMARDs replaced NSAIDs because of their comparable toxicity (33) and their better control of progression and pain symptoms, and the decreased disability (17, 33, 34) and joint damage in early RA (8, 35). Over the past two decades, conventional synthetic (cs) DMARDs were supported by biological (b) ones that block cytokines and cytokine networks or that modulate lymphocyte function (section 1.1.2) (13). Most recently, the development of small molecules is being investigated to target intracellular signaling.

Current therapies and management recommendations – The therapies available in 2017 according to EULAR are csDMARDs (MTX (36), leflunomide (37), sulfasalazine (38)), GCs, bDMARDs (TNFα-inhibitors (15, 39) (adalimumab (40), certolizumab-pegol, etanercept, golimumab, infliximab), abatacept, rituximab, tocilizumab (41), clazakizumab, sarilumab, sirukumab, biosimilar (bs) DMARDs), and targeted synthetic (ts) DMARDs (Janus kinase (JAK)-inhibitors (42): tofacitinib (40) and baricitinib) (5). Symptomatic therapy, psychological support, physical measures, and surgery may still supplement global treatment of patients (5) when conventional strategies have failed (31).

As management recommendations, MTX should first be in combined with short-term GC (5). In case of failure and in the absence of unfavorable prognostic markers (autoantibodies, high disease activity, early erosions, failure of two csDMARDs), the second strategy should consist of switching to or adding another csDMARD (in combination with short-term GC). If the prognostic markers are present, any bDMARD or JAK-inhibitor should be

added to the first strategy (5). As a final recommendation, any other bDMARD or tsDMARD should be used (5).

Therapy pathways – GCs at low doses diffuse freely across cell membranes to bind the cytoplasmic GC receptor α (cGCRα) to form a complex that migrates to the nucleus. The complex binds GC responsive elements in DNA, which induces anti-inflammatory proteins. Indirectly, however, this complex also interacts with other transcription factors, which is related to side-effects (43).

Within the group of csDMARDs, MTX has proven its efficacy and is widely used in monotherapy and in combination (44, 45). The suggested mechanisms are (a combination of) inhibition of purine and pyrimidine synthesis, suppression of transmethylation reactions with accumulation of polyamines, reduction of antigen-dependent T cell proliferation and promotion of adenosine release with adenosine-mediated suppression of inflammation. The effect on purine and pyrimidine synthesis is also responsible for the many toxicities of MTX: bone marrow suppression, liver toxicity and stomatitis. More detailed information about the affected pathway of MTX can be found in the review by Tian *et al.* (44). Another currently used csDMARD is leflunomide (5, 37, 45). It inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which is essential in the *de novo* synthesis of the pyrimidine ribonucleotide uridine monophosphate (rUMP). The lack of rUMP triggers p53-mediated pathways in autoimmune and activated lymphocytes (37), so these cannot proliferate (T-cells), or cannot produce autoantibodies (B-cells) (45). Non-lymphoid cells are less affected because these have salvage pathways (37). Sulfasalazine has a wide range of biological activities classified as antibacterial, anti-inflammatory, or immunomodulatory (38). In the large intestine this csDMARD is absorbed or split into sulfapyridine and 5-aminosalicylic acid and absorbed as sulfapyridine (45). Sulfasalazine and sulfapyridine are found in synovial fluid. The exact mechanism of their action is unclear, but sulfasalazine inhibits folate-dependent enzymes, similar to MTX. This treatment option is recommended for women who want to have children (45).

Combination therapy is the second treatment recommendation. MTX is mostly used as a baseline therapy, with which other drugs are combined (45). A triple (t) DMARD treatment of MTX, sulfasalazine and hydroxychloroquine has proven to be effective and safe, and superior to MTX monotherapy in early and highly active RA (45), but a more recent study showed no significant clinical effect (46). The combination of MTX and leflunomide has been investigated and found to be effective, although this combination comes with more gastro-intestinal side-effects and a higher hepatotoxicity risk (45).

The third treatment recommendation is the use of bDMARDs that target cytokines and the cytokine networks (15), including five TNFα-inhibitors, one inhibitor of IL-6, one of IL-1, one B, and one T cell-targeting bDMARD (45). TNFα-inhibitors initiated the development of

bDMARDs (45). The main side-effects of this treatment concern the risk of infections, so patients should be screened for tbc and HBV before initiation. The first generation includes etanercept, infliximab and adalimumab. Etanercept is the only recombinant human soluble fusion protein among the biological TNFα-inhibitors. It inhibits the interaction of TNFα and its receptor by binding TNFα itself. Etanercept has the lowest risk of tbc among the TNFα-inhibitors. Infliximab is a chimeric murine-human IgG1 monoclonal antibody against TNFα that induces the production of antibodies against the chimeric structure, so the combination with MTX is recommended. Adalimumab is the first human monoclonal antibody developed to interact with TNFα. The second generation of TNFα-inhibitors include golimumab and certolizumab and were developed after IL-1, IL-6 and B- and T-cells have been targeted (see further). Golimumab is a human monoclonal antibody against TNFα and is recommended in combination with MTX if other TNFα-inhibitors have failed. Certolizumab-pegol is an Fc-free humanized PEGylated anti-TNFα Fab' fragment. Its mechanism of action is different from the other TNFα-inhibitors, yielding a higher efficiency (45). Although it showed more side-effects and serious infections in trials, this was due to the design of these trials and not a problem of clinical reality (45). The problem with current anti-TNFα therapies is that a substantial minority of patients does not respond to treatment, which necessitated the development of other bDMARDs (15).

Tocilizumab (41) is a recombinant humanized IgG1 monoclonal antibody against IL-6 receptors. In comparison to other biologics, rare events of gastrointestinal perforations are reported. This treatment interferes with CRP, so CRP becomes inutile as marker in the follow up of RA (45). The success of tocilizumab caused the development of biologics interfering with the IL-6 pathway. Sirukumab is a human monoclonal antibody against IL-6, whereas clazakizumab and sarilumab are humanized monoclonal antibodies against IL-6 and the IL-6 receptor, respectively. Although there are no observational data from post-marketing studies yet, sarilumab has a broad efficacy among several RA subtypes and is superior to adalimumab as monotherapy. Its safety profile is similar to tocilizumab (19).

Abatacept is a recombinant human soluble fusion protein of the extracellular domain of human cytotoxic T-cell-associated antigen 4 (CTLA4) and the modified Fc portion of human immunoglobulin G1 (IgG1), that interferes with T-cell activation (15). Rituximab is a chimeric anti-CD20 monoclonal antibody targeting B-cells that can be combined with MTX or leflunomide. Especially in ACPA-positive patients, rituximab is better than a second TNFα-inhibitor after failure of a first TNFα-inhibitor (45).

Recently, orally available small molecules have been developed against JAK kinase pathways and these are classified within the tsDMARDs by the EULAR (5, 42). These pathways are found in immune cell activation, production of pro-inflammatory cytokines and cytokine signaling (40). Tofacitinib interferes with the intracellular pathways of dendritic cells,

CD4+ T-cells (Th1 and Th17) and activated B-cells (42), blocking γ-chain-containing cytokine production (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) (40). Their efficacy is shown in monotherapy and in combination with MTX. Observed adverse effects are related to infection, hematologic, hepatic and renal disorders, but monitoring post-marketing safety is recommended (42). Baricitinib can also be used in monotherapy and in combination with MTX. There is good evidence for its efficacy and tolerance up to 5.5 years. The observed adverse drug reactions so far were upper respiratory tract infections, increased low-density lipoprotein cholesterol (LDLc), nausea and thrombocytosis.

Other biologics are being investigated and developed thanks to new targets or improvements of current biologics (45) and new strategies (15). Interest goes to small molecules, because these may have a significant advantage over monoclonal antibodies (15). Examples of new targets are Th17 cells and cytokines (IL-12, IL-23) (15). Although IL-12 and IL-23 play a role in the regulation of the type 1 and 17 immune response, this has not shown any success yet. Targeting the B-cell activating factor of the TNFα family (45), interfering with cell-matrix interactions, and increasing Treg activity are also at an early experimental stage (15).

Monitor response – Modern therapy goals are the relief of signs and symptoms, the normalization or improvement of impairment in physical function, quality of life, social and work capacity, and the inhibition of structural damage of cartilage and bones (5, 14, 31). To achieve these goals, therapies have to be monitored for their efficacy as well as for their safety. There are EULAR recommendations for patient follow-up during treatment (5, 14).

DAS28 is a complex disease activity score using 28 joint counts calculated together with other components. There is also a simplified disease activity index (SDAI) and a clinical disease activity index (CDAI) (14, 47). These scores correlate with impairment of physical function or damage progression. Structural damage is measured with radiographies (14). Recently the ACR/EULAR developed new remission criteria that correlate with an absence of residual inflammatory disease activity, in contrast to previous criteria.

Monitoring the side-effects of the drugs is necessary as well (5). The main risks of bDMARDs and tsDMARDs occur in case of infections or vaccination, but each drug has a separate pathway and therefore specific monitoring recommendations (5, 48). In addition, every patient is different (49), signifying that whereas phenotypes can differ amongst patients at diagnosis, patients with the same phenotype not necessarily have the same immunological and molecular abnormalities. Although still at an experimental stage (49), thanks to individual biomarkers for early RA and biomarkers for follow-up (8), prevention of damage can be accomplished by immediate and targeted treatment with DMARDs (1) (see section 1.2 and section 3).

1.1.6 Prognosis

In this section I describe current prognostic factors of asymptomatic populations at risk of developing RA, current prognostic factors of RA-patients at risk of developing extra-articular manifestations, aggressive disease, and progression, and a few predictors of response to treatment.

At-risk patients – High titers of RF in asymptomatic people predict a risk of developing RA of up to 26 times higher if titers are more than 100 IU/ml (47). ACPA-positive, asymptomatic people are also at risk for RA (17). Combined with arthralgia, this risk becomes 40-70% within four years (3). In combination with smoking, the risk of developing RA and cardiovascular disease is also increased (47). *Porphyromonas gingivalis* antibodies titers in blood can correlate with RA and/or disease activity and the risk of RA is associated with periodontal disease (see section 3.1.1). Infections also have been suggested to trigger RA (14).

Extra-articular manifestations – The disease course of RA varies between patients. The presence of autoantibodies at diagnosis is associated with more severe symptoms and joint damage and with higher mortality, because autoantibodies lead to complement activation by binding to self-proteins (14, 47). Smoking is an additional risk factor for extra-articular manifestations, because it facilitates ACPA-formation. The presence of IgA isotype autoantibodies is associated with extra-articular manifestations (47). Additionally, HLA-DRB1 genotypes suggest a more aggressive, erosive disease and a higher mortality (50). Serum levels of IL-6 correlate with the severity of the disease, with radiological joint progression (19), and with more cardiovascular disease (19). Other prognostic factors of disease activity are CRP and ESR.

