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Identification of Metabolites as Biomarkers and Mediators of Inflammation in Inflammatory Arthritis

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Identification of Metabolites as Biomarkers and Mediators of Inflammation in Inflammatory Arthritis

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List of Abbreviations

YORA	**Y**oung **O**nset **R**heumatoid **A**rthritis
EORA	**E**lderly **O**nset **R**heumatoid **A**rthritis
PsA	**P**soriatic **A**rthritis
PsO	**Ps****O**riasis
PsD	**P**soriatic**D**isease
DMARD	**D**isease **M**odifying **D**rug
ALT	**AL**anine Amino**T**ransferase
HLA	**H**uman **L**eukocyte **A**ntigen
CRP	**C** **R**eactive **P**rotein
ESR	**E**rythrocyte **S**edimentation **R**ate
RF	**R**heumatoid **F**actor
CCP	**C**yclic **C**itrullinated **P**eptide
DAS28	**D**isease **A**ctivity **S**core including 28 joints
HAQ	**H**ealth **A**ssessment **Q**uestionnaire
PTX3	**P**entraxin **R**elated **P**rotein 3
DUSP11	**Du**al **S**pecificity **P**rotein phosphatase 11
TNF	**T**umor **N**ecrosis **F**actor
IL	**I**nterleukin
MBDA	**M**ulti-**B**iomarker **D**isease **A**ctivity
EGF	**E**pidermal **G**rowth **Factor**
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
SAA	**S**erum **A**miloid **A**
VCAM1	**V**ascular **C**ell **A**dhesion **M**olecule 1
MMP	**M**atrix **M**etalloproteinase
TNF-R	**T**umor **N**ecrosis **F**actor **R**eceptor
YKL	**H**uman **C**artilage **G**lycoprotein-39
ACR	**A**merican **C**ollege of **R**heumatology
BL	**B** **L**ymphocytes
US	**U**ltrasound
MRI	**M**agnetic **R**esonance **I**maging
FDG	**F**luoro**D**eoxy**G**lucose
PET	**P**ositron **E**mission **T**omography
CT	**C**omputed **T**omography
GRAPPA	**G**roup for **R**esearch and **A**ssessment of **P**soriasis and **P**soriatic **A**rthritis
COMP	**C**artilage **O**ligomerix **M**atrix **P**rotein
MCP	**M**onocyte **C**hemoattractant **P**rotein
NGF	**N**erve **G**rowth **F**actor
ADAMTS	**A** **D**isintegrin and **M**etalloproteinase with **T**hrombospondin **M**otifs
CXCL10	**C**hemokine (C-X-C motif) **L**igand 10
DMARD	**D**isease **M**odifying **A**nti **R**heumatic **D**rugs
DNA	**D**eoxyribo**n**ucleic **A**cid
RNA	**R**ibonucleic **A**cid
NMR	**N**uclear **M**agnetic **R**esonance

TMSP	**T**ri**M**ethyl**S**ilyl**P**ropanoic acid
MS	**M**ass **S**pectrometry
NPLC	**N**ormal-**P**hase **L**iquid **C**hromatography
RPLC	**R**eversed-**P**hase **L**iquid **C**hromatography
FLS	**F**ibroblast **L**ike **S**ynoviocytes
OXPHOS	**OX**idative **PHOS**phorylation
ATP	**A**denosine **T**ri**P**hosphate
PFKFB	**P**hos**P**ho**F**ructo**K**inase-2
PKM2	**P**yruvate **K**inase isozyme M2
GLUT1	**GLU**cose **T**ransporter 1
TCA	**T**ri**C**arboxylic **A**cid cycle
NLRP3	**N**OD-, **LR**R- and **p**yrin domain-containing protein 3
Th	**Th**elper
HIF	**H**ypoxia **I**nducible **F**actor
IDO	**I**ndoleamine 2, 3-**D**i**O**xygenase
BCAA	**B**ranched-**C**hain **A**mino **A**cids
NADPH	**N**icotinamide **A**denine **D**inucleotide **P**hosphate
mTOR	**m**echanistic **T**arget **O**f **R**apamycin
BCAT	**B**ranched **C**hain **A**mino**T**ransferase
CoA	**C**oenzyme **A**
LPS	**L**ipo**P**oly**S**accharide
FFA	**F**ree **F**atty **A**cid
OA	**O**steo**A**rthritis
DHA	**D**ocosa**H**exaenoic **A**cid
EPA	**E**icosa**P**entaenoic **A**cid
AA	**A**rachidonic **A**cid
DGLA	**D**ihomo **G**amma **L**inoleic **A**cid
PUFA	**P**oly**U**nsaturated **F**atty **A**cid
ALA	**A**lpha **L**inolenic **A**cid
COX	**C**yclo**ox**ygenase
LOX	**L**ypo**ox**ygenase
ALOX	**A**rachidonate **L**ypo**Ox**ygenase
DM2	**D**iabetes **M**ellitus type 2
SCFA	**S**hort **C**hain **F**atty **A**cid
GPR	**G** **P**rotein-coupled **R**eceptor
HDAC	**H**istone **D**e**AC**etylase
CIA	**C**ollagen **I**nduced **A**rthritis
Treg	**T** **Reg**ulator
TMAO	**T**ri**m**ethyl**a**mine-N-**o**xide
TMA	**T**ri**m**ethyl**a**mine
GPC	**G**lycero-**P**hospho**C**holine
PC	**P**hosphatidyl**C**holine
BA	**B**ile **A**cid
HETE	**H**ydroxy**E**icosa**T**etra**E**noic acid
HEPE	**H**ydroxy**E**icosa**P**enta**E**noic acid
LC/MS	**L**iquid **C**hromathography coupled with **M**ass **S**pectrometry
GC-MS	**G**as **C**hromathography coupled with **M**ass **S**pectrometry
LT	**L**euko**T**riene
NK	**N**atural **K**iller
LA	**L**inoleic **A**cid
LPE	**L**yso**P**hosphatidyl**E**thanolamine

PI	**P**hosphatidyl**I**nositol
PAF	**P**latelet-**A**ctivating actor
PG	**P**rosta**G**landin
HX	**H**epo**X**ilin
HODE	**H**ydroxy**O**cta**D**ecadi**E**noic acid
ODE	**O**cta**D**ecadi**E**noic acid
HDoHE	**H**ydroxy**Do**cosa**H**exa**E**noic
TX	**T**hrombo**X**ane
TBXA2R	**T**hrombo**X**ane **A2**isoprostane **R**eceptor
GC	**G**luco**C**orticoids
SDMA	**S**ymmetric **D**i**M**ethyl **A**rginine
ADMA	**A**symmetric **D**i**M**ethyl **A**rginine
MTX	**M**etho**T**hre**X**ate
EULAR	**EU**ropean **L**eague **A**gainst **R**heumatism
UDP	**U**ridine-**D**i**P**hosphate
cAMP	**c**yclic **A**denosine **M**ono**P**hosphate
GWAS	**G**enome **W**ide **A**ssociation **S**tudies
DLG2	Discs Large MAGUK Scaffold Protein 2
FADS	**F**atty **A**cid **D**esaturase
STAG1	**ST**romal **A**nti**G**en 1
SNP	**S**ingle **N**ucleotide **P**olymorphism
LIPC	Hepatic Triacylglycerol Lipase
Ig	**I**nmuno**g**lobulin
RT-PCR	**R**everse **T**ranscription **P**olymerase **C**hain **R**eaction
BLyS	**B** **Ly**mphocyte **S**timulator
SDF1	**S**tromal cell-**D**erived **F**actor 1
APRIL	**A** **P**roliferation**I**nducing **L**igand
OLS	**O**rdinary **L**east **S**quares
FDR	**F**alse **D**iscovery **R**ate
DMA, NN	**DM**ethylamine
THF	**T**etra**H**ydro**F**olate
IMP	**I**nosine **M**ono**P**hosphate
CTL	**C**holine **L**ike **T**ransporter
CDAI	**C**linical **D**isease **A**ctivity **I**ndex
BSA	**B**ody **S**urface **A**rea
TJC	**T**ender **J**oint **C**ount
SJC	**S**wollen **J**oint **C**ount
SDAI	**S**implified **D**isease **A**ctivity **I**ndex
BMI	**B**ody **M**ass **I**ndex
NSAID	**N**on **S**teroidal **A**nti**I**nflammatory **D**rugs
CYP	**CY**tochrome **P**450
NE	**N**on **E**nzymatic
PGFS	**P**rosta**G**landin **F** **S**ynthase
PGES	**P**rosta**G**landin **E** **S**ynthase
PGDS	**P**rosta**G**landin **D** **S**ynthase
PGIS	**P**rosta**G**landin **I** **S**ynthase
TXAS	**T**hrombo**X**ane **A**2 **S**ynthase
LTAH	**L**euko**T**riene **A**4 **H**ydrolase
MDB	**M**embrane **D**ipeptidase
HEDH	**H**ydroxy**E**icosanoid **D**e**H**ydrogenase
PGDH	hydroxy**P**rosta**G**landin **D**e**H**ydrogenase

13-PGR	keto**P**rosta**G**landin 13 **R**eductase
sEH	**s**oluble **E**poxide **H**ydrolase
Rv	**R**esol**v**in
FMO	**F**lavin-containing **M**ono**O**xygenases
CVD	**C**ardio**V**ascular **D**isease
SPM	**S**pecialized **P**roresolving **M**ediators
LX	**L**ipo**X**in
VAS	**V**isual **A**nalogue **S**cale
PLS-DA	**P**artial **L**east **S**quares **D**iscriminant **A**nalysis
PLA2	**P**hospho**L**ipase **A**2
cDAPSA	**cD**isease **A**ctivity Index for **P**soriatic **A**rthritis
PASI	**P**soriasis **A**rea and **S**everity **I**ndex
CSA	**C**linically **S**uspect **A**rthralgia

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Abstract

Inflammatory arthritis represents a great social and economic burden despite recent therapeutic advances. Although we have a better understanding of the pathogenic mechanisms, treatment election is still made on a trial basis, which leads to lack of control of disease activity in approximately 30% of patients and a high rate of side effects. The identification of disease mediators and predictors of response to treatment are needed to allow the adequate treatment and achieve clinical remission or at least low disease activity. A single biomarker is unlikely to provide sufficient information to explain these heterogeneous diseases. Metabolomics is a tool that can be used for biomarker discovery as it can identify profiles of a large number of metabolites in different types of samples. Metabolites are not just the end result of chemical processes that occur in the cell, but also play critical role in a variety of cellular processes, such as post-translational modifications and immune cell regulation. With the hypothesis that circulating metabolites reflect synovial processes, this project aimed to study circulating metabolites in relation to disease activity and response to disease modifying antirheumatic drugs. We described different metabolomic profiles in patients with inflammatory arthritis compared to controls. We also identified metabolites that correlate with disease activity and that may be mediators of disease, as well as metabolites that are associated with response to treatment.

Resumen

La artritis inflamatoria representa una gran carga social y económica a pesar de los recientes avances terapéuticos. A pesar de un mejor conocimiento de los mecanismos patogénicos, la elección del tratamiento todavía se realiza a modo de prueba, lo que conduce a una falta de control de la actividad de la enfermedad en aproximadamente el 30 % de los pacientes y una alta tasa de efectos secundarios. La identificación de mediadores de enfermedad y predictores de respuesta al tratamiento es necesarioa para permitir el tratamiento adecuado y lograr la remisión clínica o al menos una baja actividad de la enfermedad. Es poco probable que un solo biomarcador proporcione información suficiente para explicar estas enfermedades heterogéneas. La metabolómica es una herramienta que se puede utilizar para el descubrimiento de biomarcadores, ya que puede identificar perfiles de una gran cantidad de metabolitos en diferentes tipos de muestras. Los metabolitos no son solo el resultado final de los procesos químicos que ocurren en la célula, sino que también juegan un papel crítico en una variedad de procesos celulares, como las modificaciones postranslacionales y la regulación de las células inmunes. Con la hipótesis de que los metabolitos circulantes reflejan procesos sinoviales, este proyecto tuvo como objetivo estudiar los metabolitos circulantes en relación con la actividad de la enfermedady la respuesta a los fármacos antirreumáticos modificadores de la enfermedad. Describimos diferentes perfiles metabolómicos en pacientes con artritis inflamatoria comparados con controles. También identificamos metabolitos que se correlacionan con la actividad de la enfermedad y que pueden ser mediadores de la enfermedad, asi como metabolitos asociados con la respuesta al tratamiento.

Dedicated to my family

1 Introduction

1.1 Inflammatory Arthritis

Inflammatory arthritis are a group of diseases characterized by chronic inflammation of the joints, adjacent tissues, as well as other connective tissues. The most prevalent forms include rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS)[1].

Rheumatoid arthritis is an autoimmune inflammatory arthritis that affects around 1% of the population [2, 3]. RA can affect the younger population (young onset rheumatoid arthritis - YORA), with an average age less than 50, mostly affecting women, with a female to male ratio of 2-3:1 and typical symmetrical pattern of joint involvement, often affecting the small joints of the hands. Elderly onset rheumatoid arthritis (EORA) affects the older population, with an average age of more than 60 years, a more equal sex distribution, asymmetry of the involved joints and a more frequent involvement of large joints. All these characteristics lead to the need of differential diagnosis of EORA with other rheumatic diseases, such as polymyalgia rheumatica, osteoarthritis, gout, chondrocalcinosis and psoriatic arthritis.