Predictors of response to treatment – Most predictors of response to treatment are biomarkers (see section 3), but some other factors also exist. Poor response to anti-TNFα treatment can be predicted by a high disability at diagnosis, no initial NSAID or no concomitant MTX, smoking, female gender, older age, concomitant prednisolone, previous treatment with three or more DMARDs, high ESR, high tender joint, and a high Health Assessment Questionnaire (HAQ) score at diagnosis (47). Predictors of remission with anti-TNFα treatment are inversely related with a low HAQ score, age less than 53, and male gender. However, RF-negativity is a prognostic factor for remission (51), while in RF-positive RA patients, the DAS28 improved with anti-TNFα treatment (52). Poor response to rituximab is predicted by ACPA-positivity (IgM subtype in particular) at diagnosis, high levels of CD20+, and CD79+CD20- B-cells in the synovium (47). Better response to rituximab is seen in patients with RF-negativity. Better response to abatacept is predicted by ACPA-positivity, but, in comparison to other treatment options, RF-positivity has no prognostic value. Additionally, the response to abatacept is negatively influenced by prior anti-TNFα failures (47). Poor response to tocilizumab is predicted by high hemoglobin levels, a high DAS28-ESR score at diagnosis and

more previous failures of csDMARDs and bDMARDs (47). Remission after three months of treatment with tocilizumab is predicted by ESR levels of less than 30 mm/h and/or CRP levels of 10 mg/ml or more at diagnosis and extra-articular manifestations (47).

1.1.7 Prevention

The early application of DMARDs in early RA has already shown its benefits on the progression of joint damage, course of disease, extra-articular manifestations, disability, and quality of life (3). Here, I describe the possibility of preventing the development of RA in the preclinical or even the at risk-phase of RA. In this approach clinical RA can be seen as the "culmination of a whole series of well-established pathologic events" (53), and not as the beginning of the disease.

A problem for these preventive measures is how to define people who should be screened with autoantibody titers and people who are in a preclinical or even at-risk phase of RA (3). RF is also common in the normal population and autoantibodies are not always present in people who develop RA. Also, joint damage is rarely present in the preclinical phase of RA, but can best distinguish RA from other rheumatic diseases (7). Another issue is the sensitivity and specificity of current scoring systems (54): patients with low risk can score negative, but still develop RA, while patients with high risk and positive scores, might never develop RA. Despite the fact that the Study Group for Risk Factors for RA, which is part of the EULAR, made a proposal for a new nomenclature on the different phases of RA (55), the currently used ACR criteria (section 1.1.1) only focus on established RA (7). Different studies on the preclinical phase of RA are therefore not easily compared (3). Identification of high-risk individuals could be obtained through a combination of biomarkers and anamnestic information about family history, personal history of immune-mediated diseases, and environmental factors (54).

Even more, there are no official guidelines for primary prevention in RA and extra-articular manifestations other than some lifestyle measures (3): smoking cessation, dietary changes and weight reduction by dietary changes (53). Also, preventive measures of immune regulation of the oral and gut mucosa are suggested, but need more research. In general, periodontal care and treatment of periodontitis are relevant (53).

In theory, intervening in the pathogenesis of RA itself (antigen presentation and production of autoantibodies) could be preventive (3). Targeting B-cells with rituximab can be useful in a selected group of patients at-risk. Also, interfering with T-cells and antigen-presenting cells with abatacept can be investigated. Other explorable interventions are induction of tolerance by vaccination with dendritic cells, inducing autoantigen-specific Tregs, or desensitization with antigens (3).

1.2 BIOMARKER DISCOVERY AND OMICS ANALYSIS

Classification criteria are based on current evidence, but with omics analyses this knowledge will likely grow. The term 'omics' refers to the comprehensive study of molecules in a cell or organism (56). Therefore, a lot of omics analyses have been created in different research fields. Those which are well established in the literature are genomics, transcriptomics, epigenomics, and proteomics. More recently, metabolomics has joined this list (see section 1.3). The specific value of these techniques in RA are described more detailed in section 3.1. Semerano *et al.* (13) suggested that multilevel information from these techniques should be combined to identify biomarkers, followed by the development of new criteria to diagnose early and at-risk RA patients so patients can get preventive, instead of curative, treatment (7). This way, the economic, social, physical, and psychological burden of RA can decrease.

1.2.1 Exposomics

1.2.1.1 Definition

The exposome is a relatively new concept that was first defined as the "totality of exposures throughout the lifespan" (57), but the definition now consists of two elements (58): (1) the exposome consists of chemical and non-chemical agents (diet, stress, social, and behavioral factors) that are cumulatively measured, and (2) exposure (endogenous and exogenous) is measured quantitatively and repeatedly in series. The exposome thus provides a holistic measurement of all the environmental influences and exposures over a lifetime.

1.2.1.2 Value of exposomics

The genome alone cannot completely explain complex diseases (57). Understanding the interactions between the genome and the exposome can therefore help to understand disease etiology, trends and prevention.

Molecular epidemiology studies and regulatory agencies use traditional biological measurements (also known as targeted analysis) for quantification and longitudinal surveillance of known exposures in a population (58). Afterwards, this data can be used to identify subgroups with abnormal levels of exposure.

The hybrid approaches (see section 1.2.1.3) can be used for exposomic analysis and for metabolome-wide association studies (MWAS) that aim to quantify important chemicals for health and risk assessment (58).

1.2.1.3 Methods to study exposomics

The current methods to analyse the exposome are biomonitoring through traditional biological measurements and global exposomic approaches (58). Both methods use the

biomaterials described in section 1.3.3.4. The main difference between both methods is that biomonitoring aims to measure only potentially toxic agents, while exposomic approaches measure all exposures (endogenous and exogenous) of health significance.

Biomonitoring assesses exposure to certain agents that might represent a risk to human health through questionnaire data and ecological, environmental or biological measurements. The latter is preferred because the internal dose of an agent is measured. More specifically, traditional biomonitoring is the targeted analysis of particular chemicals, metabolites or reaction products in media like blood and urine. Despite the advantages of traditional biomonitoring (58), it measures mainly biologically persistent chemicals, so short-living chemicals can only be detected if they are continuously presented to the individual or measured at the time of exposure.

Exposomic approaches, also called exposomic biomonitoring or untargeted analyses, measure levels of all detectable chemicals using high-resolution metabolomics, mainly in blood and urine (58). The resulting exposure profile of an individual consists of the exposures themselves and the metabolic consequences of the exposures, such as psychological stress and other non-chemical stressors (*e.g.* noise), and nutrition. This method is similar to non-targeted metabolic fingerprinting (see section 1.3.3) and can be used for exposome-wide association studies (EWAS) that compare a large amount of chemical profiles of healthy and diseased populations gathered in databases (58).

As a part of exposomic biomonitoring, hybrid approaches such as semi-targeted analyses and suspect screening, are the preferred analytical methods (58). These utilize a combination of targeted and broader exposomic methods. When as-yet unknown chemicals are to be analysed, targeted methods cannot be applied because these cannot detect enough chemicals at once, or are limited primarily to stable chemicals (58). However, targeted methods are still valuable to assess a chemical, once discovered, with higher accuracy and depth than the broader exposomic methods. Although broad exposomic methods are first used to detect as many chemicals as possible, these are time consuming, expensive, unable to measure xenobiotics at low concentrations, and require larger sample volumes. Therefore, the number and type of agents that can be analysed remains limited.

1.2.1.4 Challenges of exposomics

The exact impact of the environment on human health remains largely unknown and is therefore essentially uncertain (58). Neither targeted nor untargeted methods currently obtain sufficiently correct and complete information. Additionally, suitable databases and associated bioinformatics tools to study the exposome do not yet exist (58).

A first issue of targeted analyses is that several potentially toxic chemicals are difficult to measure because of their lack of stability and/or their absence in the biomaterials used

(typically peripheral fluids such as blood, urine, etc.) (58). Pesticides and phthalates, for example, are only detected in urine if the individual was exposed in the days directly before sampling, so there is a need for continuous collection of samples. A second issue is the measurement of a large amount of these targeted chemicals, especially in biomaterials other than blood and urine because there are no standardized methods for these biomaterials. A third issue is the selection of the chemicals measured. Most measurements of common chemicals are based on a list of target chemicals from the Centers for Disease Control (CDC). Chemicals of potential concern are continuously added to this list. The first problem with the CDC list, is that it is based on simplicity and compatibility with the methods used. The second problem is that the level of some potentially toxic chemicals decrease because of the successful management of their release into the environment. The third problem is that the toxicity of some of these chemicals is doubtable and might be irrelevant to measure. A last issue is that different laboratories apply biomonitoring techniques in different ways, which means that results of studies are not always reproducible or accurate.

A key issue of untargeted analyses is the detection of chemicals at low concentrations, mostly xenobiotics (58). To increase their detection, semi-targeted or multiplex methods (hybrid approaches in exposomic biomonitoring) can be used.

1.2.2 Genomics

Genomics is the study of the genome of cells and organisms (59, 60). It was the first analytical method available for precision medicine. Large datasets of DNA sequences exist for diagnosis, risk prediction, and targeted therapy (56). The interest in genomics when studying RA derives from evidence that genetic factors are not only associated with the genetic predisposition to develop RA, but also with disease progression, outcome, and phenotype (61).

Genomics consists of DNA-sequencing techniques that allows candidate gene and SNP genotyping, followed by genome-wide association studies (GWAS) and subsequent meta-analysis of the GWAS datasets.

A challenge is the frequently unknown specific disease-causing genes or sequence variants, and the mechanism of the disease caused by this sequence (56). Strategies other than genomics that have tried to give scores to a combination of genes without knowing the causative gene, have not been successful. In addition, diseases caused by multiple gene mutations (including RA), are not predictable with single or multiple gene biomarkers (47). Also, GWAS can be biased because of differences in the study population, such as race, geography, and ethnicity, which can overshadow a difference in health status (16). It is thus important to select a study group with similar race and genetic structure. In RA for example, GWAS results are applicable only for seropositive patients with white European heritage. In

addition, SNPs are not necessarily causal and thus do not have a high value in risk classifications for diseases.

1.2.3 Transcriptomics

Transcriptomics analyses the expression of genes of cells and organisms by determining messenger (m)RNA levels through RNA sequencing and array-based technologies (59, 60). Large-scale RNA sequencing is possible thanks to next-generation sequencing (60). Transcriptomics provides more biological insight in RA (61) or diseases in general, which makes it a tool for risk assessment, diagnosis, and prognosis of diseases (56). Alterations in gene transcripts can lead to an imbalance of tolerance, activation of immune cells and a loss of control over the immune responsiveness (61).