Psoriatic arthritis (PsA) is an inflammatory joint disease which is part of the complex psoriatic disease (PsD), an umbrella term that is used to include the various ways in which psoriasis can affect an individual: it includes not only joint inflammation, but can also affect the nails, skin, enthesis and it is associated with the metabolic syndrome and potential extra-musculoskeletal manifestations, such as colitis and uveitis [4]. The prevalence of PsA is low in the general population, around 0.05-0.25%, however, it is common among patients with psoriasis (PsO), affecting between 6-41% of PsO patients, depending on the definitions used [5]. Most frequently, psoriasis (PsO) precedes the development of arthritis, with an annual incidence of PsA of approximately 1.8%, [6] however, up to 15-25% will develop PsA at some point of their lifetime[7, 8].

Both RA and PsA are debilitating diseases since the natural evolution of the disease is towards structural joint damage which leads to joint deformities and functional disabilities,

which are associated with a heavy burden for the patient, as well as economical consequences for the society and healthcare systems. Cardiovascular diseases and obesity are relevant comorbidities for both diseases, which result in more health consequences and contribute to a lower quality of life in these patients. Hence, there is a need to have a good understanding of their pathogenesis and of adequate medications to control disease activity, which has been proven to prevent joint damage.

1.2 Biomarkers

According to the National Institutes of Health and the Food and Drug Administration, a biomarker is a "defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" [9, 10]. Although several types of biomarkers have been described, nowadays, when making use of the term, we mostly refer to molecular biomarkers which can be identified in biological fluids or tissues.

According to their use in different stages of health and disease state management, several categories of biomarkers have been described, as can be seen in Table 1.1.

1.2.1 Biomarkers in Inflammatory Arthritis

The discovery of biomarkers in the field of arthritis is challenging and has been hindered by several factors. Inflammatory arthritis are heterogeneous diseases, not only in their pathophysiology, but also disease course (variation in the severity and distribution of affected joints) and response to treatment. This makes it highly improbable that a unique characteristic/molecule could be useful as a biomarker. Moreover, the site of the disease is represented by the synovial tissue, which is accessible only by procedures that have been so far considered invasive, such as synovial biopsies, arthroscopy or remnant surgical tissue in patients who undergo joint replacement. Another added challenge is the multi-factorial nature of arthritis. In oncology, most of the predictive biomarkers are related to the fact that many cancers are caused by a single gene mutation, such as, for example, the presence of a specific mutation in breast cancer is related to the response to a particular treatment [12].

Biomarker categories	**Use**
Diagnostic	Detects or confirms the presence of a disease or condition of interest, or identifies an individual with a subtype of the disease
Monitoring	A characteristic that can be measured serially to assess the status of a disease or medical condition for evidence of exposure to a medical product or environmental agent, or to detect an effect of a medical product or biological agent
Pharmacodynamic/response	A characteristic that changes in response to exposure to a medical product or an environmental
Predictive	The finding that the presence or change in the biomarker predicts an individual or group of individuals more likely to experience a favorable or unfavorable effect from the exposure to a medical product or environmental agent
Prognostic	Used to identify the likelihood of a clinical event, disease recurrence, or disease progression in patients with a disease or medical condition of interest
Safety	A characteristic measured before or after an exposure to a medical intervention or environmental agent to indicate the likelihood, presence, or extent of a toxicity as an adverse event
Susceptibility/risk	Indicates the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition

TABLE 1.1: **Categories of biomarkers** [11]

There is a continuous struggle for biomarker discovery in the field of inflammatory arthritis with efforts being directed towards the discovery of molecular biomarkers that can be identified in easily accessible specimens, such as peripheral blood, urine, saliva or other readily available biospecimens. Nonetheless, one of the most important developments of the last years is the easier accessibility to obtain synovial tissue, through ultrasound synovial biopsies performed by rheumatologists. The experience of European rheumatologists has shown these procedures are safe and well tolerated by patients[13, 14]. In spite of the large number of studies that employ the newest technological advances (whole genome sequencing, proteomics, transcriptomics, immunology techniques), very few biomarkers have actually made it into clinical practice. These techniques generate large amounts of information which require advanced bioinformatics analyses. Another reason is the lack of validation, a concept with 2 meanings. One refers to the rigors of the techniques used for biomarker discovery,

which should ensure its reproducibility in different laboratories and over the years. Unfortunately, due to different constraints, most of the studies have not been able to perform validations in different laboratories/cohorts, to evaluate the reproducibility of the reported results. Another meaning for validation refers to the usefulness in clinical practice. A statistically significant result is not equivalent to a clinically significant results. A large majority of biomarker studies in the rheumatic field haven't properly evaluated the clinical usefulness of their findings [15].

The search for biomarkers in inflammatory arthritis has four main objectives:

1. Early diagnosis - to allow early initiation of treatment, which has been proven to prevent structural joint damage.

2. Prognostic indicators - to identify patients with aggressive forms of disease

3. Monitoring - disease activity to evaluate treatment efficacy; but also specific biological parameters (ALT - alanine aminotransferase) to evaluate treatment toxicity.

4. Selection of treatment - to identify biomarkers predictive of response or side effects to treatments [16].

1.2.2 Biomarkers in Rheumatoid Arthritis

Despite the considerable number of studies that have been exploring biomarkers, a low number is being used in the management of RA at the moment. Some of these are clinical, such as the number of swollen joints, while others are genetical (human leucocyte antigen - HLA) or biological, such as C reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) or anti-cyclic citrullinated peptide antibodies (CCP).

The existing biomarkers are being employed in different stages of the process of RA management, as can be observed in Table 1.2[17].

Nevertheless, not even the few biomarkers included in the table have real clinical usefulness. The human leucocyte antigen gene HLA-DRB1 is associated with an increased risk of RA development. However, the increase in risk conferred by carrying this gene is only of 1-3%, which explains why the identification of carriers of this gene has not been introduced as a screening method for RA development in the general population [18].

In other cases, the significance of the presence of specific biomarkers can change over time due to the interventions developed in RA. For example, the presence of RF and anti-CCP antibodies is considered to be associated with more aggresive forms of RA. Studies seem to

Purpose	Biomarker
Susceptibility/risk factor assessment	HLA-DRB1, smoking
Diagnostics	number of swollen joints, elevated CRP or ESR, presence of RF or anti-CCP antibodies
Monitoring	swollen joint count, CRP and ESR
Prognostics (relates to natural history of the disease)	presence and higher titers of RF and anti-CCP antibodies
Predictive (relates to benefit from a specific therapy)	seropositivity and response to certain treatments
Monitor drug safety	liver function, cell blood count
Surrogate endpoints	remission according to DAS28 score
Development of drug target	cytokine expresion in serum and synovial membrane

TABLE 1.2: **Current uses of biomarkers in RA management** [17]

agree that seronegative RA patients have higher disease activity at baseline [19, 20, 21], but the response to treatment was found to be different. Choi et al, as well as Barra et al found no significant differences in outcomes between seropositive and seronegative RA patients at 1 and 2 years of treatment[20, 21], while Bird et al found that seronegative patients had lower remission rates and lower improvements in physical functioning [19]. In the same vein, Matthijssen et al found that both seropositive ans seronegative RA patients had a good response to treatment, but only seropositive patients had improvement in long term outcomes: sustained DMARD-free remission, mortality and functional disability measured by yearly Health Assessment Questionnaire (HAQ)[22].

A considerable number of exploratory methods have been and continue to be employed for the identification of biomarkers in rheumatoid arthritis. Trying to improve diagnosis of seronegative RA, a new type of auto-antibodies were described, anti-carbamylated protein antibodies[23]. Their existence was found to predate the development of inflammatory arthritis by years [24, 25], similar to RF and anti-CCP antibodies. Several studies also observed their value as a factor for poor prognosis, relating them to more radiological progression [26]. Their usefulness in the management of pre-clinical RA is still being studied, especially in RF and/or anti-CCP negative patients[27]. Other auto-antibodies such anti-acetylated peptides (lysine, ornithine), anti malondialdehyde-acetaldehyde, anti malondialdehyde antibodies, anti-PTX3 (pentraxin related protein 3) and anti-DUSP11 (dual specificity protein phosphatase 11) have also been described, however, their usefulness in the management of RA is still to be decided [28, 29, 30].

Several cytokines are known to be involved in the pathogenesis of RA, and part of the available treatments are directed against them, such as anti-tumor necrosis factor (TNF) alpha or anti interleukin-6 (IL-6) therapies. So far, the circulating or even tissue concentrations of these cytokines have not been useful to evaluate disease activity or to monitor therapy, although some studies found that higher synovial expression of TNF-α was associated with a better response to TNF inhibitiors [31]. Using a combination of cytokines and other molecules, a multi biomarker disease activity score was proposed, MBDA or VECTRA-DA, with 12 molecules included: EGF (epidermal growth factor), VEGF-A (vascular endothelial growth factor A), leptin, IL-6, SAA (serum amyloid A), CRP (C reative protein), VCAM-1 (vascular cell adhesion molecule 1), MMP-1 (matrix metalloproteinase 1), MMP3, TNF-R1 (TNF receptor 1), YKL-40 (human cartilage glycoprotein-39) and resistin[32]. The score can be used to classify patients by disease activity (low, moderate and high disease activity)[33] and change in MBDA score was shown to correlate with change in clinical disease activity [34]. It is a measure that is recommended for regular use by the American College of Rheumatology (ACR)[35]. Moreover, it seems it is a stronger predictor for radiographic progression, compared to other classical factors such as disease activity score 28, CRP or seropositivity[36], but not a good predictor of response to treatment[37]. Although it's been available for several years, it's use is still not widely recommended, since it needs validation in larger cohorts and also it's price.

Genetic analyses, including gene polymorphisms and epigenetics, in association with flow citometry studies, are other techniques being employed in biomarker discovery in RA, either on synovial tissue or peripheral blood components, most often immune cells. As an example, Liebold et al found different methylation patterns in peripheral blood mononuclear cells from RA patients compared to controls, and the degree of methylation correlated with disease activity[38]. Rodriguez et al identified that a baseline B lymphocyte/CD4+ lymphocyte ratio (BL/CD4 ratio) <0.2 in the peripheral blood is associated with not achieving remission after treatment with TNF inhibitors[39].

Imaging biomarkers, employing ultrasound (US), MRI but also 8 F-fluorodeoxyglucose (FDG)-positron emission tomography (PET)/computed tomography (CT) techniques are another study field for biomarkers in RA. Although US can be performed in office by the rheumatologist and the inter-observer agreement is high, no US score has yet been validated for use in clinical practice[40]. MRI and FDG-PET/CT are expensive and less accessible in

the clinics[41, 42, 43], although they might be useful in the earlier diagnosis of progression.

A recent development in the field of biomarkers in RA is the description of synovial pathotypes, which involves a detailed description of the histopathological characteristics and the molecular and cellular signatures of the distinct cell types involved in the inflammatory process[44, 45, 46, 47, 48]. Based on histological characterization, 3 synovial pathotypes have been described: lympho-myeloid, characterized by well-organized B or plasma cell aggregates and rich in macrophages; diffuse-myeloid, with predominant macrophages within the sublining tissue and lacking B/plasma cell aggregates, and pauci-immune, with scant infiltration of immune cells and prevalence of resident fibroblasts [49], and each have specific molecular signatures. Once the pathological tissue has been described, ongoing research is looking into the predictive potential of these characteristics with regards to response to different types of treatments. The R4-RA group, based in the United Kingdom, found that the synovial pathotypes are associated with disease severity and response to treatment [50, 51]. One of their recent findings shows that the patients with a low number of synovial B cells have a lower probability of response to B cell targeted therapies (Rituximab)[52].

1.2.3 Biomarkers in Psoriatic Disease

The study of biomarkers in PsD lacks behind the field of RA. At this moment, mostly clinical characteristics are being employed as biomarkers of poor prognosis. A delay in diagnosis, symptom duration of more than 1 year before diagnosis, age more than 50 at diagnosis, female sex, smoking, dactylitis, polyarticular disease and the presence of nail psoriasis [53, 54, 55, 56, 57, 58] are all factors considered to be associated with worse physical function and disease progression.Erythrocyte sedimentation rate (ESR) is the only biological biomarker that was found to be associated to radiological progression in one study. However, the validation of these studies has not been performed properly and it's discriminatory value appears not to be sufficient for use in clinical practice.

The Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA) has specific groups of experts working on the unmet need of developing biomarkers in PsD, however none have yet reached the clinical setting. The combination of genetic, clinical and biological biomarkers, with the inclusion of the system biology approach, has been proposed by this group to facilitate biomarker discovery, since, due to heterogeneity of the disease,

it's unlikely that unique biomarkers would be useful. So far, the bone resorption and cartilage destruction markers and combinations of different proteins and genetic factors are the strongest candidates as biomarkers useful in differentiating PsA and PsC, predicting disease activity and response to treatment[4, 59, 60, 61, 62].

Amongst the circulating markers, a combination of 4 molecules: cartilage oligomerix matrix protein (COMP), resistin, monocyte chemoattractant protein-1 (MCP-1) and nerve growth factor (NGF,) was found to discriminate PsA from osteoarthritis patients, but it lacks validation in larger independent cohorts [63].

Efforts are also being directed towards the identification of biological markers that predict the development of arthritis in PsO patients. IgG autoantibodies against 2 novel antigens, LL-37 and ADAMTS-L5, have been described to be significantly increased in patients with psoriasis compared to healthy controls. Importantly, both autoantibodies were also significantly elevated in PsO patients with PsA compared to those without PsA, suggesting that these molecules may be involved in the pathogenesis of arthritis in these patients[64, 65]. Chemokine (C-X-C motif) ligand 10 (CXCL10) levels are elevated in PsO patients who develop PsA and decrease after the arthritis onset[66]. Moreover, recently, tissue-resident memory CD8+ T cells derived from the skin were shown to be enhanced in the circulation of PsA as compared to PsO patients[67].

1.2.4 The Need for Biomarker Development in Inflammatory Arthritis

In the case of RA, patients can benefit of the existence of a large number of disease modifying drugs (DMARDs), both synthetic(s) and biological(b) agents, nonetheless, no clear criteria on the election of the most adequate drug for each patient are available. Moreover, in spite of the myriad of available drugs, up to 30% of RA patients do not present a good control of the disease [68]. A poorly controlled disease represents a high burden for the society and is associated with higher health care costs [69, 70]. Hence, identifying biomarkers of response to treatment would help in reducing the number of treatments a patient needs to try and would lead to a faster achievement of therapeutic goals, either low disease activity or remission.

Another potential use of biomarkers in RA would be in the diagnosis of pre-clinical disease, although at the moment there are no strategies of preventing progression to clinically manifest arthritis.

In the case of PsA, rheumatologists can also choose from a large pool of DMARDs. The issue they come across in this case is the divergent response to these treatments of the skin and joint components. More research into the pathogenesis of psoriatic disease is required to help scientists develop better targeted therapies. Another main concern is the discovery of predictive biomarkers of the development of PsA in patients with PsO.

1.3 Metabolomics

In the recent years we have witnessed the development of a new type of research, centered on system biology, which studies the interactions between a system's components, as opposed to studying isolated components. The study of systems biology comprises several sciences, including genomics (the study of the complete sequence of DNA in a cell/organism), transcriptomics (the study of the complete set of RNA transcripts from DNA), proteomics (the study of the complete set of proteins of a tissue/cell) and metabolomics [71]. Metabolomics is the study of small molecules in a biological system and it reflects the actual state of the studied system, as a result of all the processes occurring upstream, at gene transcription, translation, post-translation and chemical reactions levels, as can be seen in Figure 1.1 [72]. The term and its definition was proposed by Fiehn in 2001 [73].

Among all the "omics" sciences just mentioned, metabolomics has had the largest growth in the past years due to the development of analytical techniques, as well as data analysis tools. The biological specimens that can be used for analysis include blood, serum, plasma, urine, synovial fluid or tissue. The heterougenous types of matrices, along with the need to quantify metabolites which are present in low concentrations in these types of specimens lead to the development of highly sensitive analytical techniques, making possible the use of metabolomics not only in the clinical setting, but also in the study of disease pathogenesis and, more recently, as exploratory method of biomarker discovery, due its high throughput nature [74].

Lipidomics is a branch of metabolomics which involves "the full characterization of lipid molecular species and their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" [75].

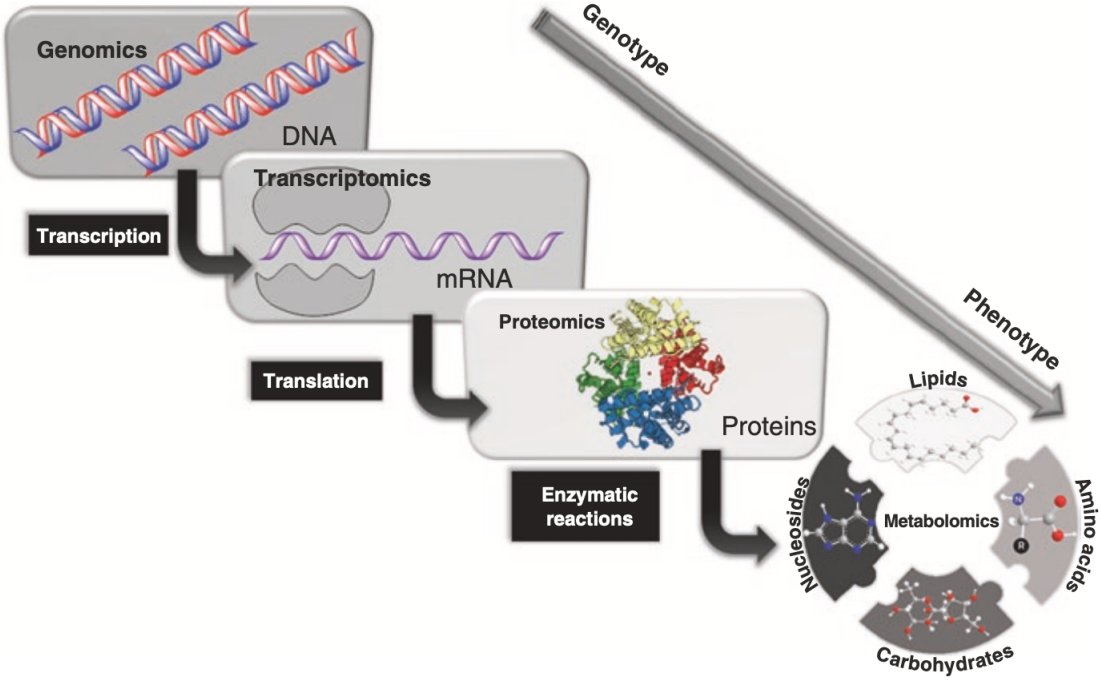

FIGURE 1.1: **The relation between the main omics strategies used in systems biology studies**. [72]

1.3.1 Techniques Employed in Metabolomics Analysis

Metabolomics studies employ two main techniques for metabolites detection: mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy, each with its advantages as well as disadvantages. Mass spectrometry is usually coupled with a separation technique. The most frequently used separation techniques is chromatography, which performs the separation of a mixture by passing it in a solution or a gas through a medium in which the components move at different rates.

There are 2 approaches to metabolomics studies: targeted and untargeted. The targeted approach represents a quantitative analysis, where concentrations are determined, or a semi-quantitative analysis, where relative intensities are registered, and it identifies a number of predefined metabolites, usually a few hundreds, which either belong to selected metabolic pathways or to common chemical classes. The untargeted approach represents a qualitative or semiquantitative analysis of a large number of metabolites which belong to diverse biological and chemical classes from a biospecimen, some of which can be identified afterwards[74]. The two approaches use different techniques for metabolite extraction, depending on the targeted molecules they aim to identify and/or quantify.

Lipidomics is largely considered a targeted approach, since it focuses on the study of

specific metabolites, the lipids. Nonetheless, lipids are complex molecules and can be classified in several chemical classes. This lead to the development of a targeted approach, which focuses on specific classes (ie phospholipids, fatty acids, oxylipins), as well as a global or untargeted one, which performs an exploratory analysis of all lipid classes [76] .

1.3.2 Nuclear Magnetic Resonance

NMR is the gold standard method for structural characterization of chemical compounds. This method doesn't require separation, so it has the advantage that the sample can be reused after analysis. It is considered a powerful tool for metabolomic studies, offering highly reproducible and quantitative analyses, however, it is able to detect fewer metabolites compared to mass spectrometry. The field of NMR metabolomics has been greatly aided by the development of modern spectrometers and software, allowing high-throughput analysis with near real-time feedback. Whilst one-dimensional proton (1D-1H) NMR analysis is best described and remains most widely used, several other alternative NMR techniques are now available that offer additional chemical and structural information and resolve many of the limitations of conventional 1D-1H NMR such as spectral overlay. An advantage of this approach is the linearity of the NMR signal with respect to the metabolite concentration, which makes it suited to quantify metabolite concentrations in complex mixtures with a large range of concentrations of the different molecules.So far, the 1D-NMR technique has been employed in the majority of metabolomics studies [77, 74].

NMR metabolomics involves a specific step in sample processing, represented by the addition of a phosphate buffer solution and of an internal chemical shift standard such as TMSP (Trimethylsilylpropanoic acid) or DSS (Sodium trimethylsilylpropanesulfonate). Deuterated water (D2O) is added to all aqueous NMR samples to a final concentration of 5 %. NMR systems use the D2O signal as a lock frequency to compensate for long-term magnetic field drifts [74].

1.3.3 Mass Spectrometry

Mass spectrometry is defined by the International Union of Pure and Applied Chemistry as the "study of matter through the formation of gas-phase ions that are detected and characterized by their mass and charge"[78]. It is considered the most suitable technique for metabolomics studies, especially non targeted approaches, due to its high sensitivity and

ability to detect a high number of metabolites. Samples can be directly injected in a mass spectrometer, however, this leads to the loss of a high number of metabolites and the necessity of a high resolution mass spectrometry, which is highly expensive, hence this method is rarely done. Most frequently, mass spectrometry is coupled with a separation method, such as gas or liquid chromatography. In this way, the compounds in a specimen, once they are separated, are injected one by one in the mass spectrometer, leading to a higher sensitivity and facilitating identification.

A mass spectrometry system (Figure 1.2) has 4 main components: an inlet, which is represented by the separation part, an ion source, the mass analyzer and the ion detector. The sample inlet, once the separation has been performed, introduces the compounds into the system, the ion source generates gas-phase ions via ionization, the mass analyzer separates the ions according to their mass to charge (m/z) ratio and the detector generates an electric current from the ions, which is proportional to their abundances[79]. The mass analyzer can be used alone or combined. The combination of mass analyzers technique is called tandem mass spectrometry (MS/MS). In MS/MS, the ions that arrive at the first mass analyzer (precursor ions) are isolated, subsequently fragmented, and finally those fragment ions are separated according to their m/zin a second mass analyzer and detected.

With regards to the separation techniques, liquid chromatography is most often employed due to its feature that allows the separation of different classes of compounds, from very polar up to very non-polar compounds. The separation in the chromatographic system depends on properties such as hydrophobicity, molecular size and polarity of the compounds. The separation of compounds occurs into a chromatographic column composed by a stationary phase with polar or non-polar properties. In chromatography using polar stationary phase columns, the solvent used to elute the compounds from the stationary phase(mobile phase) presents higher polarity than the stationary phase, which is called normal-phase liquid chromatography (NPLC). However, in chromatography using non-polar stationary phase columns, the mobile phase presents lower polarity than the stationary phase, which is called reversed-phase liquid chromatography (RPLC). Then, non-polar compounds, such as lipids, elute first in NPLC, whereas polar compounds, such as aminoacids, elute first in RPLC [74].

Clinical samples contain very polar compounds (aminoacids) and also compounds with high hydrophobicity (phospholipids). Thus, the stationary phase can be chosen based on the compound classes of interest, if the aim of the study is targeted metabolomics. However,

if the interest is to reach the most information as possible (untargeted metabolomics), more than one type of column might be necessary [74].

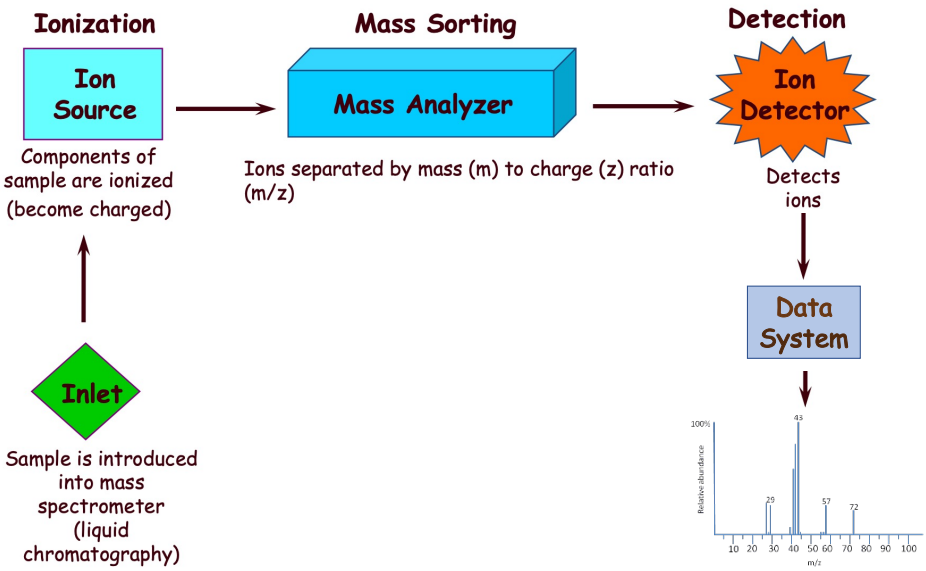

FIGURE 1.2: **The components of a mass spectrometry system**

Gas cromatography performs the separation of volatile (or made more volatile by chemical derivatisation) and thermally stable metabolites. The chemical classes that can be considered naturally volatile are: ketones, aldehydes, alcohols, esters, furan and pyrrole derivatives, heterocyclic compounds, sulphides, some lipids, isocyanates, isothiocyanates and hydrocarbons with 1–12 carbons. The classes that can be made volatile by derivatisation are sugars, sugar phosphates, aminoacids, lipids, peptides, long-chain alcohols, amines, amides, alkaloids, sugar alcohols and organic acids. This separation method is mostly employed in metabolomics studies that quantify short and medium chain fatty acids, which are volatile compounds [74].

1.4 Metabolic Alterations in Inflammatory Arthritis

Several studies have shown the presence of metabolic alterations in inflammatory arthritis. The most relevant studies for these alterations involve RA, since it's more prevalent, followed by PsA and other types of inflammatory arthritis [80, 81].