A challenges is the dynamic process of gene expression depending on the stage of disease, time course of treatment, type of tissue, cell type, and other influencing factors (62). Therefore, in RNA sequencing, samples must be taken at the same time and from the same source to have a useful outcome. Also, although transcriptomic analysis is mostly done on peripheral blood in RA patients, researchers find the use of synovial tissue more accurate. This brings the problem of having to use invasive means to obtain biomaterials, so research on more accurate ways to investigate more easily accessible samples is required.

1.2.4 Epigenomics

Epigenomics is the study of the regulation of gene transcription and takes part in both the genome and the transcriptome (59). It may serve as a link between exposomics, genomics and transcriptomics. The study of this interactions is part of a more holistic approach, called systems biology (see also section 1.2.3). The environment influences gene transcription through epigenetic factors such as DNA methylation, post-translational modifications of histones, and expression of micro (mi)RNAs and long non-coding (lnc)RNAs (56, 61). When the promotor region of a gene is methylated, transcription is suppressed (56, 61), while transcription is facilitated in case of histone acetylation, which is the most common post-translational modification of histones (61). Sequencing-like techniques are used to study epigenetics at the genome level and histone modifications are typically studied using proteomics methods (see below). Genome modifications are heritable, making this an interesting field for research on the etiology of diseases. With this information, therapeutic options can be developed to interfere with specific epigenetic mechanisms in the clinical, but also in the early and at-risk phase of RA. In the transcriptome, miRNA regulates protein expression by binding similar mRNA sequences. Because alterations in miRNA were found in RA patients compared to a control population (61), miRNAs can be used as biomarkers for follow-up and monitoring responses to treatment (61). These are detected traditionally with

northern blotting, reverse transcription polymerase chain reaction (RT-PCR), and microarrays (61).

1.2.5 Proteomics

Proteomics includes techniques to determine the protein content and composition of cells and organisms (59). Originally, it was performed using high-resolution gel electrophoresis and mass spectrometry (MS), but this has now been superseded by gel-free (or peptide-centric) MS analysis, which can identify and quantify thousands of proteins in one sample (60). Other important methods that are based on protein analytics are immuno-phenotyping and flow cytometry. Olivier *et al*. (56) also mention affinity-based protein arrays as a commonly used technique, and additionally nuclear magnetic resonance (NMR) and X-ray crystallography as complementary methods to define the structure of proteins and protein complexes in cells and tissues. However, there is no methodology available to assess all aspects of the proteome, because of the complexity and diversity in posttranslational mechanisms (56).

The proteome is not a mere translation of mRNA (56). First, posttranslational modifications such as phosphorylation provide activity or signaling control at the protein level. Second, folding and posttranslational processing of pre-proteins, and the formation of multi-protein complexes is often needed before a protein (or group of proteins) can execute certain, or all, of their cellular functions. Finally, the localisation of the protein within the cell determines its final activity. Because proteins are the targets for nearly every therapy, understanding these mechanisms is an important step in the research, prevention and treatment of diseases. Proteomics can thus have an important role in RA, by detecting proteins for early diagnosis, but also because of the therapeutic relevance of proteins (61).

The enormous diversity of the proteome, coupled with the limited analytical resolution of the current approaches necessarily limits our view on the actual proteome in a sample.

1.2.6 Systems biology

1.2.6.1 Definition

Systems biology is a holistic approach for the study of living systems in which several omics analysing methods are combined (59). For example, extracellular vesicles that are influenced by extracellular cell-cell communication can change intracellular gene expression (inflammation, cell proliferation) (1), or environmental factors (exposome) can change the acetylation pattern of several genes (epigenome) and thus change transcription (transcriptome), which in turn results in a change in the proteome and metabolome of an organism (56).

1.2.6.2 Value of systems biology

Exposomics, genomics, transcriptomics, proteomics, and metabolomics complement each other in the comprehensive analysis of biological systems in health and disease (8, 58, 63). Epigenetics, metabolomics, and the study of the oral, respiratory, and gastrointestinal microbiome may provide new biological mechanisms to link genetic and environmental risk factors in the pathogenesis of rheumatic diseases (16). Organism-environment-interactions can thus help explain disease mechanisms (16, 64). As such, many of the biomarkers discovered to predict treatment responses are cellular markers identified by immunohistochemistry, synovial cytokines, chemokines, and gene-expression profiles (1).

1.2.6.3 Methodologies of systems biology

The methodologies of systems biology consists of the integration of data obtained through exposomic, genomic, transcriptomic, epigenomic, proteomic, and metabolomic research. Several tools and algorithms are being developed to address this challenge. Cambiaghi *et al.* (65) reviewed several such software packages that are useful for experimental researchers.

1.2.6.4 Challenges of systems biology

The integration of the enormous amounts of data obtained across different high-throughput methods remains a problem (65). While information from the different omics fields is readily available, the entire process from handling to integrating this information requires specialized tools that are not yet mature. Statistical and bioinformatics aspects need to be improved further to enable substantial progress in systems biology approaches to complex diseases.

1.3 METABOLOMICS

In analogy with the other analytical methods, I define metabolomics, explain its possible value, elaborate the analytical techniques, and list current challenges in metabolomics.

1.3.1 Definition

The modern approach of metabolomics dates from only two decades ago. Both metabolomics and metabonomics are used, which are either considered linguistically different (59), or considered different terms. Priori *et al.* (63) distinguished metabolomics and metabonomics, respectively, as: “the nonbiased identification and quantification of all metabolites in a biological system”, and “the quantitative analysis of metabolites in response to biological perturbation (*e.g.,* disease or therapeutic treatment) or genetic modification”. Generally, metabolomics is defined as the comprehensive and systematic identification and quantification of small molecules (metabolites) in a biological sample at a specific moment

(59). Some studies describe metabolites as molecules of less than one kiloDalton (kDa) (8), while others refer to molecules of less than 1.5 kDa (59). In general, the metabolome consists of endogenous metabolites such as carbohydrates, amino acids, oligopeptides, organic acids, nucleotides, or lipids, and of exogenous molecules (xenobiotics) such as drugs, food, toxins (66), and other molecules introduced and modified by environmental exposure and coexisting organisms (8). These molecules are intermediates of biochemical processes that occur in living organisms (8), hormones, other signaling molecules, and secondary metabolites (63).

1.3.2 Value of metabolomics

The metabolome is seen as the reflection of the current biochemical status of the organism and represents underlying changes in genome, transcriptome, and proteome (59). In the perspective of systems biology, the metabolome shows the association between the functions of specific genes, and the impact of the metabolome on the activity of proteins and genes (8). It is therefore said that metabolomics can serve as the link between genotype and phenotype (59). It can measure short and rapid responses of the metabolic pattern to any physiological change in the organism, which can in turn provide a greater understanding of the mechanisms of disease (8, 59). This has already been proven useful in cancer, diabetes, and cardiovascular and pulmonary disorders (59).

Thanks to metabolic biomarkers, metabolomics offers an efficient method to diagnose diseases, to differentiate between disease subtypes based on disease activity (8, 59), to make a prognosis based on several prognostic markers, and to detect metabolic changes before symptoms occur (59), thus in the preclinical phase. Additionally, biomarkers can be used therapeutically to predict the response to a particular treatment approach (8).

1.3.3 Methodologies of metabolomics

Here, I describe the approaches and methods to measure metabolites, the way the resulting large amount of data is collected in databases, and their statistical analysis, the biomaterials used for metabolomic research, and the current contribution of metabolomics to our understanding of human health and disease.

1.3.3.1 Data acquisition

Three approaches are frequently used to obtain data: metabolic fingerprinting, metabolic profiling, and metabolic footprinting (59). Each approach uses a different order or combination of methods to obtain data and achieve its goals (63).

Fingerprinting, a non-targeted approach, refers to an initial differentiation based on an unbiased, detailed and reproducible analysis (8). It consists of the detection of the complete metabolome or of panels of several substances (*e.g.* lipids, including phospholipids, amino compounds, sugars and bile acids) without focusing on a specific compound (8). It is used for

the classification of samples and as a screening tool to discriminate between samples of different conditions (*e.g.* biological status or origin). While this fingerprint represents many of the diverse compound classes of metabolomes (8), there is no universal analytical platform to determine the entire fingerprint (59).

Metabolic profiling, a targeted approach, identifies and quantifies metabolites that have been selected *a priori* based on the similar biochemistry of known metabolites (*e.g.* carbohydrates, amino acids, organic acids, nucleosides), the same biochemical pathway, and/or previous non-targeted studies (8).

Metabolic footprinting is more often used in microbiological or biotechnological studies (59). This particular approach will not be discussed any further here, because it has little direct relevance for the perspective of this thesis.

There are two main methods to measure the metabolome in biofluids and –tissue and both methods can be used in targeted and untargeted approaches to acquire data. MS measures ionized molecules based on their mass-to-charge ratio (59), while NMR provides one- or two-dimensional structural information. 1D-NMR targets the proton (H1) alone to identify metabolites, whereas 2D-NMR targets carbon or nitrogen isotopes along with H1 to increase specificity of metabolite identification. The latter has the capacity to identify unknown metabolites and determine the structure of new molecules, such as drugs or even small proteins. Ideally, a combination of methods is used in a multiplatform approach (66).

Each technique has its positive and negative aspects. NMR and MS both need a minimal amount of fluid or tissue (less than 1 ml for liquids and 1 mg for solids) and can measure tens to hundreds of metabolites in spectroscopic patterns (8). Compared to MS, NMR does not require much sample handling and is non-destructive, so multiple analyses can be done on one sample (8). MS is also more expensive, less reproducible, and more difficult (66), more platform dependent, and susceptible to variability (8). It requires sample pretreatment, which consists of the separation of metabolites into different classes of components by chromatography (63). Specific applications of chromatography on different biomaterials are liquid chromatography-MS (LC-MS), gas chromatography-MS (GC-MS), and capillary electrophoresis-MS (CE-MS) (59). Despite these challenges for MS, it is superior in sensitivity and is therefore more frequently used (59). A combination of both techniques, called LC-NMR-MS, combines high-throughput analysis of NMR with the high sensitivity and resolution of LC-MS (59).

LC-MS uses MS after separation of metabolites by high-performance LC (HPLC). It is widely used and applicable for non-volatile, thermally unstable, high-, or low-molecular-weight compounds with a wide polarity range (urine, blood, and tissue extracts). This technique is faster than GC-MS, because it does not require the derivatization step (59). It distinguishes

between metabolites based on their chemistry in the stationary phase in the chromatographic column (59).

GC-MS uses MS after separation of metabolites by high-resolution capillary columns instead of HPLC (59, 66, 67). It is applicable for volatile and thermally stable metabolites, for which a long and complex derivatization is necessary. This can cause metabolite loss during the procedure. Metabolites can be identified through comparison to structural and mass spectral libraries that are universally available, and this process is powered by the high reproducibility of the approach.