1.4.1 Altered Metabolic Pathways in Rheumatoid Arthritis

In RA, the synovial membrane is the main site of the pathological process. The inflamed synovial membrane contains multiple cell types, both immune, such as T and B cells, macrophage like synoviocytes (type A synoviocytes) and mast cells, as well as non-immune cells, such as stromal cells (type B synoviocytes - fibroblast like synoviocytes FLS) and endothelial cells, which all play a role in the pathogenesis of RA. These cells present an activated phenotype and their interaction creates and maintains a pro-inflammatory environment in the joint, requiring high energetic demands, which leads to changes in metabolic pathways; moreover, the involved metabolic pathways can be different depending on the cell type. One of the biggest unknowns is if the metabolic changes are a consequence or drivers of the disease, since the resulting metabolites are not only end-products of cellular processes, but also function as signaling molecules and participate in regulating other processes, such as transcription of different genes involved in inflammation and immune functions [82, 80].Several studies have employed different analytical methods (MS, NMR) to characterize the metabolomic profile in the blood (serum or plasma), urine, or synovial fluid in patients with rheumatoid arthritis. Due to the heterogeneity of the methods that were used, the results of most of the studies are not comparable; however, there are metabolites with similar changes across multiple studies (Figure 1.3 and Table 1.3).

Glycolysis

Alterations of the glycolytic pathway have been detected in several studies on animal models, as well as human metabolomics studies, with particularities specific to each cell type.

RA FLS are characterized by an aggressive phenotype, which consists of high proliferation rate, migration and invasive capacity, as well as increased production of pro-inflammatory cytokines and matrix degrading enzymes. This phenotype has high metabolic requirements which are obtained by a shift of the aerobic oxidative phosphorylation (OXPHOS) to a glycolytic state, in which less adenosine triphosphate (ATP) is produced but at a faster rate. The RA joint hypoxic environment and pro-inflammatory cytokines, such as TNF-α, stimulate an increase of the glycolysis:OXPHOS ratio and is accompanied by an amplification of the aggresive phenotype and an increase of indirect markers of glycolysis (PFKFB3, PKM2 and GLUT1)[90, 82].

Type of sample/study	Number of participants	Metabolite changes
Plasma		
Prospective. RA patients vs controls	47 RA patients on DMARDs (23 active and 24 in remission) and 51 controls. Sample collected at 0, 2, 4 weeks and 6, 12 months.	Elevated metabolites in RA patients compared to controls: choline, cholesterol, acetylated glycoprotein, lactate and unsaturated lipid. Decreased HDL in RA patients compared to controls
Cross-sectional	24 RA patients on methotrexate and less than 10mg prednisolone daily	Positive correlation with fatigue in RA: Fructose, arachidonic acid, glycerol-3-phosphate, indole-3-acetic acid, proline. Negative correlation with fatigue in RA: 2-oxoisocaproate, cystine, hydroxyproline, decosahexaenoic acid, tryptophan, pipecolic acid, valine, ornithine, arginine, urea, tyrosine, linoleic acid
Cross-sectional. RA patients vs controls	132 established RA patients and 104 controls	Metabolites increased in RA vs control: prolyglycine. Metabolites decreased in RA vs control: 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, sarcosine. * Steroids in those with past corticosteroids treatment vs those who never received them or are currently taking it
Serum		
Cross-sectional. RA patients vs controls	14 healthy controls 16 established RA patients, and two groups of early RA patients (89 and 127 RA patients)	High in RA patients compared to controls: 3-hydroxybutyrate, lactate, acetylglycine, taurine, glucose. Low in RA patients compared to healthy controls: LDL-CH3, LDL-CH2, alanine, methylguanidine, lipid
Cross-sectional RA patients vs controls	33 established RA patients and 32 controls	Metabolites increased in RA compared to controls: glycerol, citrate, pyruvate, cholesterol, fatty acids. Metabolites decreased in RA compared to controls: glucose, urate, alanine, serine, methionine, threonine, leucine, valine, isoleucine, aspartate, phenylalanine, tyrosine, proline, urea
Cross-sectional. RA on GC vs RA that did not receive GC	281 RA patients 73 Males taking GC 42 Females taking GC	Higher in female patients on GC: lysophosphatidylcholines and lysophosphatidylethanolamines. In male, lysophospholipids levels were similar between GC users and non-users
Cross-sectional. RA and pSS patients vs controls	30 active RA patients and 30 pSS as a disease control 32 controls	Metabolites increase in RA vs pSS and control: L-Leucine, L-phenylalanine, glutamic acid, L-proline, 4-methoxyphenylacetic acid. Metabolites decrease in RA vs pSS and control: Tryptophan, argininosuccinic acid, capric acid

TABLE 1.3: **Factors involved in circulating metabolic profile in patients with RA.** Several factors influence the circulating metabolites levels. Not only dietary factors or local synovial metabolites, but also comorbidities, treatment and individual factors, such as sex, age and genetics, will modify their metabolism and gut microbiome and hence, the circulating metabolic profile [83, 84, 85, 86, 87, 88, 89].

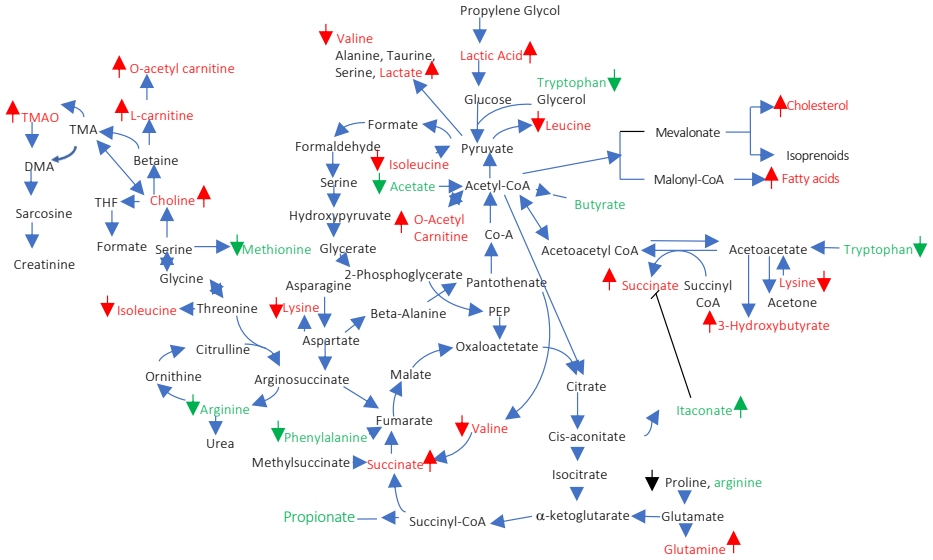

FIGURE 1.3: **Pro and anti-inflammatory circulating metabolites described in RA patients.** The red color indicates pro-inflammatory metabolites and the green color indicates anti-inflammatory metabolites. The arrows indicate increased/decreased concentrations of the metabolites compared to healthy controls [83, 84, 85, 86, 87, 88, 89].

The different types of T cells undergo modifications of different metabolic pathways depending on their functions. Naive T cells, in a rest state, rely on fatty acid oxidation as an energy source. Following activation, similar to the RA FLS, T cells rely on increased glycolysis. This also applies to the T cells which migrate to the joint to promote and amplify inflammation, which are called T effector cells. However, the memory T cells also rely on fatty acid oxidation. The activation of the glycolytic pathway in T effector cells in RA occurs years before the initiation of symptoms and it is related to a dysfunctional mitochondria[91].

Synovial monocytes and macrophages are other critical players in the RA synovium that suffers profound metabolic alterations during inflammation. They also present an enhanced glycolysis. High glucose concentrations (thorough a (NLRP3)/inflammasome-dependent mechanism) [92] and increased levels of α enolase in RA macrophages, [93] along with an increased activity of hexokinase I, also a glycolytic enzyme, stimulate the secretion of the pro-inflammatory cytokine IL1-*beta* [94].

Lactate is the end product of glycolysis. High concentrations of lactic acid are found in both blood and synovial fluid from inflamed joints in RA patients. Past studies have shown that lactate promotes the aggressive phenotype of FLS [95] and the pro-inflammatory properties of macrophages [96, 97, 98], stimulates IL-17 secretion by CD4+ T cells and,

at the same time, decreases CD4+T migration, which is related to the maintenance of a chronic inflammatory infiltrate [99, 100]. Moreover, recently, a new lactate induced histone modification was described, lactylation, which correlates with the levels of lactate and is different from acetylation [101]. These findings require further study to evaluate their role in disease states, since altered epigenetic marks have been recently described in RA FLS [102].

Succinate is elevated in the synovial fluid of patients with RA [103]. The TCA metabolite promotes inflammation by stimulating IL-1 secretion in murine macrophages through HIF-1 [96]. Moreover, succinate activates NLRP3 inflammasome inducing IL-1β secretion by synovial fibroblasts in a rat model of RA [104]. It seems that succinate also plays a role in innate and adaptive immune responses, since the genetic deficiency of Sucnr1, a succinate receptor expressed by immune cells, decreases trafficking of dendritic cells and reduces expansion of Th17 cells in the lymph nodes, reducing the symptoms of arthritis in the mouse antigen-induced arthritis model [105].

Itaconate, a macrophage activation marker, is thought to play an anti-inflammatory role, since it inhibits the succinate dehydrogenase-mediated oxidation of succinate, and through this, exerts anti-inflammatory effects in activated macrophages, as shown in an in vivo model of ischemia-reperfusion injury [106]. However, in an animal model of RA, higher levels of itaconate were found to be associated with high disease activity [107]. It would be interested to explore how these changes translate in humans with RA.

Aminoaacid metabolism

Glutamine is an amino acid used as a source to fuel metabolism. Glutaminase 1, the enzyme responsible of glutaminolysis, is upregulated in RA synovial fibroblasts and inhibition of this enzyme decreased the aggressive phenotype of the FLS and improved the severity of arthritis in the SKG murine model of arthritis [108].

Tryptophan metabolism. Tryptophan is an essential amino acid that must be provided in the diet. It has been described that microbes-derived tryptophan metabolites can exert systemic and anti-inflammatory effects [109]. Moreover, tryptophan and its catabolic metabolites generated through the kynurenine pathway are involved in inflammation. Kynurenine has known anti-inflammatory effects that are toxic to T cells and induce cell death by apoptosis. Kynurenine is formed from tryptophan by the activity of indoleamine 2, 3-dioxygenase (IDO). The activation of IDO is involved in the resolution of arthritis in mice associated with an increase in kynurenine metabolites [110]. Kynurenine itself has been identified as

a ligand for the aryl hydrocarbon receptor, which is important in the maturation of immune cells, and its addition promotes the differentiation of regulatory T cells and suppresses the differentiation of pathogenic Th17 cells [111].

BCAA (Branched-chain amino acids). Decreased levels of valine, leucine, and isoleucine were found in RA patients. Decreased levels of BCAA could be explained by low dietary consumption or by a higher intake of these amino acids by inflamed tissue. These are essential amino acids, so their source is the diet, and lately, they have been related to inflammation by inducing oxidative stress (via NADPH and Akt-mTOR signaling) and promoting the secretion of proinflammatory cytokines (IL-6, TNF) as well as the migration of peripheral blood mononuclear cells [112]. Branched chain aminotransferases 1 (BCAT1), an enzyme that initiates BCAA metabolism, is the predominant isoform in human primary macrophages. Its action on leucine produces acetyl-CoA and glutamate, which enter the TCA cycle. Treatment of LPS and TNF stimulated human macrophages with ERG240, a leucine analogue that blocks BCAT1 activity, decreased oxygen consumption and glycolysis. Moreover, oral administration of ERG240 reduced the severity of collagen-induced arthritis in mice [113].

Lipid metabolism

Cholesterol comes from the diet and its levels are increased in RA patients [83, 84, 85, 86, 87]; this was found to be predictive of RA in women, but not men [114]. Lipid metabolism is altered in RA, but cholesterol metabolism in RA has not been specifically studied. Interestingly, cholesterol was recently found to be high in OA chondrocytes, due to an increased uptake, upregulation of cholesterol hydroxylases, and increased production of oxysterol metabolites [115].

Free fatty acids (FFA) can be either taken from the diet (essential FA, alpha-linolenic acid -an omega-3 FA- and linoleic acid -an omega-6 FA-) or synthesized in the organism. It was suggested that they were proinflammatory, since they contribute to low-level inflammation in obese patients. FFA levels were higher in the serum of RA patients than in control subjects, and correlated with disease activity [116]. Frommer et al showed that FFA contribute to the pathogenesis and damage in RA, OA, and PsA, since stimulation of FLS with oleic, palmitic, and linoleic acid induced the secretion of proinflammatory cytokine IL-6, the chemokines IL-8 and MCP-1, as well as the matrix metalloproteinases pro-MMP1 and MMP3 [117]. Arachidonic acid (ARA) is the precursor of classically described prostaglandins (PGE2), which are known to be involved in inflammation in general, but also in arthritis [118, 119].

Polyunsaturated Fatty Acids (PUFA) Related Metabolites. Docosahexaenoic acid, DHA, and eicosapentaenoic acid, EPA have anti-inflammatory properties, mainly because they compete with AA for the action of the enzymes (cyclooxygenase-COX, lypooxigenase-LOX, cytochrome P450) which results in a decreased production of AA derived proinflammatory oxylipins and an increased production of DHA and EPA derived anti-inflammatory oxylipins (Figure 1.4) [120, 121]. Several studies have described improved outcomes in RA patients after dietary intervention [120, 122, 123]. Decreased levels of EPA and DHA were described in Spanish RA patients, and were associated with higher disease duration, positivity for rheumatoid factor, erosive disease and with a worse response to TNF inhibitors [124]. Gene variants of the enzymes involved in the PUFA metabolism can determine the metabolic and clinical response to dietary intake of PUFA. For example, 5-lipoxygenase (ALOX5) gene variants were found to influence response to fish oil supplementation, changing the oxylipin profile and, consequently, having a different effect on cardiovascular risk [125]. Another study checked the association between genetic variants of ALOX5, ALOX12, ALOX12B, and ALOX15, and type-2 diabetes mellitus (DM2), and found that ALOX12 and ALOX12B genetic variants increased susceptibility to DM2 development, possibly though alterations in PUFA/ARA metabolism[126].