Due to recent improvements, the use of CE-MS in metabolomic research steadily increases (59). It is applicable for water-soluble and charged molecules, so its value lies in complementing the other methods. Urine samples are already being analysed, and a lot of research effort is now being put into the analysis of serum samples.

1.3.3.2 Databases

The results obtained by the metabolomics methods are assembled in online databases, digital libraries containing data on metabolite concentration, obtained by different methods in different biomaterials of different species under different physiological or disease conditions (66). Several databases already exist and each database is constructed for a specific research question (58). Even though the Human Metabolome Database (HMDB) lists more than 41 000 metabolite entries in its latest version, only 3000 have been associated with diseases to date. Other metabolomics databases include the Kyoto Encyclopedia of Genes and Genomes (KEGG), Lipid Maps, PubChem, ChEBI, Metabolite and Tandem MS Database (METLIN), and the Madison Metabolomics consortium database (59, 66). The largest reference databases are METLIN and HMDB (58).

1.3.3.3 Statistical analysis

As metabolomic data is most valuable in combination with genomic, transcriptomic, epigenomic, and proteomic data, and because of the complexity of biological samples and the sensitivity of analytical techniques, a multivariate data analysis is suggested in statistical research of metabolomic data (59). A multivariate approach creates a holistic view on the biological system. First, the data obtained by MS or NMR is coded, which means that each variable of one sample or patient is given a number, identifying its characteristic (63). Second, the combination of all variables of each sample or patient is arranged into matrices that can be analysed by multivariate statistical techniques (63).

Two techniques are used to analyse differences between patients to distinguish pathologies, or differences between samples of one patient, to discover the evolution of its metabolome: principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) (63). Correlation analysis is at the core of both these techniques. PCA

defines new coordinates that represent a linear correlation between metabolite levels of a known variable and an experimental variable from MS or NMR data. Such a coordinate is thus a 'biomarker' for a relationship between biological substances (metabolic profile) that changes in different known conditions (*e.g.* health and disease). PLS-DA associates the experimental matrix (MS or NMR) to a response matrix that is known (the class or pathology of each sample or patient), to classify an unknown sample. The response matrix thus discriminates the experimental data (discriminant analysis). For more detailed information about these statistical techniques, I refer to other publications (65, 68).

1.3.3.4 Biofluids and other biomaterials

The type of material in metabolomics largely determines the techniques employed (59). Typical biomaterials that are often studied are blood (plasma/serum), urine, saliva, tissue extracts, and exhaled breath (59).

Blood metabolites provide a lot of information about the physiological and pathophysiological state of an organism (59). Metabolomic analysis of blood samples has been successful in diagnosis, prognosis, and management of breast cancer (69). It also gives a possibility to discriminate pancreatic from biliary tract cancer, and to develop screening biomarkers for cancer in general (70).

Saliva contains enzymes, cytokines, hormones, antibodies, lipids, amino acids, and nucleic acids for its physiological functions (59). Many of the metabolites in saliva have been transported from blood by passive intracellular diffusion, by active transport, and by extracellular ultrafiltration, causing a similar value for those metabolites in blood and saliva. In contrast to blood, saliva is noninvasive to sample, easy and quick to obtain, low in cost, and stable during short- and long-term storage.

Urine metabolomics has similar advantages to saliva (59), but midstream urine after perineal cleansing is preferred, and in contrast to blood and saliva, metabolite levels should first be normalized (mostly versus creatinine concentration, urine osmolality or locally weighted scatterplot smoothing (LOESS)). Normalization is not necessary with a 24-hour urine sample, but this technique consumes more time and is more difficult to perform.

Exhaled breath sampling is also noninvasive, easy and quick (59). Exhaled breath contains volatile organic compounds (alcohol, hydrocarbons, ketones, aldehydes, esters) and can thus be analysed with metabolomics.

Tissue sampling is more difficult and invasive and metabolomic analysis of tissue extracts is more difficult, because of the complex pretreatment procedure (59). Most tissue samples are therefore analysed with proteomics.

1.3.4 Challenges of metabolomics

Although metabolomics has made numerous positive contributions to research, some challenges are still encountered. Gupta *et al.* (66) mention that metabolomics measures the average or overall status of metabolites, but different cells may be in different phases in a pathway, and may have different, even antagonistic pathways operating at the same moment. This would mean that some metabolites are not measured, causing a gap in information. They also note that a dynamic process cannot be measured in one sample, because the metabolome is very sensitive to minor changes in the environment, such as diet, drugs, time of day, and more obvious but unavoidable factors, such as age and gender. This causes a risk of over-interpreting changes, and by consequence the need for analysis of timed and controlled sample collections with appropriate age, gender, and environmentally matched controls.

Despite the large amount of data, the composition of the metabolome is not fully defined (59). An additional problem is that the human metabolome's size is possibly overestimated, because of the influence of diet and drugs, but also because of contributions of the gut microbiome (59).

Many software packages exist to analyse and integrate metabolomic data, but there are still some problems in this field. In three recent publications (65)77, 78), a structured summary of tools, software, and databases developed for primary metabolomics was assembled, in an attempt to create a clear perspective on the different possibilities and to illustrate the need for more powerful, and more standardized methods in metabolomics.

1.4 THE INTENTION OF THIS THESIS

Metabolomics can play a crucial role in precision medicine thanks to better biomarkers for diagnosis, monitoring, prognosis of disease, and predicting drug responses (66). Also, the discovery of new metabolites and pathways in disease pathogenesis can open up a path to prevention and early diagnosis and targeted drug development (66). Another advantage of metabolomic biomarkers is the possibility to obtain much information in a non-invasive way from, for example, a blood sample, saliva, or breath (47).

In this thesis, I specifically focus on the value of metabolomics in RA for several reasons. RA is intensively studied and is a common and debilitating disease. Moreover, I have witnessed the onset and effects of RA in my own family, which made me extra interested in studying this specific disease. Because RA is a very complex disease, it is difficult to increase our knowledge of its mechanisms and progression, which in turn means that we are not yet able to, for example, prevent and cure this disease. This is why metabolomics drew my interest, as it might be of substantial value in our search for understanding RA, and to deliver diagnostic, preventive, and treatment options for RA.

In my opinion, it is unlikely that metabolomics alone will succeed in explaining all questions about RA, because the complexity of the disease requires a holistic approach. Therefore, I present an overview of the current knowledge on RA in relation to exposure and environment (exposomics), in the other omics research fields, and in the systems biology approach. I end this thesis with the promises and challenges of metabolomic research in the study of RA.

2 METHODS

For the introduction section, I focused on finding reviews on PubMed. I searched for the most recent recommendations for RA and the most relevant reviews of each topic in the omics technologies, to have a concise and up-to-date starting point. MeSH terms used are 'Rheumatoid Arthritis', 'Metabolomics', 'Rheumatology' and 'Precision Medicine'. From there, I started to select more detailed topics and structured these into subtopics, resulting in the current structure of this thesis. Additionally, I turned to Google Scholar and Web of Science for additional articles. When publications were not directly available, I turned to Ghent University Library to order and consult these publications.

To construct the results section, I studied articles referred to by relevant reviews, as well as additional articles directly found on PubMed, Google Scholar, Web of Science, or Embase with more specific search terms. The structure of the results section of this thesis follows the structure of the introduction, aiming to answer the main question 'Can metabolomics provide promising perspectives for future patients with Rheumatoid Arthritis?'

The discussion and conclusion sections were created during the research process. While writing and reading, I gathered important information that I wanted to point out and discuss in more detail, and selected the highlights that I wanted to include in the conclusion.

All references are combined in the reference section in chronological order of mention. The reference style is Vancouver, conform to the guidelines of the Faculty of Medicine and Health Sciences at Ghent University.

3 RESULTS

Here, I describe new perspectives on RA thanks to biomarkers obtained with several omics analyses giving a brief overview of biomarkers of RA that are part of the exposome, genome, transcriptome, epigenome, and proteasome, followed by a view on systems biology and a more extended focus on metabolomics.

3.1 BIOMARKERS OF RHEUMATOID ARTHRITIS AND OMICS ANALYSIS

3.1.1 Exposome

Environmental factors for very early risk of RA include high birth weight, obesity, lower economic status (53) and lower educational attainment (14). Exposure to ultraviolet light and silica dust also increases the risk of RA (53). In contrast, protective factors appear to be breastfeeding and moderate alcohol intake.

In the past, a high body mass index (BMI) was suggested to be only an influencing, but not an etiologic risk factor for RA (53, 71). In contrast, two recent studies mentioned a causal association between high BMI and the risk of RA (72, 73), which is stronger in women (73). Obesity negatively influences disease activity, patient-reported outcome during therapy, and the remission rates (71). The role of diet is intensively studied in prevention and alteration of disease activity of RA, because many (if not all) patients are interested in a way to control their disease (74). A vegan diet may be beneficial because of the antioxidant constituents, lactobacilli and fibers, and changes in intestinal flora (74, 75). Another protective diet is the Mediterranean diet, because omega-3 polyunsaturated fatty acids (PUFAs) and vitamins, and their influence on the gut microbiome, are anti-inflammatory. Gluten-free and elemental diets show some benefits, but these are still unclear. Fish and other sources of long-chain PUFAs are protective for development of RA. Also, vitamin D decreases disease activity. More research is needed on the role of fasting, anti-oxidant supplementation, flavonoids, probiotics, and the role of fish oil and vitamin D supplementation. While moderate alcohol intake is protective against RA development, and associated with a lower systemic inflammation (76-78), a recent study did not show improvement on local joint inflammation on MRI (77).

Air pollution in general is a risk factor for developing RA. Non-smoking patients, in particular, are more at risk to develop RA when they live within 50 meters of a highway (79). Occupational inhaled pollutants are a risk factor for men working in the metal, mining, and construction industry (80). A larger risk has been found for people exposed to silica dust, who also smoke (79).

Reproductive factors and hormonal exposure have also drawn some interest in the development of RA (section 3.2). Early age at menarche and irregular menstruation negatively

influences the development of RA (81). While the prevalence of RA during pregnancy is low, there is an increased risk for seronegative RA three months until two years postpartum, or in women who are at a young age at first birth (82).

In the preclinical phase, there is an increase of antibodies caused by several factors that precede, but contribute to autoimmunity development (53). In addition, smoking contributes to cardiovascular disease and other extra-articular manifestations (21), and it reduces the response to several DMARDs (21). In contrast, an etiologic role of the lung mucosa independent of smoking is suggested, because lung parenchymal abnormalities are more frequent in ACPA-positive than in ACPA-negative early RA patients (53).

There is an etiologic link for RA with the condition of the oral mucosa when the patient is a current smoker and has susceptible genes (53). Periodontal disease (PD) caused by the bacterium *Porphyromonas gingivalis* in particular, is frequently observed in the personal history of patients with RA. *P. gingivalis* causes citrullination of arginine residues, and triggers ACPA levels in lung mucosa in a similar way as smoking (14, 53). Food containing citrullinated proteins can trigger ACPA in the presence of *P. gingivalis* (83). Citrullinated proteins can be derived from viruses, bacteria, fungi, and plants (83). It should be noted that only ACPA-positive RA patients show this link between RA and PD, but as saliva is easy to obtain, this link can be of future research interest.

An intestinal dysbiosis is seen in early disease (53). Whether this is a consequence of the CRP and ACPA status of RA patients (84), or an etiologic factor for autoimmunity, remains unclear (53).

3.1.2 Genome

According to Castro-Santos *et al.* (61), genetics explain the risk of RA for 50% especially for the ACPA-positive RA patients, of which 80% have HLA-DRB1, compared to 49% of the ACPA-negative RA patients. Additional elements of the genome that can explain RA have been identified through GWAS (61) resulting in a total of 101 RA non-HLA risk loci (85-87). SNPs currently explain a small amount of the variability of RA-susceptible twins (88).

Although some of the genes found to be associated with RA are already targeted by existing drugs, this was not known when these drugs were developed (61). Therefore, focusing drug discovery on gene signatures is a founded option (89).

Dennis *et al.* (90) identified four phenotypes using gene expression profiles of synovial tissue from patients with clinical RA. However, patients did not strictly show one affected biological process, which suggests a continuum instead of a strict distribution of phenotypes. In order to put targeted gene-based therapy decision into practice, serum biomarkers are needed to easily identify the relevant phenotypes.

3.1.3 Transcriptome

Measuring response to treatment by investigating the genome-wide transcriptional effects can help to find characteristic RNA sequences and mechanisms of diseases, including RA. Walsh, *et al.* (46) studied tDMARD treatment of MTX, sulfasalazine and hydroxychloroquine, and characterized RNA sequences for RA. They show that tDMARD downregulates genes involved in T-cell activation and signaling, and in plasmablast/plasma cell differentiation, but the specific biological pathways remain unknown (15).

The type I IFN signature, which is an increased expression of several IFN genes, increases the predictive value of ACPA and RF to define the risk of RA, and is an independent risk factor for RA (53, 91). Additionally, a low B-cell signature together with a high IFN I signature is predictive of an even higher risk of RA than the IFN I signature alone, and a high B-cell signature is protective (53). Patients with the abovementioned IFN I signature also show a good response to bDMARDs, including TNFα-inhibitors, tocilizumab, and rituximab (62).

3.1.4 Epigenome

Despite the genetic risk (14, 54), epigenetic regulation and interactions between genes and environmental risk factors are more important for RA prediction (54). Epigenomics causes the variability in symptom severity, remission and relapse rates, response to therapy, and progression over time in twins, but also the global increase of RA prevalence (88).

Methylation signatures, for example, can differentiate between RA and Osteoarthritis (61). Also, inhibiting enzymes that cause histone deacetylation, can change the immune response in RA, because histone deacetylation controls the development of Treg cells (92). miRNAs are key regulators of lymphocytes, macrophages and synovial fibroblasts. Although, the therapeutic utility is still unclear (93), miR-146a in synovium is associated with increased RA disease activity, and an increase of miR-155 in circulating mononuclear cells is associated with RA (47). Also, miRNAs repress the translation of TNFα in a negative feedback loop during the disease (15, 18). Epigenetic changes in FLSs of the synovial fluid, reviewed by Doody *et al.* (61, 88), can be of use in early diagnosis, prognosis and targeted therapy.

3.1.5 Proteome

An extensive immunophenotyping of different Th cell subsets in lymph node tissue and peripheral blood samples obtained from RA risk individuals, early RA patients, and healthy controls, suggested that not Th1 cells, but Th17 cells play the main role in RA (17). In contrast, citrulline-specific Th1 cells are increased in peripheral blood of RA patients and their frequency is influenced by disease duration and therapy (94).

Elevated levels of inflammatory cytokines and chemokines and adaptive immune system activation are predictive of RA development (54). Studies generally conclude that the overall increase of cytokines is more predictive than individual cytokines and that a higher level

of cytokines is correlated to a closer onset of RA (54). In contrast, most CD4+ T-cells produce a decreased amount of cytokines in lymph nodes in preclinical and clinical RA patients, while still maintaining B-cell antibody production, possibly due to exhaustion of T-cells (17).

The incomplete resolution of lymphocytic infiltrates after the combination treatment with infliximab and MTX, indicates that TNFα-independent pathways are active in RA (95) and explains the low grade disease activity in RA patients that received this treatment.

The potential of anti-carbamylated protein (anti-CarP) antibodies as early diagnostic and prognostic factors for RA has been troubled by two conflicting reports (96). Whereas anti-CarP antibodies were detected in ACPA-positive and -negative patients in the one study, no anti-CarP antibodies were present in ACPA-negative patients in the other (96). Additionally, whereas anti-CarP antibodies were found to be less sensitive and specific than ACPA and RF in the first study (104), a higher specificity was found for anti-CarP antibodies than for ACPA in the second (96).

A recent study has determined antibody isotypes that increase the sensitivity of diagnosis based on serum for seropositive and seronegative RA patients (97).

The general response to any therapy is a decrease in synovial macrophages, detected by immunohistochemistry (1). However, Kim and Moudgil (18) showed that an increase in Th1 and Th17 cells in the peripheral blood is seen in response to anti-TNFα therapy. This does not correlate with an increase in joint inflammation because the migration of these cells to the joint is inhibited. Also, the response to MTX and prednisolone can be monitored by measuring synovial lymphocytes (1). The response to infliximab is represented by synovial lymphocyte aggregates (98). Leflunomide and MTX modulate the synovial tissue inflammation and metalloproteinases expression in patients with active RA (99).

New therapeutic options can also be developed thanks to proteomics. For example, peripheral blood mononuclear cells carry mGCR and are upregulated in RA, so these can be a target for new, (more) specific GCs (43).

3.1.6 Systems biology

Although many more possibilities would arise if a multivariate approach of all omics analyses would be possible (60), some examples where omics techniques have been combined are already available. By comparing proteomics and metabolomics insight has been gained in energy metabolism disorders, as a contributing factor for RA (100). Exposomic and epigenomic research combinations can potentially reveal promising links in the pathogenesis and thus the possible prevention of RA (section 3.1.1 and 3.1.4). Several environmental factors change the hormonal status (menstrual cycle, menopause, stress, GC use, oral contraception, *etc*.) and can trigger RA development. A genetic polymorphism causing an altered function or level of these sex hormones can thus contribute to RA development (101). For instance, the

effect of breastfeeding on RA development is protective, but there is a group of patients with severe RA, because their breastfeeding was combined with a genetic susceptibility (a linkage between HLA-DRB1 alleles and the prolactin gene on chromosome 6) (102).

Hypotheses on the causes of lipid changes detected in RA are based on systems biology. For example, the association of particular SNPs with changes in LDLc levels (103), the correlation of PTNP22, TRAF1/C5, STAT4, and HLA-SE with different lipid changes, the link between regulatory genes of immune functions and lipid changes (104), or the correlation of high-density lipoprotein cholesterol (HDLc) with vitamin D deficiency, which is related to a decreased Treg production and an inflammatory environment (105).

3.2 METABOLOMICS AND RA

Based on the idea that the serum metabolome reflects the overall condition of an organism, there is a growing amount of studies focusing on metabolomics analyses of RA. One single biomarker is extremely unlikely to define RA, but thanks to statistical analysis, patterns of metabolomic changes could in the future diagnose and classify RA, distinguish RA from other diseases, and predict the risk for RA, disease outcome, response to therapy, and disease activity. In this section, I therefore give an overview of the known metabolic changes which can bring new perspectives for future patients with RA.

3.2.1 Diagnostic biomarkers

To understand the large amount of changes in molecules, I give a short explanation on relevant metabolic pathways. Then I describe preclinical and clinical biomarkers, and biomarkers to distinguish RA from other diseases.

3.2.1.1 Metabolic pathways

The healthy metabolism is complicated and so are the changes of metabolism in RA due the changes in total energy expenditure, resting energy expenditure, and physical activity. Resting energy expenditure is 12% higher in RA patients, while their physical activity is much lower, and their metabolic rate is 8% higher (105).

In normal proliferative conditions, energy in the form of ATP is mainly obtained by glycolysis for pro-inflammatory CD4+ T-cells or by producing mitochondrial ATP (106). However, metabolic stress and nutrient deficiency leads to a shift to the catabolism of cell organelles and proteins, providing amino acids and other substrates for alternative pathways, and to survival mechanisms that protect mitochondria and the reducing environment of the cell. Yang *et al.* (100) confirmed an enhanced anaerobic catabolism and reduced aerobic oxidation of glucose and fatty acids (FAs) in synovial fluid. This leads to an acidic environment, which damages the joint and thus could explain RA development and progression.

An important survival mechanism in cells is the pentose phosphate pathway (PPP) (106). This pathway provides ribose-5-phosphate for energy and NADPH to reduce oxidative stress. However, inflammation can cause oxidative stress with the depletion of NADPH and CD4+ T-cells of RA patients upregulate the PPP so that NADPH levels stay normal. Other elements that reduce oxidative stress are glutathione and cysteine and these are decreased in peripheral blood and synovial T-cells of RA patients.

Narasimhan *et al.* (107) gave some interesting insights in amino acid metabolism as studied in cancer, to better understand the changes in RA. In general, several amino acids play a crucial role in protein, lipid, and other biosynthetic pathways, and in providing energy for mitochondrial metabolism, for example in the tricarboxylic acid cycle (TCA) cycle for lymphoid cell proliferation and survival. Especially glutamine, glutamic acid, proline, aspartate, and alanine are important. Valine, leucine, and isoleucine metabolism has shown to be increased in tumor cells. Although serine and threonine are nonessential amino acids for energy provision, these nevertheless are important substrates in the anabolic pathways of glutathione, nucleotides, phospholipids, and other compounds. Also, these are important for protein synthesis and support cell growth and proliferation.

3.2.1.2 Preclinical diagnosis

According to Young *et al*. (108) useful biomarkers of inflammation in early RA are CRP together with lactate and lipid changes, although the sensitivity and specificity of these biomarkers are low. Metabolic profiling also proved to be of value for ACPA-negative patients with preclinical or clinical RA, who would have been missed with the ACPA test alone (109). Although the specificity of metabolic profiling is lower than the ACPA test, the sensitivity is 93%. In another study, glutamic acid, related to early bone erosion, has been found to have an equal value to RF and ACPA (110).

Lipid and metabolic changes occur in blood before RA becomes clinical (105). These changes are an increase in lysophosphatidylcholines, tryptophan metabolism, disturbed FA β-oxidation, and oxidative stress (110). Mysoedova *et al.* (111) compared non-RA lipid profiles to lipid profiles from a period of five years before onset of RA. Total cholesterol and LDLc levels decreased in this period, in contrast to HDLc and triglycerides (TG). The hypothesis for the decrease in lipids is an increased catabolism or increased subendothelial deposition.