Oxylipin Related Pathways. Prostaglandins, thromboxanes and leukotrienes are the classically described oxylipins involved in the pathogenesis of RA. The newer methods of LC/MS and NMR make described oxylipins involved in the pathogenesis of RA. The newer methods of LC/MS and NMR it is possible to identify several other oxylipins, e.g., 8-HETE, 12-HETE, and 12-HEPE are products of the make it possible to identify several other oxylipins, e.g., 8-HETE, 12-HETE, and 12-HEPE are products 12-lypoxygenase pathway. Liagre et al. demonstrated the presence of 12-LOX in type B synoviocytes of the 12-lypoxygenase pathway. Liagre et al. demonstrated the presence of 12-LOX in type B and found that IL-1β and TNF stimulation increased 12-HETE production, while IL-6 and IL-4 did not have the same effect [127]. This pathway was also studied by Kronke et al., who showed that the deletion of 12/15-LOX in two models of arthritis (the K/BxN serum-transfer and a TNF transgenic mouse model) led to uncontrolled inflammation and tissue damage [128]. LTB4 and 5-HETE are products of AA via 5-LOX pathway; 5- and 15-LOX are expressed in both OA and RA synovium in the lining and sublining macrophages, neutrophils, and mast cells, and have been shown to be involved in RA pathogenesis, promoting inflammation [129].

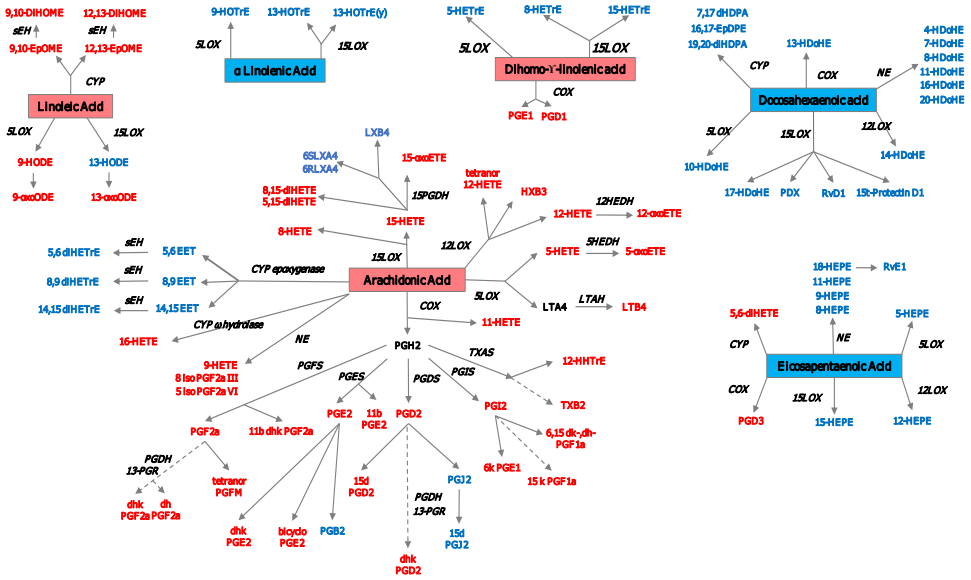

FIGURE 1.4: **Oxylipins derived from PUFA.** Pro-inflammatory oxylipins are marked in red, while anti-inflammatory ones are marked in blue. The precursor n3-PUFAs are marked in a red square, while the n6-PUFAs are marked in a blue square. COX – cyclooxygenase; LOX – lypooxigenase; CYP – cytochrome P450; NE – non-enzymatic; PGFS – prostaglandin F synthase; PGES – prostaglandin E synthase; PGDS – prostaglandin D synthase; PGIS – prostaglandin I synthase; TXAS – thromboxane A2 synthase; LTAH – leukotriene A4 hydrolase; MDB – membrane dipeptidase; HEDH - hydroxyeicosanoid dehydrogenase; PGDH - hydroxyprostaglandin dehydrogenase; 13-PGR – 15-ketoprostaglandin13 reductase; sEH – soluble epoxide hydrolase.

Short Chain Fatty Acids (SCFA) are byproducts of the metabolism of dietary fiber by the gut microbiome. They modulate immune and inflammatory responses via the activation of free fatty acid (FFA) receptors type 2 and 3 (FFA2 and FFA3 receptors) and G protein-coupled receptor 109A (GPR109A), and via inhibition of histone deacetylases (HDACs). A metabolomic study performed on a CIA rat model found decreased levels of acetate, propionate, butyrate, and valerate in fecal samples of arthritic rats compared to controls [130]. The administration of butyrate inhibited collagen-induced arthritis via Treg/IL10/Th17 axis [131].

Choline Trimethylamine-N-oxide (TMAO) Metabolites related to the choline pathway were identified in several studies in synovial tissue, synovial fluid and bloodd (serum and plasma) samples in both animal models of arthritis and human studies. Diet is the main source of choline [132],whose metabolites (trimethylamine-N-oxide, TMAO) have already been related with cardiovascular inflammation [133, 134]. Choline and other dietary trimethylamine

(TMA) containing species like carnitine are metabolized to TMA by the gut microbiota. TMA is subsequently oxidized by at least one member of the flavin-containing monooxygenases, FMO3, forming trimethylamine-N oxide (TMAO), which is then released into circulation [135].Despite being so well studied in relation to cardiovascular inflammation, no studies evaluating the role of TMAO in RA have been yet performed. TMAO, as well as choline, was found to be increased in serum samples in the murine K/BxN model of arthritis compared to control mice [136]. Choline is also a nutrient uptaken by the cells and metabolized via the Kennedy pathway, during which several phospholipids that function as signaling molecules are produced, such as glycero-phosphocholine (GPC), phosphocholine, phosphatidylcholine (PC), lyso-PC, diacylglycerol, and lysophosphatidic acid [137]. Importantly, choline metabolism has been related to the RA FLS phenotype [138] and IL-1β secretion in macrophages [139].

Bile acids (BA) seem to have anti-inflammatory properties. Primary BAs are synthesized in the liver and are liberated in the gastrointestinal tract to help with lipid digestion. Gut bacteria metabolize primary bile acids and can deconjugate them, synthesizing secondary BAs. BAs have been detected in the systemic circulation, where their concentrations vary with diet, and have been related to insulin resistance [140]. High concentrations of BA can actually kill intestinal bacteria to prevent colonization, but they also regulate the mucosal immune functions through several receptors. A recent study showed the role of BA in the maintenance of the homeostasis of the mucosal immune function in the gut, through the vitamin D receptor [141]. An in vitro study found that taurolithocholic acid suppressed the expression of genes involved in mediating pro-inflammatory effects, phagocytosis, interactions with pathogens and autophagy, as well as the recruitment of immune cells, such as NK cells, neutrophils and T cells [142]. BA exert their actions through both specific and nonspecific receptors. The activation of TGR5 receptor by endogenous BA suppressed the production of LPS induced inflammatory cytokines in macrophages, while no effect was seen in macrophages that lacked this receptor [143, 144]. Of interest, a study described an anti-inflammatory role of taurochenodeoxycholic acid in RA FLS [145]. Most studies focused on the effects of the BA on the gut mucosal immunity, and hence, future studies are needed to elucidate the roles of circulating BAs in disease states.

The existing data that we just described is still not sufficient to support the certain involvement of metabolites in RA pathogenesis. However, the explosive growth of the field of

tissue immunometabolism and its description of multiple critical metabolic pathways in the activation and differentiation of immune cells such as T and B lymphocytes, macrophages, dendritic cells, and fibroblasts, among others, suggests that most of the metabolites involved in the immune response can also be important in RA. Figure 1.5 presents a summary of pro- and anti-inflammatory metabolites associated with RA pathogenesis.

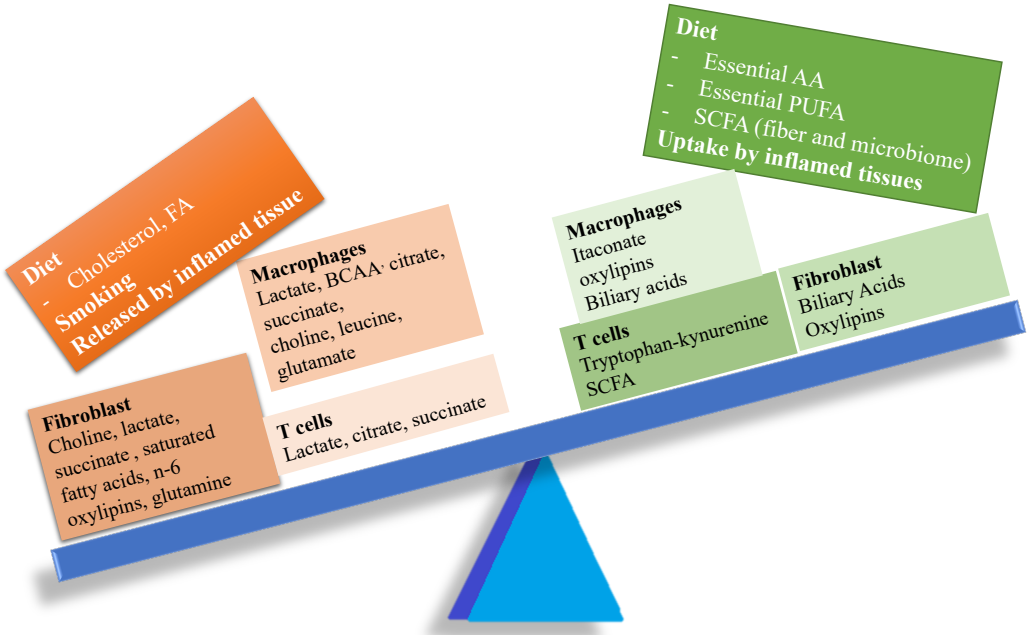

FIGURE 1.5: **Imbalance between pro- and anti-inflammatory metabolites in RA.** Several pro-inflammatory metabolites (left side of the balance) might play a key role in RA pathogenesis modulating the function of several cell types involved in synovial inflammation. [83, 84, 85, 86, 87, 146, 139, 147, 103, 96, 138, 112, 113, 108, 95, 101, 104, 105, 107, 116, 117, 118, 119, 122, 123, 124, 125, 127, 128, 130, 144, 145, 110, 111]

1.4.2 Altered Metabolic Pathways in Psoriatic Arthritis

PsA is an inflammatory disease associated with metabolic syndrome and epidemiological studies have found that the prevalence of metabolic syndrome in patients with PsA is around 30%[148, 149]. This makes the possible metabolic alterations encountered in these patients more difficult to interpret, since they might be associated to arthritis or to the comorbidities. Metabolomics has been less employed in the study of PsA, however, several studies have looked at metabolic alterations in PsO, specifically at the lipid metabolism and a few studies explored metabolomic changes in patients with PsO only compared to PsA patients.

Lipid metabolism

The majority of studies focused on the alteration of lipid metabolism in blood samples (serum, plasma, cells) in PsO and PsA. In PsO, the study of local alterations in the lesional skin has been possible due to the easier access to obtaining skin from patients. However, local alterations in the tissues involved in PsA, such as synovium and enthesitis, have not yet been performed, due to the more invasive procedure required to obtain tissue.

The level of free fatty acids (LA, DHA and AA) was found to be decreased in plasma of both PsO and PsA patients, compared to controls, with higher values in PsO compared to PsA. Phospholipids were also altered in these patients compared to controls: different species of lysophosphatidylethanolamine (LPE) were lower in PsO and PsA patients compared to controls, while phosphatidylinositol (PI) species levels were upregulated [150]. Moreover, the activity of enzymes involved in the metabolism of free fatty acid and phospholipids ((phospholipase A2, acetylhydrolase PAF, cyclooxygenases 1 and 2) was upregulated.

Classical oxylipins have been studied extensively in PsO. Prostaglandin E2 (PGE2) is the most abundant prostaglandin in the skin and has a role in the pathogenesis of PsO, since it was shown to be important in the IL (interleukin)-23 dependent generation of pathogenic Th (helper) 17 cells, which are related to PsO pathogenesis [151, 152]. However, it is also involved in the activation of the resolution of inflammation, by stimulating the secretion of specialized pro-resolving mediators [153]. PGE2 and 12- and 15-HETE were described to be higher in psoriatic skin compared to skin from healthy controls, in contrast to serum concentrations that were found to be lower in PsO patients compared to healthy controls [154, 155]. HXB3 was also described to be higher in lesional skin compared to skin from healthy controls [156]. The same studies also found that the concentrations of EPA (5-, 12-, 15- and 18-HEPE), LA (13-HODE, 9-oxoODE, 13-oxoODE), and DHA (4-, 7-, 14- and 17-HDoHE) derived oxylipins were higher in lesional skin compared to normal skin, but lower in serum of PsO patients versus healthy controls.