Steroids also show important changes. Risk factors for RA are hypogonadism for men (79), and low cortisol levels which are pro-inflammatory (110). Women with preclinical RA who also have low cortisol levels, show adrenocortical insufficiency, reflected by lower androstenedione levels compared to healthy controls (112). Additionally, contradictory information of the effect on estrogens exists. On the one hand, women with decreased estrogen levels (postmenopausal or taking anti-estrogen agents) are more at risk of RA and

high estrogen levels (oral contraception and hormone replacement therapy) are associated with protective effects (79). On the other hand, every situation in which estrogen levels increase could be a trigger for an inflammatory response and the development of RA (113). Such a situation could be: an exposure to environmental estrogens, polymorphisms of genes coding for hormone metabolic enzymes or receptors, gonadal disturbances causing stress system activation *via* the hypothalamic pituitary adrenocortical (HPA) axis, and physiological changes of the menstrual cycle, pregnancy, the postpartum period, and menopause (113). The risk is higher with a younger (less than 45 (102)) age of onset of the menopause (79, 114). It becomes even more complex, however, because some studies associate early menopause with seropositive RA (114), while others found the association with seronegative RA (79, 102). And oral contraception use shows different information depending on the doses, and types of response of the cytokine balance in Th1 cells (101).

3.2.1.3 Clinical diagnosis

Zhou *et al.* (115) studied serum metabolite profiles of RA patients and healthy controls. They found 35 significantly different metabolites that suggested highly active glycolysis metabolism, TCA cycle, urea cycle, amino acid metabolism or FA metabolism. In similar studies, Li *et al.* (110) found most differential serum metabolites to be part of protein synthesis, linoleic acid metabolism, and glutathione metabolism, whereas Madsen *et al.* (109) found increases in glyceric acid, D-ribofuranose and hypoxanthine, and decreases in histidine, threonic acid, methionine, cholesterol, asparagine and threonine in serum of RA patients compared to healthy controls.

Yousri *et al.* (116) studied plasma of RA patients and found 32 metabolites with a 91.6% sensitivity and 88.4% specificity. A large amount of steroids were discovered, together with alterations in amino acid metabolism, FA metabolism, purine metabolism, and the decrease of a xenobiotic, iminodiacetate.

When 4-methoxyphenylacetic acid, L-phenylalanine, and L-leucine would be combined into a biomarker, RA could be diagnosed with a sensitivity of 93.30% and a specificity of 95.20% (110). Similarly, ornithine, citrulline, succinate, fumarate, asparagine, lysine, and glutamine are suggested to be major biomarkers of RA (117).

Lipids – Serum free fatty acids (FFA) and glycerol, both part of the FA metabolism, were increased in RA patients (115). Significantly changed FFAs were palmitelaidate, oleate, trans-9-octadecenoate, cis-5,8,11-eicosatrienoate, docosahexaenoate, 2-ketoisocaproate, and 3-methyl-2-oxovalerate. Some of these FFAs are pro- and some anti-inflammatory. Docosahexaenoate, for example, has protective effects and counteracts pro-inflammatory eicosanoids (115). In contrast, capric acid (also called decanoic acid), which normally reinforces the immune system, is decreased in RA patients (110).

ACPA and RF were negatively correlated with serum TG, and in male patients, positively correlated with LDLc levels (118). Therefore, clinical diagnosis could be ameliorated through lipid measurement.

Synovial fluid normally consists of hyaluronic acid, interstitial fluid and a low number of cells (23). Lipoproteins and apolipoproteins are found in very small amounts in synovial fluid in healthy populations. However in RA, many changes to the lipid profile of synovial fluid have been described. An increase is seen in prostaglandin levels as well as a change in phospholipid composition (23), and cholesterol, lipoprotein, and apolipoproteins A-I, B and E increased (105). PUFAs, hydroxylated FAs, and lipoxygenase products are found in synovial fluid of RA patients and hydroxyeicosatetraenoic acids (HETEs), in particular an isomer of LTB4 (a lipid mediator), reflect an active trans-cellular biosynthesis in platelets and neutrophils (23). Sphingolipids, including ceramides, sphingomyelins, and lactosylceramides, are increased in synovial fluid of RA patients (119). In general, sphingolipids are pro-inflammatory lipids and play a modulating role in apoptosis, cell cycle, and inflammatory responses.

Amino-acids – Serum amino acids were significantly different between RA and control patients (115). In general, urea and amino acids decrease as a consequence of a highly active urea cycle. The decrease in leucine, isoleucine and valine implies an increased energy metabolism. Threonine and alanine were decreased, possibly because they were increasingly converted into glucose. The proline decrease can be linked to TCA and urea cycle activation.

In contrast, Yousri *et al.* (116) attributed the decreases in amino acids in plasma to cartilage destruction. They found decreases in 4-methyl-2-oxopentanoate and 3-methyl-2-oxovalerate, part of the branched-chain amino acid pathway, and in N-methylglycine, a glycine-serine-threonine metabolite. They also found an increase in prolylglycine. Other significant amino acids, were part of the leucine, isoleucine and valine pathway, and the phenylalanine and tyrosine pathway.

In the study of Li *et al.* (110), glutamic acid, L-leucine, L-phenylalanine, and L-proline were increased, whereas tryptophan and argininosuccinic acid were decreased. L-leucine provides energy in conditions of constant energy consumption. Proline is important for protein synthesis, and its metabolism is abnormal in RA causing cartilage and bone damage.

In the study of Madsen *et al.* (109), histidine was the most specific serum biomarker of RA. Although it is generally suggested that histidine catabolism is increased in RA, no histidine metabolites were found, nor was there another explanation for histidine decrease. Other than histidine, also methionine, asparagine and threonine were decreased.

To summarize, three studies consent that the decrease of leucine is due to an upregulation of energy metabolism (110, 115, 116), and that the serine-threonine pathway is altered (109, 115, 116) (see section 3.2.1.1). Metabolites of glutamine (pyroglutamate and

glutamic acid) a provider of energy, and a change in phenylalanine and tyrosine pathway were found in two studies (110, 116).

On the changes and role of proline, however, the studies give contradictory information. One study saw a decrease due to TCA and urea pathway activation (115), while another saw an increase due to an abnormal metabolism with cartilage and bone damage (110). Moreover, changes in amino acids can be explained in different ways. Suggested explanations are joint damage (110, 116), upregulation of a highly active urea cycle to provide energy (107, 115), or changes in muscle and tissue due to the chronic, systemic inflammation (110).

Steroids – Sixteen of the significant serum metabolites, found by Yousri *et al.* (116), were steroids. Steroid levels among RA patients were lowest during GC treatment, due to a suppression of the HPA axis. The levels were higher when the patient had never received GC treatment, and highest when the patient had received GC treatment in the past. The fact that patients who never received GC treatment still had more deficiency in steroids than patients treated in the past, could be explained by a persistent deficiency as a part of RA, or by the existence of a subgroup of RA patients who are prone to this deficiency. The most significant steroids found in this study (116) (dehydroepiandosterone sulfate (DHEAS), 4-androsten-3β,17β-diol monosulphate, 4-androsten-3α,17α-diol monosulphate, and 4-androsten-3β,17β-diol disulphate), were classified in the pregnenolone to cortisol pathway, and the pathway in which sulfated forms and α- and β-isomers of adrenal androgen, dehydroepiandosterone (DHEA), androstenedione, androstenediol, androsterone and epiandrosterone, are found. Eleven other serum metabolites were unknown, but were identified as steroids by the software Metabolon. 16-hydroxy-DHEA, converted from DHEA, is also decreased in synovial fluid of RA patients, and is a precursor of 16-hydroxy-estrogen.

Decreased androgen levels (testosterone, dihydrotestosterone (DHT), DHEA and DHEAS) and a decreased androgen/estrogen ratio are found in synovial fluid of RA patients (101). Serum testosterone is decreased in men with RA, but contradictory information is published about women with RA (79, 102). In contrast, other studies have shown a decrease in serum androstenedione in women with RA (112), or a decrease in adrenocortical androgens and an adrenal androgen-to-cortisol imbalance in a minority of women with premenopausal RA (120). Both men and women with RA, show a decreased serum androgen/estrogen ratio (79).

Estrogens can have both a stimulating and an inhibiting influence on the immune system, but the effects in RA are not well understood (79). Although there is a lot of contradictory information, there is a consensus that androgens have immunosuppressive effects and play an important role in local immune responses, and estrogens have pro-inflammatory effects (79, 101). Estrogen possibly has suppressive effects on cellular immunity, and stimulating effects on humoral immunity (102). This makes a decrease in estrogen levels

a stimulating factor for T-cell differentiation towards Th1 cells (102). However, in clinical RA, many cytokines are present in synovial fluid, which causes an increased activity of aromatase, and this converts androgens to estrogens (101).

Other metabolic pathways – Significant changes in serum were found in mannose, ribose, scyllo-inositol, glycerol and 1,5-anhydrosorbitol (115). In particular, serum glucose levels were decreased and lactate increased, which reflects the higher glucose metabolism of FLS in the hypoxic joints of RA patients (106, 107, 115).

Pyruvate (glycolysis) and citrate (TCA cycle) were also increased in serum of RA patients (115). Succinate is a substrate in the TCA cycle and a crucial part of mitochondrial ATP production. In RA, serum succinate was positively correlated to the synovial production of IL-1 and to post-translational modification of synovial proteins, but this correlation was not significant (107).

Choline metabolism is related to inflammation, and trimethylamine (TMA) and trimethylamine N-oxide (TMAO) are metabolites derived from choline (107). Also, TMA and TMAO are both related to cardiovascular inflammation. In RA patients, TMA is increased and choline is decreased.

3-hydroxybutyrate was correlated with IL-1 and IL-8, probably because of its signaling function (107). This metabolite inhibits histone deacetylases apart from its energy transporting function from liver to peripheral tissue.

Bilirubin seemed to be an important molecule in the study of Li *et al.* (110). Normally, it binds HLA-DR4 molecules, which blocks the binding of antigenic peptides and thus inhibits immune response. In RA, bilirubin is decreased.

3.2.1.4 Differentiation between RA and other diseases

Madsen *et al.* (109) have found biomarkers to distinguish RA from PsA with a sensitivity of 90% and a specificity of 94%.

4-methoxyphenylacetic acid, L-phenylalanine, and L-leucine can distinguish RA from primary Sjogren's syndrome patients, according to Li *et al*. (110). L-leucine is higher in RA patients compared to pSS patients. Also, cortisol, bilirubin and capric acid levels were lower in RA patients compared to pSS patients.

Kim *et al.* (117) discovered twenty metabolites to distinguish RA from Ankylosing Spondylitis, Behçet's disease, and Gout with a sensitivity of 92.3% and a specificity of 68%. Higher amounts of succinate, octadecanol, asparagine, terephthalate, salicylaldehyde, glutamine, citrulline, tyrosine, uracil, lysine, ribitol, tryptophan, xylose, and ribose were found, in contrast to lower amounts of isopalmitic acid, glycerol, myristic acid, palmitoleic acid, hydroxylamine, and ethanolamine.

Interestingly, the metabolites found by Kim *et al*. (117) are intermediates of the urea (ornithine and citrulline) and the TCA cycle which are highly activated in synovial fluid. Succinate and fumarate including their derivative amino acids asparagine, lysine, and glutamine are part of the TCA cycle. Isopalmitic acid, myristic acid, and palmitoleic acid were lower than in other diseases, so the FA metabolism is less active in RA than in Ankylosing Spondylitis, Behçet's disease and Gout.

CD4+ T-cells show diminished glycolytic activity in comparison to SLE, and use mainly the PPP to produce NADPH (106). Thus NAPDH levels are elevated in contrast to SLE.

A difficulty concerning diagnosis, is the similarity of the lipid profile of RA to the lipid profile of other (chronic) inflammatory diseases such as cancer, sepsis, and postoperative state (105).

3.2.2 Prognostic biomarkers

Research on possible biomarkers to classify patients within the RA group into subgroups according to their phenotype and to relate disease outcome to these phenotypes remains scare.

Phenotypes – Ketone bodies in blood, acetoacetate and its metabolites 3-hydroxybutyrate and acetone, are negatively correlated with a fibroblast phenotype in synovium (107). The hypothesis is that fibroblasts need more ketones to support their invasive phenotype.

According to Chinese medicine classification, there is a heat pattern and a cold pattern RA phenotype, of which the plasma metabolome is significantly different (121). The heat pattern phenotype shows increased glycochenodeoxycholate, proline, saturated and mono-unsaturated phosphatidylcholine (PC) in plasma compared to the cold pattern RA phenotype. Also, a decrease of urea, FFA and polyunsaturated PC is found in plasma of the heat pattern phenotype. In addition, higher levels of 11 acylcarnitines and of DHEAS were found in urine of the heat pattern phenotype.

Disease outcome – There is an association between early menopause (before 45 years) and a milder type of RA (102). Although a lot of contradictions on oral contraceptive use exist, it seems to be correlated with milder RA. Low cortisol levels predict a more severe course of disease (110). There is an association between the duration of breastfeeding and a more severe type of RA (102).

While RA patients show lower total cholesterol and LDLc, their cardiovascular risk remains elevated (105). This is called the lipid paradox, which could be explained by an increased subendothelial deposition of LDLc (111). This phenomenon was confirmed by Giles *et al.* (25), who scored the coronary artery calcium (CAC) as this was known to correlate with cardiovascular disease. RA patients with low LDLc had higher CAC than non-RA controls at

high LDLc level up to 160 mg/dl, and a similar CAC to non-RA controls with LDLc higher than 160 mg/dl, especially in RA patients who were white race, smokers, and non-obese. The hypothesis of Giles *et al.* was inflammatory-induced lipid retention by macrophages and hepatocytes, or oxidation which facilitates uptake of LDLc by macrophages. Despite the fact that low LDLc levels correlate with high cardiovascular risk in RA, high HDLc and low TG correlate with lower cardiovascular risk for both RA and non-RA patients (25). These biomarkers can be monitored in RA patients to control the need for cardiovascular prevention.

A higher fatigue score is associated with the increase of uric acid and the down-regulation of metabolites from the urea cycle, FAs, tocopherols, aromatic amino acids, and hypoxanthine (122). Decreased tryptophan, phenylalanine, and tyrosine are due to oxidative stress. Tocopherol, an antioxidant related to disease activity, was also decreased. Decreased hypoxanthine and increased uric acid were associated with xanthine oxidase activation, an enzyme that also produces reactive oxygen species (ROS) (123).

3.2.3 Therapeutic biomarkers

Targeted therapy – The main goal of “treat-to-target” therapy is to treat the patient aggressively enough and to tackle the right aspect in the pathogenesis of the individual RA patient (1). Ideally DMARDs could be administered at a very early disease stage thanks to biomarkers (8). So far, there are no established biomarkers to predict response to therapy, but differences in metabolome before treatment have been studied between responders and non-responders (107).

Wang *et al.* (124) identified eleven biomarkers of response to MTX, using NMR in blood samples of early RA patients: increased uric acid, taurine, histidine, glycine, hypoxanthine, methionine, and decreased uracil, trimethylamine-N-oxide, tryptophan, aspartate and α-oxoglutarate. Therefore, pathways related to MTX response could be nucleic acid metabolism, homocysteine metabolism, one-carbon metabolism.

Cuppen *et al.* (39) conclude that the combination of metabolome with clinical parameters is the most effective predictor of response to TNFα-inhibitors. Increased sn1-lysophostphatidylcholine (15:0) and lysine, and decreased sn1-lysophosphatidylcholine (18:3-ω3/ω6) and ethanolamine at baseline predicted a good response to TNFα-inhibitors.

Kapoor *et al.* (125) studied urine samples of RA patients prior to anti-TNFα therapy. Responders and non-responders could be distinguished with a sensitivity of 88.9% and a specificity of 85.7%, in particular by histamine, glutamine, xanthurenic acid, and ethanolamine. The histamine increase could be caused by its production in mast cells or through histidine degradation. Ethanolamine, xanthurenic acid, and glutamine could originate from tryptophan and other amino acid degradation.

Pathways for drug development – Restoring the changes in sex hormones is suggested to be effective to improve quality of life (101, 126). Androgens, in particular, are the most attractive option because of their decreased levels in RA and their immuno-suppressive effects (113). Estrogens are less interesting, because they are possibly pro-inflammatory (101, 113). Especially in male RA patients, androgen replacement improves disease symptoms and possibly has a non-significant disease modifying effect (113, 127). However, female patients exhibit too many side effects from available androgen treatments, although there might be a benefit of DHEA in some female RA patients. Androgens together with MTX stimulate apoptosis of monocytic inflammatory cells and reduces cell growth *in vitro* (101).

Monocytes and FLS have a more activated mechanistic target of rapamycin (mTOR), which makes them a potential target for therapeutic intervention of rapamycin (106). mTOR is activated by the accumulation of branched amino acids, glutamine, kynurenine, and histidine, and the depletion of glutathione and cysteine.

Increasing bilirubin levels could control inflammation, which has been confirmed in collagen-induced arthritis (CIA) rats (110). However, Li *et al.* (110) doubt the safety of this treatment, because of the toxicity of high bilirubin levels.

Capric acid is decreased in RA and can reinforce the immune system. It is found in coconut oil together with polyphenols and lauric acid, and is sometimes prescribed for RA. Capric acid and polyphenols have beneficial effects on joint damage and arthritic pain, respectively (110).

3.2.4 Biomarkers for follow up

3.2.4.1 Monitoring response to treatment

csDMARDs – Pang *et al.* (119) studied the metabolome changes after successful MTX treatment in CIA rats, which show similarities in pathology and immunology of RA. MTX changed the arachidonic acid, linoleic acid and sphingolipid metabolism. Nineteen metabolites and eight metabolic pathways were significant to differentiate response to treatment. Ceramides, sphingomyelins, and lactosylceramides are sphingolipids that decreased in blood of CIA rats after MTX treatment. Linoleic acid can be converted to arachidonic acid and epoxides of linoleic acid (EpOMEs). Epoxyoctadecenoic acids (EETs), prostaglandins, leukotrienes, thromboxane A2 (TXA2), and hydroxyeicosatetraenoic acids (HETEs) are metabolites derived from arachidonic acid that were altered after MTX treatment. EETs are anti-inflammatory, anti-hypertension, and organ protective lipid mediators, and were increased in blood of CIA rats after MTX. Prostaglandins and leukotrienes which cause pain and cytokine production in RA patients, were decreased. TXA2 also decreased and HETEs, which play a role in inflammation, altered with a general increase in anti-inflammatory, and a decrease in pro-inflammatory lipids. EpOMEs are pro-inflammatory and toxic to leukocytes. These lipids

decreased in blood of CIA rats after MTX, in contrast to the increased, anti-inflammatory linoleic acid. However, MTX decreases the dietary absorption of linoleic acid, so it is not sure that this effect would be the same in RA patients (119).

bDMARDs – Lowered IL-6 and TNFα levels are a biomarker of the response to several bDMARDs (105), and treatment with tocilizumab (IL-6 receptor blocker) showed a decreased level of LDLc and anti-inflammatory modifications of HDLc.

After twelve weeks of anti-TNFα treatment, a distinguishing urine metabolome between etanercept and infliximab was found (125). An increase of hippuric acid, citrate, and lactic acid were specific for infliximab, whereas choline, phenylacetic acid, urea, creatinine, and methylamine increases were specific for etanercept. While TNFα-inhibitors restore the DHEAS deficiency in blood, including the physical function associated with this deficiency (116), this was not yet achieved after twelve weeks of TNFα-inhibitors (127, 128). After one year of treatment with infliximab or etanercept, serum DHEAS levels were indeed increased in another study (129). However, ACTH, cortisol, LH, estradiol, or testosterone did not change in this study over two years. Priori *et al.* (63) found biomarkers of a good response to etanercept after six months including higher serum levels of isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose and lower levels of 3-hydroxybutyrate. Lysine increase also is a marker of response to TNFα-inhibitors, together with a decrease in sn1-lysophosphatidylcholine (18:3-ω3/ω6) (39). Whereas a decrease of sn1-lysophostphatidylcholine (15:0) is a marker of non-response to TNFα-inhibitors (39). High TG and low HDLc is a poor response profile to TNFα-inhibitors (130). Long-term treatment with TNFα-inhibitors showed increased HDLc and total cholesterol, but no change in LDLc and arthrogenic index (105).

3.2.4.2 Monitoring disease activity and progression

Synovial fluid shows twelve metabolites correlated with disease activity: 2-hydroxyvalerate, fucose, tryptophan, indole-3-lactate, isothreonate, thymine, phenylalanine, lactose, arabitol, mannose-6-phosphate, citrate, and oxoproline (131). This could predict six associated pathways: fructose and mannose degradation, phenylalanine and tyrosine metabolism, citric acid cycle, galactose metabolism, tryptophan metabolism, and pyrimidine metabolism.

There is a significant difference in lactate, total cholesterol, acetylated glycoprotein, and HDLc between patients with active RA and in remission (132). Low total cholesterol, high TG and low HDLc levels were correlated with systemic inflammation (118, 130). Lysophosphatidylcholines and lysophophatidylethanolamines, signaling phospholipids in pathological pathways, negatively correlated to disease activity (39). Oxylipins, specifically produced by reactive oxygen species (ROS), lipoxygenase (LOX) and cyclooxygenase (COX), positively correlated to disease activity. These are pro-inflammatory, except for two negatively

correlating oxylipins produced by LOX and COX. All oxylipins synthesized by cytochrome P450, were anti-inflammatory, and negatively correlated with disease activity (39).