Using an animal model of skin psoriasis, Ueharaguchi et al observed that another eicosanoid, TXA2 (AA derived via COX2 and precursor of TXB2) signaling through its receptor, TBXA2R, may facilitate psoriatic dermatitis by promoting IL-17 production in psoriatic lesions [157]. Furthermore, mRNA expression of TBXA2R was significantly increased in skin biopsies from psoriatic lesions compared to skin from healthy controls.[157] While IL23 induces the production of PGE2 by Th17 cells, PGE2 acts back on its receptors, EP2 and EP4 on these cells, to increase Il23r expression in a positive feedback manner. Interestingly, EP4 receptor

(PTGER4) was overexpressed in human psoriatic lesional skins (15)

Glucose metabolism

Patients with PsA not only have a higher prevalence of type 2 diabetes mellitus (DM2), but are also at increased risk of developing DM2 [158, 159]. Glucose and glucose metabolism related metabolites levels are lower in lesional psoriatic skin, due to high energetic consumption by proliferating cells. The substrates of anaerobic enzymes are also decreased (myoinositol and lactic acid) in psoriatic lesions. This has not been studied in the synovial tissue in the PsA joint, however, it could be inferred that the inflamed PsA joint is similar to the RA joint, which is characterized by enhanced glycolysis and increased levels of lactic acid.

Aminoacid metabolism

Glutamine and asparagine levels were found to be lower in patients with psoriasis than in those without. However, levels of alpha-ketoglutarate are higher in patients with psoriasis vulgaris but lower in those with psoriatic arthritis. This suggests may be related to the consumption of glutamine due to overactive immune cell proliferation. However, the decrease in asparagine is due to spontaneous deamidation and catabolism in an inflam- matory, oxidative stress environment. a-Ketoglutarate is involved in the synthesis of proline, a major substrate for collagen synthesis, and in the oxidative supply of the tricarboxylic acid cycle as a citric acid cycle intermediate

1.5 Factors That Influence Circulating Metabolites

Due to hightroughput metabolomics approaches, this "omic" science is adequate for biomarker exploratory analysis, followed by more thorough studies of specific metabolic pathways, employing the targeted approach [160]. Over the past years, an increasing number of studies have employed metabolomics to study the relation between disease activity, certain disease characteristics and response to different treatments on one hand, and metabolites on the other hand. The most frequently used biospecimens for metabolomics are blood (both serum and plasma), synovial fluid and urine. So far, only a handful of studies have analyzed the relation between circulating and tissue metabolites. Due to different techniques: NMR versus LC-MS/MS versus GC-MS used for metabolites quantification, the results of the studies are difficult to compare, nonetheless, metabolites with similar changes across different studies have been identified.

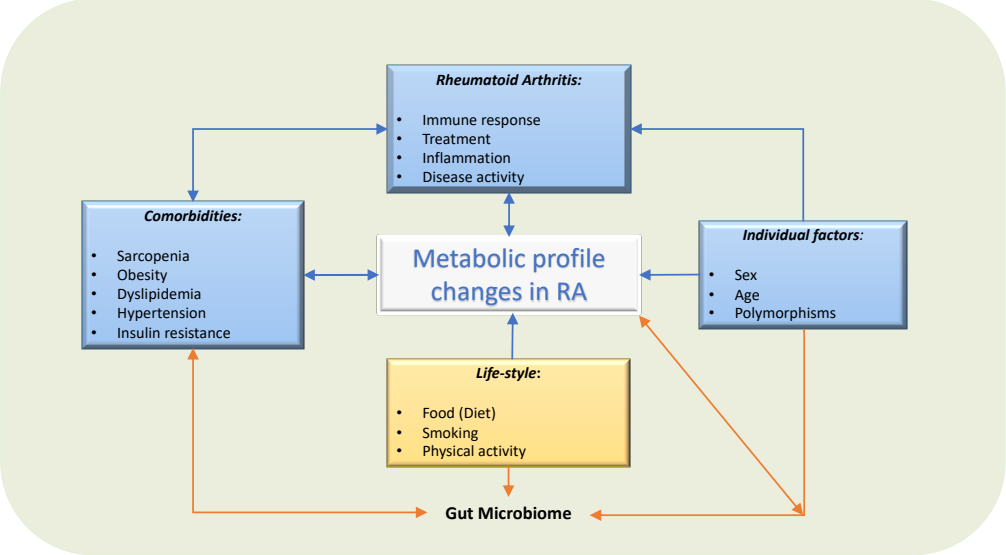

FIGURE 1.6: **Factors involved in circulating metabolic profile in patients with RA.**Several factors influence the circulating metabolites levels. Not only dietary factors or local synovial metabolites, but also comorbidities, treatment and individual factors, such as sex, age and genetics, will modify their metabolism and gut microbiome and hence, the circulating metabolic profile.

Metabolites reflect an organism's state, which results from the interaction of internal and external factors, such as genetic and environmental/lifestyle factors, respectively. In disease states, the circulating metabolites are also affected by the pathological processes, and there are already well-studied metabolites that are considered to be disease reporters, like the increase of blood glucose levels in diabetes mellitus. In systemic diseases such as RA, the abnormal circulating metabolomic profile might reflect genetic predisposition, local inflammation, comorbidities, and several environmental factors including diet, smoking, or microbiome (Figure 1.6).

Diet. Amongst environmental factors, diet is one that directly affects circulating metabolites. For example, essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and essential fatty acids (alpha-linolenic acid and linoleic acid) come from the diet. Of interest, some of these essential nutrients were found to be low in RA patients, including linoleic acid, and several amino acids (Table 1.3), suggesting a link between diet and inflammation in RA.

Epidemiological studies have shown a relationship between diet and RA; thus, some of the metabolomic changes observed in several fluids (serum/plasma or urine) in early arthritis

could be related to differences in dietary patterns between RA patients and the healthy population. A study that included a large number of patients (15770 adult males and females) found that patients with arthritis (including RA and osteoarthritis) had lower quality diets compared to people without arthritis, based on HEI-2015, a healthy eating index created by the USDA and based on the Dietary Guidelines for Americans [161]. Patients with arthritis consumed less fruit, vegetables, greens and beans, whole grains, seafood, and plant protein, but more added sugars, saturated fats, and empty calories compared to those without arthritis [162]. The association of poor dietary quality with RA was also observed in other studies, in which RA patients had an inadequate intake of fruit, vegetables, dairy, fatty acids, and whole grains [163, 164, 165]. A study in a Chinese population found that RA patients were consuming different amounts of chicken, fish, mushrooms, beans, citrus, dairy products, and organ meats than healthy controls [166]. Another study that included a white population found that both women and men on a nonvegetarian diet were at higher risk of developing RA [167]. Hu et al analyzed the cohort of women included in the Nurses' Health Study and Nurses's Health Study II that were followed from 1984 to the present-day, and found that good dietary quality, moderate alcohol consumption, and low intake of red meat were associated with a lower rate of RA incidence [165].

The identification of food biomarkers is an ongoing process; a consensus has not been reached as to which metabolites would be the most adequate biomarkers for different types of foods. Moreover, some metabolites are markers of categories of food, not being able to discriminate between the exact types of foods being analyzed (1-methylhistidine and 3-methylhistidine are found in meat and are not useful in discriminating between types of meat). It is possible that for some foods, a combination of metabolites would be more suited as a marker than a single metabolite. Unfortunately, as of now, the metabolomic studies in RA (Table 1.3 have not collected food intake data nor used the same metabolomic platforms, making it difficult to associate specific food intake with metabolic changes in RA patients. However, some metabolites from Table 1.3 suggest an interaction between circulating metabolites and diet. For instance, some of these studies [86, 168] showed higher levels of carnitine and taurine in RA patients, which are potential biomarkers of meat intake.

Drugs. Researchers have used a metabolomics approach to evaluate the changes in circulating metabolites from drugs used in RA treatment (Table 1.4). The study of these changes might help to understand RA pathogenesis, since the therapeutic effects of these drugs could

Drug	Decreased	Increased
Methotrexate		
Plasma Samples	Taurine, aspartate, alanine, hypoxanthine, cytosine, uric acid, uracil, lactic acid, S-adenosyl-L-homocysteine, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate	Tryptophan, threonine, histidine, methionine, and glycine, carnitine, guanine, adenosine
Glucocorticoids		
Serum Samples	None reported	Lysophosphatidylethanolamines and lysophosphatidylcholines (Females)
Plasma Samples	Asymmetric dimethyl arginine, symmetric dimethyl arginine	None reported.
Anti-TNF		
Serum samples	3-hydroxyisobutyrate, lysine, acetoacetate, acetylphosphocholine, creatine sn-glycero-3-phosphocholine, histidine and phenylalanine	Leucine, acetate, betaine and formate
Serum samples	3-hydroxybutyrate	Isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose
Urine Samples	Eanolamine, p-hydroxyphenylpyruvic acid, and phosphocreatine	Hippuric acid, citrate, and lactic acid (Infliximab). Choline, phenylacetic acid, urea, creatine, and methylamine (Etanercept). Histamine, glutamine, phenylacetic acid, xanthine, xanthurenic acid, and creatinine

TABLE 1.4: **Metabolic profile modifications by drugs used in the treatment of RA**

potentially be driven by metabolic changes either by normalizing their abnormal values or by increasing anti-inflammatory metabolites. For instance, using a targeted metabolomic approach, Fu et al. compared the effect of oral glucocorticoids (GC) on serum polar lipids and observed an increase in lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE) in females but not in male patients with RA [88]. GC inhibits phospholipase A, a key enzyme that hydrolyzes membrane phospholipids which is increased in inflammatory tissues. The effect of GC on phospholipase A will likely modify the phospholipid profile. Of interest, polyunsaturated acyl LPC and LPE presented an anti-inflammatory effect on animal models [169]. The effect of low dose GC (<10 mg/day) on arginine metabolism and cardiovascular risk in RA patients was also studied [170]. This study from Australia that included 36 RA patients, 18 of which were on GC (GC users) and 18 that were not receiving GC (non-GC-users), found that asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA) levels were lower in patients on chronic GC compared to non-GC users, suggesting that long-term treatment with GC had an improved endothelial function and a cardiovascular protective effect by modulating arginine metabolism [170].

Wang et al. [168] studied the change of the plasma metabolic profile in 29 RA patients after the initiation of treatment with methotrexate (14 patients) or a combination of

methotrexate with a Chinese medicinal herb (15 patients). They found decreased levels of several amino acids (tryptophan, threonine, histidine, methionine, and glycine) as well as other metabolites (carnitine, hypoxanthine, cytosine, uracil, and uric acid), while taurine, aspartate, alanine, lactic acid, adenosine, and guanine were significantly increased in RA patients compared to controls. Interestingly, the treatment with methotrexate brought the levels of all these metabolites back to normal levels, suggesting a causative role of these amino acids in RA pathogenesis. The combination of MTX with tripterygium glycosides tablets was more effective in obtaining these results compared to monotherapy with MTX. Although more data is needed to link amino acid changes to abnormal immune response in RA, data in immune cells suggest a direct link between amino acid metabolism and T cell and macrophage responses by promoting and modulating inflammation, which could potentially be involved in RA pathogenesis [171, 172, 173, 174]. In RA, tryptophan is the substrate of indoleamine-2,3-dioxygenase IDO2, which was demonstrated to be required for the activation of CD4+ Th cells, the production of pathogenic autoantibodies, and the subsequent development of arthritis in a KRN mouse model of arthritis [175, 176, 177]. This offers a possible explanation for the decrease in tryptophan levels that is then reversed by the addition of methotrexate. On the other hand, levels of S-adenosy-L-homocysteine, 5-formyltetrahydrofolate, and 5-methyltetrahydrofolate were similar between controls and RA patients before treatment, and decreased after 3 months of methotrexate, pointing to these methotrexate-associated metabolites as adherence biomarkers [168].

TNF is a potent pro-inflammatory cytokine that plays key role in cell metabolism, including glucose and lipid metabolism [178]; thus, changes in metabolic profile are expected after the administration of a TNF inhibitor. The first study that evaluated the changes in the metabolic profile of 16 RA and psoriatic arthritis (PsA) patients after TNFi treatment (etanercept and infliximab) used urine samples. The study described increases in hippuric acid, citrate, and lactic acid after infliximab treatment, while increases in choline, phenylacetic acid, urea, creatine, and methylamine were seen after etanercept treatment [179]. Another group evaluated the serum metabolomic profile in 20 patients with RA before and after treatment with TNFi (etanercept or adalimumab). Of the 20 patients, 55% of patients had a moderate EULAR response, while only 20% reached a good response. At 3 months

posttreatment, 3-hydroxyisobutyrate, lysine, acetoacetate, acetylphosphocholine, creatine sn-glycero-3-phosphocholine, histidine, and phenylalanine levels decreased, while leucine, acetate, betaine, and formate levels increased, but they did not reach those of the healthy control [177]. The changes of the serum metabolic profile in response to treatment with a TNFi, etanercept, in 27 patients with active RA were also evaluated by Kapoor et al. These patients were receiving concomitant therapy with GC and disease-modifying antirheumatic drugs. After 3 months of treatment, isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose levels were found to be increased in good responders as defined by EULAR-ESR criteria, whereas 3-hydroxybutyrate levels were reduced . The decrease of 3-hydroxybutirate, acetoacetate, and acetylphosphocholine levels suggests a modulation of lipid metabolism after TNF inhibition, especially in responders. In addition, the increase of glucose and other amino acids suggests a decrease of glucose and amino acid metabolism by the inflamed tissues[180].

Comorbidities. RA patients present several comorbidities including obesity, metabolic syndrome, and sarcopenia, probably triggered by a disbalance of proinflammatory cytokines including TNF and IL-6 among other causes [181, 182, 183, 184, 185], that will modify the circulating metabolites [186]. Several studies have investigated circulating metabolic changes related to the metabolic syndrome and obesity [187, 188]. Of interest, a lot of circulating metabolites that are different in RA patients compared to controls could be related to associated metabolic syndrome, since choline metabolism (especially TMAO and carnitine), aminoacids (alanine, glutamine, glutamate, arginine, aspartate, asparagine, histidine, methionine, cysteine, lysine,branched-chain amino acids (BCAA), phenylaniline, tyrosine, and tryptophan) and phospholipids (phosphatydilcholines) also change in those with metabolic syndrome [188]. Several works on muscle mass have also suggested that some circulating metabolites can be biomarkers of muscle mass and sarcopenia [189]. Even though both fat tissue and muscle, as well as associated immune cells in these inflamed tissues, can be sources of metabolites, it is unknown how much they can contribute to the pool of circulating metabolites. For example, studies measuring the metabolomics profile in visceral adipose tissue and serum from obese patients found low correlations between serum and adipose tissue metabolites [190]. On the other hand, we can speculate that there might be a competition between inflamed tissues (adipose tissue vs. synovial tissue) for the uptake of circulating anti-inflammatory metabolites.

Sex and Age. Several epidemiological studies have shown differences in metabolite concentrations according to sex and gender. A cross-sectional study in urine samples showed that some metabolites from the tricarboxylic acid cycle (TCA) cycle such as citrate and fumarate were elevated in women, while carnitine, acetylcarnitine, acetone, and creatinine were higher in men [191]. In addition, Fan et al. found that 2-hydroxyglutaric acid, -ketoglutarate, and 2-oxyglutaric acid were higher in women. However, UDP-glucoronic acid was higher in men, suggesting that this could be linked to sex hormones [192]. Another study showed differences between sex and metabolic profile in serum, suggesting that glycine, serine, and sphingomyelines are upregulated in women, and ornithine, arginine, acyl carnitines, and amino acids derived from glutamine pathway are elevated in males [193]. Finally, a longitudinal cohort of adults showed a positive correlation of levels of glutamine, tyrosine, long chain fatty acids, acyl-carnitines, and sphingolipids, and a negative correlation of histidine, tryptophan, threonine, serine, and leucine levels with age [194].

Smoking and Exercise Smoking is a known risk factor for RA and is associated with an increased risk of more severe arthritis, and less likelihood of achieving remission. Smoking also decreases the effectiveness of some disease-modifying antirheumatic drugs (DMARDs) [195, 196]. The exact reason of these associations is not well understood, although the effect of smoking on immune cells, and cytokine production, and the increase of oxidative stress that it causes, have been described,[197, 198, 146] and these likely affect the immune response in RA. Metabolomics has also identified blood biomarkers associated with chronic tobacco smoking. One study performed on a large number of healthy participants (892) from around the world found an association between smoking and three well-established nicotine metabolites (cotinine, hydroxycotinine, and cotinine N-oxide), and an additional 12 xenobiotic metabolites involved in benzoatic (e.g., 3-ethylphenylsulphate) or xanthine metabolism (e.g., 1-methylurate), three amino acids (o-cresol sulphate, serotonin, indolepropionate), two lipids (scyllo-inositol, pregnenolone sulphate), four vitamins or cofactors, and one carbohydrate (oxalate) [199]. Several of these metabolites, especially nicotine-derived metabolites, have been described to modulate the immune response [198], and other metabolic changes could be involved in smoking-induced methylation changes in immune cells [200]. Another study looked at the immediate effects of smoking on the metabolic profile. Thirty-one metabolites were shown to be acutely affected by cigarette smoking, including menthol-glucuronide, the reduction of glutamate, oleamide, and 13 glycerophospholipids. Moreover,

detailed analysis revealed changes in 12 cancer-related metabolites, notably related with cAMP inhibition [201]. Since a known mechanism of methotrexate in treatment of RA is to induce an increase of cellular cAMP [202], the inhibition of this metabolite by smoking could explain the decrease in the effectiveness of this drug in RA.

Exercise is another factor that might change the metabolomic profile. However, these changes depend of the quantity and type of exercise. For example, in people who exercise more than 2 h per day, some metabolites, including medium and long fatty acids, ketones, sulfated bile acids, palmitate,linoleate, stearate, and palmitoleate, increased two-fold in their plasma concentration. Decreased levels of pyruvate and lactate, among others intermediates of TCA, have been reported after a short running period [203]. The reader can find an extensive review of these changes in a recently published review [203] about metabolic changes after exercising.

Genetics: Polymorphisms Associated to Metabolism. Genome-wide association studies (GWAS) uncovered multiple loci that are associated with the level of metabolites, which involve a large number of metabolic pathways, indicating widespread genetic influences on the human metabolome (Figure 1.7). The loci that have been described involve amino acids, intermediates of lipid metabolism, including sterols, carnitines, and intermediates of inositol and fatty acid metabolism, intermediates of purine and pyrimidine metabolism, glucose homeostasis, and vitamin and cofactor levels [204, 205, 206, 207]. Polymorphisms in these metabolite-associated genes were also described in RA GWAS. In Figure 1.7, we put together a summary of metabolism-related genes described in genome-wide association studies (GWAS; https://www.ebi.ac.uk/gwas/). Polymorphisms in the genes underlined in red were found to be associated with RA. These genes are mostly related to lipid metabolism. Interestingly, lipid metabolites are considered pro-inflammatory metabolites, and higher levels of lipids were described in serum of RA patients compared to control subjects (Table 1.3). DLG2 (Discs Large MAGUK Scaffold Protein 2), which was found to be associated with glycerophospholipid metabolism [208], was also found to be related to response to TNF inhibitors in RA patients [209]. FADS1 and 2 (Fatty Acid Desaturase) and BLK (BLK Proto-Oncogene, Src Family Tyrosine Kinase), involved in fatty acid metabolism, and STAG1 (Stromal Antigen 1) and FCGR2B (Fc Fragment Of IgG Receptor IIb), involved in lipoprotein metabolism, were found to be associated with susceptibility to developing RA in several studies [210, 211, 212, 213, 214]. SLC22A4, a transporter related to isovaleryl/carnitine,

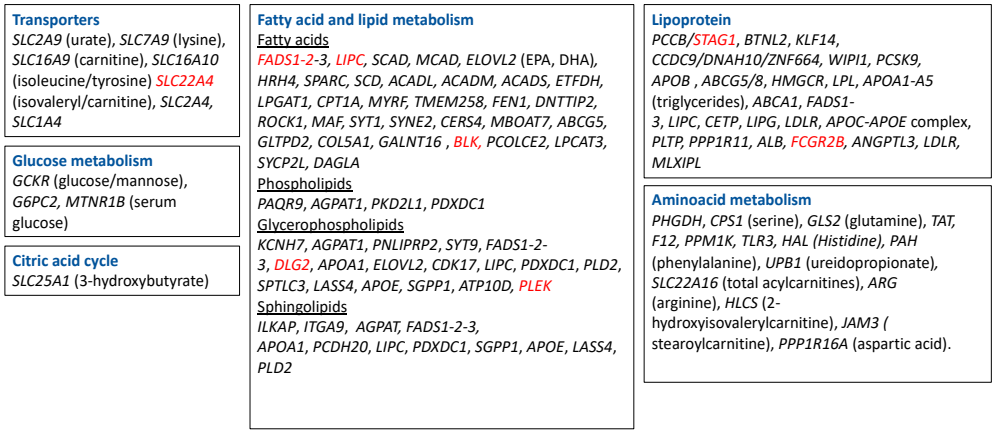

FIGURE 1.7: **Metabolism-related genes described in GWAS.**

was found to be associated with RA in a Japanese population [215], but not in a Canadian one [216]. Finally, Geiger et al. described 2 SNPs (single nucleotide polymorphism), rs9309413 and rs4775041, found on PLEK (Pleckstrin) and LIPC (Hepatic Triacylglycerol Lipase) genes, associated with sphingomyelin associated and phosphatidylethanolamine (PE) [217], that were associated with RA in a previous study [218]. Little is known about the role of these genes in inflammation and autoimmunity, so more studies are needed to determine whether some of these pathways are critical for RA pathogenesis.

Gut Microbiome/Absorption. The gut microbiome represents the collection of microbes that inhabit the intestines. Its composition is shaped by several factors, like genetics, age, delivery pattern, diet, antibiotic use, and other treatments [219, 220, 221, 222]. It can also be modulated by prebiotics [223, 224], probiotics [225], and fecal microbiota transplantation. Bacteria in the gut are important not only in the absorption of certain vitamins and in the synthesis of bile acids, but they also have the potential to modify circulating pro- or anti-inflammatory mediators, since they are involved in the metabolism of some dietary components [226]. For example, trimethylamine-N-oxide, a pro-inflammatory metabolite that derives from choline and carnitine present in red meat, eggs, and dairy products, is produced by *Prevotella copri* among other bacteria [227, 133]. An increased abundance of Prevotella copri was found in new-onset untreated RA patients, suggesting P. copri may be pathogenic in this disease [228]. In contrast, bacteria that have an almost exclusive saccharolytic metabolism, such as lactobacilli and bifidobacterial, are considered potentially beneficial [229], since they produce a variety of tryptophan catabolites (indole, tryptamine, indoleethanol (IE), indolepropionic acid (IPA), indolelactic acid (ILA), indoleacetic acid

(IAA), skatole, indolealdehyde (IAld), and indoleacrylic acid (IA)) which are critical for intestinal homeostasis by decreasing intestinal permeability [109]. In addition, some of these catabolites enter the bloodstream and may have anti-inflammatory and anti-oxidative effects [109]. The microbial degradation of whole-grain complex carbohydrates increases short-chain fatty acids (SCFA; butyrate, acetate and propionate), which were also shown to be beneficial to the intestinal immune response [230]. Microbial bile acid metabolites have recently been linked to colonic homeostasis [141].

The modulation of the microbiome through diet interventions is a potential strategy in the treatment of diseases, since microbiome alterations are related to disease, i.e., inflammatory bowel disease, obesity, cardiovascular diseases, autoimmune diseases, and others. It seems that the microbiome response to diet is variable and is highly influenced by the subject's baseline microbiome. Several studies found differences in the baseline microbiome of responders versus nonresponders to different diet interventions. Additionally, individuals with differing bacterial gene richness appear to have differing baseline gut microbiota communities that respond distinctively to a given dietary intervention which will influence the diversity of the gut microbe-derived specialized metabolites and circulating metabolites [231, 232, 233, 234, 235, 236, 237, 238, 239, 240].

Metabolites Released from or Uptaken by Inflamed Tissues. Another potential factor that determines the concentrations of the circulating metabolites is represented by the release of metabolites from the inflamed joint or their uptake by the synovium. Little is known about metabolic or lipidomic profiling of synovial tissue [241, 242]. In addition, no study has, to date, evaluated the relation between circulating metabolites in serum or plasma and synovial metabolites, although there might be a correlation. For instance, the synovial tissue of RA patients presents an enhanced level of lactate compared to noninflamed synovial tissue [138], which suggests an increase in the anaerobic cellular metabolism of resident cells [243, 244]. Lactate has also been one of the metabolites described to be upregulated in patients with RA [86]. Of note, inflammatory pathways increase the expression of nutrient transporters [245, 139, 246, 247]; therefore, this highly metabolic tissue will consume high amounts of metabolites, either to feed the increased metabolism of activated cells (pro-inflammatory metabolites) or to resolve inflammation (anti-inflammatory metabolites); this could be reflected by a decrease of circulating metabolites described in RA (Table 1.3 and Figure 1.3): such as glucose and amino acids (alanine, serine, methionine, threonine, leucine,

valine, isoleucine, aspartate, phenylalanine, tyrosine, and proline) [248, 87].

Fibroblast-like synoviocites (FLS), key cells in the pathogenesis and progression of RA, have an activated metabolism and can potentially release metabolites into the bloodstream [249, 250] characterized the intracellular metabolic profile of RA and osteoarthritis (OA) by an untargeted metabolomic approach using GC/TOF-MS. The results revealed that a high number of metabolites were increased in RA compared to OA FLS; these metabolites were amines (inosine, urate, 5-deoxy-5-methylthioadenosine, guanine, benzamide), fatty acids (behenic acid, palmitoleic acid, arachidic acid, oleic acid, myristic acid, stearic acid, palmitic acid, octadecanol, linoleic acid, lauric acid), phosphates (glucose-6-phosphate, phosphogluconic acid, adenosine-5-monophosphate, phosphate, -fructose-6-phosphate), organic acids (aspartate, adipate, 2-ketoisocaproate 3-phenyllactate, 2-hydroxyvaleric acid, phenylacetate, glycolate, oxalate, benzoate), amino acids (asparagine, glutamine), sugars and sugar alcohols (lactose fucose, mannose) and salicylaldehyde. Other metabolites, mostly amino acids (isoleucine, leucine, histidine, valine, ornithine, lysine, methionine sulfoxide, tryptophan, N-methylalanine, tyrosine, phenylalanine, citrulline, oxoproline, threonine, serine) were decreased in RA compared to OA FLS. At the same time, the glycolysis and pentose phosphate pathways were more activated in RA than OA FLS. RA FLS are aggressive cells, similar to cancer cells, and require high amounts of energy to fulfill their pathogenetic functions in RA, which include proliferation, migration, and invasion [90, 251].