Long chain FAs, precursors of pro- and anti-inflammatory molecules, and glutathione associate with lower disease activity (39). Other biomarkers of higher disease activity are a high albumin/creatinine ratio in urine (125), the upregulation of leucine, threonine, tyrosine, and aspartate in serum (107), and of 3-hydroxyburyrate (107). DHEAS deficiency is linked to a more severe disease and a longer duration of RA (116).

4 DISCUSSION

The complexities of Rheumatoid Arthritis, omics analyses, biomarker discovery, and metabolomics in particular are tackled in this thesis. I have focused predominantly on providing useful information to answer the question 'Can metabolomics provide promising perspectives for future patients with Rheumatoid Arthritis?' To me, patients should always be the focus of (bio-)medical research, because I witness the burden and limitations on daily activities that this lifelong disease imposes on a member of my family every day.

Rheumatoid arthritis is a complex disease, and the literature on its pathogenesis reflects this complexity. For example, several studies present contradictory conclusions on the level, role, or importance of cytokines in the disease. Also, most studies have focused on one or two elements in synovial fluid, while RA does not start in joints (13) and every element is part of a cascade of pathways. As a result, there is now a trend to study blood samples, and also urine and lymph node analyses are increasing. These new analytical strategies will provide new perspectives on the pathogenesis, and hopefully on the causative factors and mechanisms underlying the disease.

Because of the protective properties of Treg cells, and the fact that these showed a decrease in lymph nodes and an increase in affected joints which is not high enough to provide protective effects, increasing the amount of Treg cells could be a new therapeutic option. Therefore, Treg cells in RA specifically, are an important focus of existing (133, 134) and likely future studies.

In section 1.1.3, the comparison between a study on a Korean (20) and an Indian population (22) shows differences concerning information on extra-articular manifestations and comorbidities across countries (22). It is thus important to confirm whether this information in one population can be ported to other populations. For example, myocardial infarction (MI) or angina, pulmonary tbc, asthma, thyroid disease, depression and hepatitis B are more common in the Korean study population, but some of these have not yet been confirmed for European or, even more specifically, Belgian patients.

In section 3.2.1.2, I described that estrogen levels can have suppressive effects on cellular immunity, but stimulating effects on humoral immunity. This could explain the link between the lower prevalence of breast, ovary, and endometrial cancer in patients with RA, but should be confirmed in other studies. Similarly, an association could be made between the lower prevalence of prostate cancer in male RA patients because of their decrease in testosterone.

To describe the management of RA, I used the EULAR recommendations of 2017 (5). It should be noted that other guidelines within European countries as well as outside of Europe

exist. Here too, it is important to consider possible differences between populations. Also, every guideline should be evaluated and used as a source of information, but not as a strict management for every patient in every country. Therapeutic decisions should rely on available information about current management as well as experimental therapies, together with individual biomarkers, and socio-economic aspects of the patient and the community.

Section 1.2 and 3.1 are not very detailed, which reflects both the complexity of, as well as the limited knowledge that I have on these topics. Although each omics technique is important and very interesting, it would take several theses to adequately describe these techniques and their contribution to RA. Additionally, I found obvious associations between omics techniques, most notably for genome and exposome, which are linked through the epigenome. It could also be questioned whether every molecule that I described in section 3.2 is part of the metabolome in its narrow definition. Here too, there could be an overlap with other omics analyses. Based on such obvious overlaps, I would like to stress the importance of systems biology and a holistic view on RA, in which all these (macro-)molecules are considered as a linked whole. Many gaps in systems biology analysis still exist, so additional work on the development of statistical approaches are needed, including the optimisation of multivariate and multifactor analysis techniques.

In section 3.1.1, I mention the presence of PD in saliva of future ACPA-positive RA patients. Also, I mention interest in the gut microbiome. However, I encountered only few studies on saliva and faeces samples. Although these samples are promising and very easy to obtain, they currently remain understudied. It would therefore be interesting to invest more efforts in such analyses, and investigate saliva and faeces samples of at-risk and clinical RA patients with omics analyses.

Proteomics is of growing importance in research. Research on the contribution to RA of Th17 cells, anti-CarP antibodies, and mGCR on peripheral blood mononuclear cells are very valuable for pathway-discovery, diagnosis of seronegative patients, and drug-development, respectively. There is controversy on the role of Th17 in RA. Ramwadhoebe *et al.* (17) suggested that Th17 cells, instead of the previously thought Th1 cells, play the main role in RA. Anti-CarP antibodies showed some promise as diagnostic biomarker, but contradictions exist (53, 96, 135). Also, peripheral blood mononuclear cells are upregulated and carry mGCR which can serve as a specific target for new drug development (43).

The majority of metabolomics studies focus on biomarkers of clinical RA. And while this is already showing some promising perspectives for RA patients, more research is needed on other marker-driven aspects such as preclinical and seronegative RA diagnosis, prevention, prognosis, and therapy.

Prevention and early diagnosis are both very important, but are also difficult because the exact cause of RA is not known. Moreover, some preventive measures appear to be

conditional. For example, breastfeeding is protective, unless the person is genetically susceptible to severe RA. But sequencing of asymptomatic people to determine risk remains cumbersome, thus requiring a transparent preventive measure or communication to patients without having to sequence every healthy person. In this context, it is important to note that not many metabolomic studies have been focusing on risk biomarkers and preventive measures, nor on preclinical biomarkers. Even so, I could find some relevant information on these topics. Glutamic acid could be a serum biomarker for seronegative or preclinical diagnosis. A decrease of total cholesterol and LDLc could serve as lipid biomarkers, and low cortisol and low androstenedione as steroid biomarkers for preclinical diagnoses of RA. The role of estrogens in prevention and preclinical diagnosis, as well as in clinical RA is to be investigated more thoroughly, because there is a lot of contradictory information regarding these hormones.

Seronegative patients at risk or with clinical RA also need to be researched in more detail. In section 3.2.3.1, I mention a study that questions the utility of ACPA and/or RF antibodies to make a therapeutic decision. Also, Madsen *et al*. (109) have found that metabolic profiling is useful in seronegative RA. It might be useful to step away from these antibodies, and treat patients based on their clinical condition and individual biomarkers. Even though these patients sometimes do not strictly correspond to the definition of RA, it can help prevent damage to patients with early disease.

Clinical RA is characterised by changes in metabolism which can easily be measured in blood and urine samples. Saliva and faeces samples have not been abundantly investigated in this context, however. Synovial fluid and tissue have been studied many times but are not so easily obtained.

A problem with metabolomic studies is the manner of reporting metabolites. Sometimes, the exact same metabolite is reported with a different name, or a metabolite may not be identified properly or precisely. This is due to the use of different databases and software packages to identify the small molecules found by MS or NMR. It is therefore important to standardize the nomenclature employed in metabolite identification to be able to compare different study results. This does not guarantee that these molecules are comparable. In other cases, closely related metabolites are studied, which actually map very closely to the same pathway. This makes it harder to compare studies and makes the discovery of possible important metabolites less valuable. However, studies mostly give the metabolic pathway in which the metabolite plays a role, making a more global, higher-level comparison of studies possible.

General changes of clinical RA are lipid changes that reflect systemic inflammation in blood, and the presence of lipids in synovial fluid from which these are normally absent. Overall, amino acids decrease, but there is discussion on the underlying causative

mechanisms. Steroid changes are quite similar across studies, but should be interpreted in the context of patient sex as well as previous GC treatment. Interesting metabolites to investigate further are 3-hydroxybutyrate, succinate, and bilirubin. 3-hydroxybutyrate and succinate both show correlations with IL-1, a pro-inflammatory cytokine. 3-hydroxybutyrate also inhibits histone deacetylases which can influence gene expression and maybe alter the course of disease. Succinate also plays a role in post-translational modification of synovial proteins, which can be of interest in RA since antibodies act against modified (citrullinated) proteins. Bilirubin is decreased in RA and normally inhibits immune response by binding HLA-DR4. HLA-DR4 is linked to an allelic variant of HLA-DRB1, which is specific for RA.

Finally, interesting metabolites for drug development are given in section 3.2.3.2 and are merely experimental. Especially bilirubin, mTOR, and capric acid provide ideas for drug development based on recent studies (106, 110). Should the complexities regarding androgens and estrogens in RA be unravelled, these molecules could also deliver interesting leads for drug development.

5 CONCLUSIONS

Metabolomic research on preclinical and seronegative RA diagnosis, prevention, prognosis, and therapy remains scarce. Evidence on the role of breastfeeding in the prevention of RA, and the role of estrogens in therapy, prevention and preclinical RA is contradictory and needs clarification. A more detailed investigation on glutamic acid in blood as a possible risk biomarker could also provide new insights in prevention. Research on saliva and faeces samples could provide an easy way of opening up new ways to investigate RA. Further, the causative mechanism of observed amino acid decreases should be investigated as this can provide new information on the pathogenesis of RA. There could be a role for 3-hydroxybutyrate in RA to alter the course of disease by its effect on histone deacetylases. Changes in succinate, which modifies proteins in synovium, and the interaction of bilirubin and the genome susceptible for RA should also draw the interest of researchers. Bilirubin, mTOR, and capric acid (or analogues thereof) could offer new therapeutic options and should be studied in this context in the future.

While metabolomics can thus provide promising perspectives for future patients with RA, it will nevertheless need to be seen holistically with other omics analyses in a systems biology context, especially when investigating the pathogenesis of RA. A change in the metabolome is ultimately the result of many upstream mechanisms that likely influence each other, so unravelling this network of interacting systems could increase knowledge on the (possibly heterogeneous) onset and cause(s) of RA. Progress on statistical analyses to combine the different kinds of data from the various omics methods will dramatically aid integrative systems biology approaches. Other topics of importance for further investigation are additional research on Treg cells as a new therapeutic option and more fine-tuned therapeutic guidelines for RA patients of different origins. Proteomics research should focus on elements such as the role of Th17 cells in RA, anti-CarP antibodies as possible diagnostic biomarkers for seronegative RA, and therapeutic targeting of mGCR of peripheral blood mononuclear cells.

As new answers bring new questions, the many enigmas of RA remain far from being resolved. A complete understanding of this systemic autoimmune disease can only be accomplished by combining all omics analyses and this combined analytical power should open up new ways to improved therapy development, early diagnosis, and even prevention, which also constitute the logical next steps in the future medical care of RA patients. As new answers bring new questions, the many enigmas of RA remain far from cracked, thus leaving ample room for future researchers to improve the perspectives of RA patients.

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