Macrophages and T cells are the other dominant type of synovial cells in the inflamed joint, and are important in the progression of the disease, with their abundance being correlated with disease activity but also response to treatment [147]. Similar to the FLS, activated macrophages and T cells also rely on glycolysis and have alterations of the TCA cycle [147], which is consistent with the high levels of lactic acid, citrate, and succinate found in the synovial fluid of RA patients [103]. Although metabolic profiling of RA synovial macrophages and T cells hasn't yet been undertaken, they are probably a source of circulating metabolites, while metabolites also exert their effect on synovial cells [96, 97, 98, 99].

2 Hypothesis

We hypothesize that circulating metabolites reflect metabolic perturbations of the inflamed synovial tissue and are potential biomarkers of the synovial pathological processes, disease activity, and response to treatment.

3 Objectives

The main objective of this work is to characterize the circulating metabolomics profile in patients with inflammatory arthritis in relation to certain disease phenotypes, disease activity, and response to treatment.

Specifically, we aimed to:

- study the correlation between circulating metabolites and markers of synovial inflammation

- study the serum metabolomic profile in patients with psoriatic arthritis and psoriasis

- study the serum metabolomic profile in patients with rheumatoid arthritis

4 Compendium of Publications

4.1 Serum metabolomic profiling predicts synovial gene expression in rheumatoid arthritis

This research was originally published in *Arthritis Research Therapy*: Narasimhan R, **Coras R**, Rosenthal SB, Sweeney SR, Lodi A, Tiziani S, Boyle D, Kavanaugh A, Guma M. Serum metabolomic profiling predicts synovial gene expression in rheumatoid arthritis. *Arthritis research therapy*. 2018 Dec;20(1):1-1.

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Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

Serum metabolomic profiling predicts synovial gene expression in rheumatoid arthritis

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Abstract

Background: Metabolomics is an emerging field of biomedical research that may offer a better understanding of the mechanisms of underlying conditions including inflammatory arthritis. Perturbations caused by inflamed synovial tissue can lead to correlated changes in concentrations of certain metabolites in the synovium and thereby function as potential biomarkers in blood. Here, we explore the hypothesis of whether characterization of patients' metabolomic profiles in blood, utilizing ^{1}H-nuclear magnetic resonance (NMR), predicts synovial marker profiling in rheumatoid arthritis (RA).

Methods: Nineteen active, seropositive patients with RA, on concomitant methotrexate, were studied. One of the involved joints was a knee or a wrist appropriate for arthroscopy. A Bruker Avance 700 MHz spectrometer was used to acquire NMR spectra of serum samples. Gene expression in synovial tissue obtained by arthroscopy was analyzed by real-time PCR. Data processing and statistical analysis were performed in Python and SPSS.

Results: Analysis of the relationships between each synovial marker-metabolite pair using linear regression and controlling for age and gender revealed significant clustering within the data. We observed an association of serine/glycine/phenylalanine metabolism and aminoacyl-tRNA biosynthesis with lymphoid cell gene signature. Alanine/aspartate/glutamate metabolism and choline-derived metabolites correlated with TNF-α synovial expression. Circulating ketone bodies were associated with gene expression of synovial metalloproteinases. Discriminant analysis identified serum metabolites that classified patients according to their synovial marker levels.

Conclusion: The relationship between serum metabolite profiles and synovial biomarker profiling suggests that NMR may be a promising tool for predicting specific pathogenic pathways in the inflamed synovium of patients with RA.

Keywords: NMR, Metabolomics, Biomarkers, Synovium, Rheumatoid arthritis, Gene expression

Background

The hallmark of rheumatoid arthritis (RA) is chronic synovitis that affects multiple joints and invades cartilage causing bone erosions and joint destruction [1]. As the synovium is the principal target of inflammation in RA, and the resident synoviocytes (fibroblast-like synoviocytes and macrophages-like synoviocytes) along with recruited cells (myeloid cells and lymphocytes) are implicated in the pathogenesis of synovitis, special interest has been given to the study of synovial tissue in this disease. These studies not only aim to clarify RA pathogenesis and provide insight into the mechanisms of action of therapeutic interventions [1, 2], but are also a promising approach to search for biomarkers in the inflamed synovial tissue [1]. Changes in the cellular infiltrate or biomarkers such as cytokines or growth factors in RA-affected synovial tissue have long been known to be associated with the clinical course of disease and have been used to identify specific responses to RA therapies [1–4]. Recently there has been increasing interest in synovial biopsies to obtain inflamed synovial tissue from joints and thereby gain a better

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understanding of the pathogenic events in these diseases [5]. Histopathotype and pathological pathways-based patient stratification prior to therapeutic intervention could be exploited to identify biomarker predictors of clinical outcomes and responses to therapy [6, 7].

Tissue pathology and pathogenic pathways cannot yet be reliably explored through noninvasive circulating or imaging biomarkers. Given the complexity and heterogeneous nature of RA, it is unlikely that a single cytokine will provide sufficient discrimination between patients and thus be a good biomarker [8, 9]. Global biomarker signatures may represent a more appropriate approach for improving treatment protocols and outcomes for patients with RA. Metabolomics is the science of identifying and quantifying the biochemical byproducts of metabolism in a cell, tissue, or organism [10]. Metabolomics is an emerging field of biomedical research that can offer a better understanding of the mechanisms underlying disease and help to develop new strategies for treatment [11]. Unlike genes and proteins, which are epigenetically regulated and post-translationally modified, metabolites are direct signatures of biochemical activity and thus it may be easier to test whether they are correlated with phenotype [12].

The fundamental rationale in metabolomics is that perturbations caused by a disease in a biological system will lead to changes that are correlated with the concentrations of certain metabolites [13, 14]. Metabolite patterns represent the final response of biological systems to disease status, or in response to a medical or external intervention [12]. ^{1}H-nuclear magnetic resonance (NMR) can delineate patterns of changes in biomarkers that are highly discriminatory for the observed disease or intervention [15]. We propose in this work, that the study of metabolomics in serum from patients with RA, using NMR, can be used to predict synovial pathology. We hypothesize that perturbations caused by inflamed synovial tissue will lead to changes that correlate with the concentration of certain metabolites in the synovium. These changes will then be reflected in blood serum and function as potential biomarkers of different synovial markers. Here we describe the first study that defines metabolite signatures in serum that correlate with gene expression profiling in synovial tissue from patients with active RA.

Methods

Patients

The Assessment of rituximab's immunomodulatory synovial effects (ARISE) clinical trial (registered at ClinicalTrials.gov, NCT00147966) has been described in detail [3]. Briefly, the study enrolled people between 18 and 70 years of age with an established diagnosis of RA and a positive serum test for rheumatoid factor (RF). Patients had to have active disease (defined as a tender joint count > 8/68, a swollen joint count > 6/66, and either early morning stiffness > 45 min in duration, or an elevation in erythrocyte sedimentation rate (ESR) > 28 mm/h or C-reactive protein (CRP) > 1.5 mg/dL), despite the concomitant use of methotrexate (MTX) at a dose of > 12.5 mg/week for at least 12 weeks. One of the involved joints had to be a knee or a wrist that could be appropriately examined by arthroscopy. Concomitant use of non-steroidal anti-inflammatory drugs and oral prednisone at doses of 10 mg/day or less were permitted, provided dosing was stable for at least 4 weeks before the study. Patients previously treated with tumor necrosis factor (TNF-α) inhibitors were permitted to enroll in the study provided they had been off therapy for > 2 months for etanercept and > 3 months for adalimumab or infliximab. Patients meeting eligibility criteria underwent baseline arthroscopic synovial biopsy of an affected knee or wrist. Nineteen patients for whom both baseline synovial biopsy gene expression data and baseline serum metabolomics data were available were analyzed in the current study. Clinical disease parameters, including disease activity score (DAS), health assessment questionnaire (HAQ), pain, joint swelling and tenderness, ESR, RF, and anti-cyclic citrullinated peptide (anti-CCP) are described in Additional file 1: Table S1.

Synovial gene expression analysis

Synovial RNA was extracted from pools of six tissue fragments and complementary DNA (cDNA) was synthesized. TaqMan PCR was performed using predeveloped reagents (Applied Biosystems, Foster City, CA, USA) as described previously [3]. Gene expression, utilizing quantitative reverse transcriptase (RT)-PCR, was performed to measure inflammatory mediators and B cell survival factors, including IgM (heavy chain), IgG (heavy chain), IgKappa (light chain), CD3E, TNF-α, interleukin (IL)-1β, IL-6, IL-8, matrix metalloproteinase (MMP)-1, MMP-3, B lymphocyte stimulator (BLyS), stromal cell-derived factor 1 (SDF1), and a proliferation-inducing ligand (APRIL). Synovial gene expression for the 19 patients analyzed in this study are summarized in Additional file 1: Table S2.

ELISA

TNF-α, MMP3 IL-1β, and IL-6 from serum were evaluated by DuoSet enzyme-linked immunosorbent assays following the manufacturer's protocol (R&D systems).

Metabolomics analysis

Frozen serum was obtained from the Division of Rheumatology, Allergy, and Immunology at the University of California (UC) San Diego School of Medicine (San Diego, CA, USA). Lipid and protein fractions were removed via ultrafiltration (Nanosep 3 K OMEGA, Pall Corporation, Ann Arbor, MI, USA) at 4 °C. The filtered biofluid was

used for NMR analysis. An aliquot of 160 μL of filtered serum was mixed with 20 μL D_2O and 20 μL of phosphate buffer (100 mM final concentration) containing TMSP-d4 (0.1 mM final concentration) and sodium azide (0.05% (*w*/*v*) final concentration). The prepared samples were centrifuged to remove any remaining particulates and a 180 μL aliquot was transferred to a 3 mm NMR tube (Norell, Landisville, NJ, USA) prior to acquisition. NMR spectra were acquired with a 16.4 T (700 MHz) Bruker Avance spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) equipped with a 5 mm TCI cryogenically cooled probe and autosampler at 30 °C. Following acquisition, spectra were processed using NMRlab and MetaboLab [16]. Metabolite assignment and quantification was performed using several databases [16]. Metabolite assignment and quantification was performed using Chenomx NMR Suite (Chenomx Inc., Edmonton, AB, Canada), the Birmingham Metabolite Library [17], and the Human Metabolome Database [18]. The NMR results were recently published [19] and are summarized in Additional file 1: Table S3 for the 19 patients analyzed in this study.

Data analysis

The data, consisting of 19 patient samples measured across 18 synovial markers and 49 metabolites, were processed using Python. Hierarchically clustered heatmaps were generated for correlation between synovial markers and metabolites separately. Hierarchical clustering and visualization was performed using the scientific computing package SciPy, and the visualization package Seaborn (https://seaborn.pydata.org/). Dendrograms were divided into flat clusters using a cophenetic distance metric. Linear regression was performed between each cytokine-metabolite pair, controlling for patient age and gender using the ordinary least squares (OLS) method from the Python package StatsModels. Normally distributed independent variables were standardized so they had a mean of zero and a standard deviation of one. Discriminant analyses were performed to determine coefficients for linear combinations of variables that assigned cluster membership to individual cases. Basic descriptive statistics used to describe the patient population and discriminant analysis were performed using the SPSS software version 15.0.

Results

Synovial marker and blood metabolite clustering

We first analyzed whether synovial markers clustered into different groups (Fig. 1). IL-6, MMP1, and MMP3 are strongly correlated among themselves but are inversely correlated with TNF-α, which interestingly, is strongly correlated with CD3E. MMP1 and MMP3 are also inversely correlated with another cluster that includes IL-1β and IL-8. In addition, there was a big cluster comprising B and plasma cell markers, and growth factors, including SDF1, APRIL, CD138, CD19, CD79A, IgG and IgM heavy chains, and IgKappa.

We also characterized the blood metabolites. As shown in Additional file 1: Table S3 and Fig. 2a, most of the metabolites were downregulated compared to reference values, suggesting that these metabolites might be consumed by the inflamed synovium due to an increase in its metabolic demand. A few metabolites were upregulated compared to reference values, including glycolytic metabolites such as lactate and pyruvate. This likely reflects the increased bioenergetic and biosynthetic demands of sustained inflammation. Choline metabolism has recently been strongly related to inflammation [20]. Dietary intake of choline, through two circulating metabolites, trimethylamine (TMA) and trimethylamine N-oxide (TMAO), are mechanistically linked to cardiovascular inflammation [20]. Interestingly TMA was also elevated in our patients with RA. In addition, 3-hydroxybutyrate, a ketone body, and select amino acids, such as leucine, threonine, tyrosine, and aspartate were upregulated in patients with active RA. We also analyzed whether blood metabolites could be clustered in groups. Metabolites primarily clustered into groups according to their biological function or chemical classification (Fig. 2b). As expected, the group of metabolites that were elevated in patients, namely lactate, methylmalonate, xanthine, and 3-hydroxybutyrate, were inversely correlated with the most of metabolites.

Linear regression analysis between grouped synovial markers and metabolites

Linear regression was analyzed between each synovial marker-metabolite pair; age and gender were controlled for by including these factors as covariates in the model. The regression coefficients for each cytokine-metabolite pair were used to form a clustered heatmap to lend insight into which groups of synovial markers were correlated with which groups of metabolites. We observed significant clustering structures in the data (Fig. 3a). The color bar along the top of Fig. 3 preserves the synovial marker clusters from Fig. 1. Interestingly the clusters of synovial markers almost correspond to the clusters observed within synovial markers (Fig. 1), suggesting that cytokine clusters in the synovial tissue have a similar metabolite signature in blood. The most striking difference is seen in the cluster comprising SDF1, APRIL, CD138, CD19, CD79A, and IgG and IgM heavy chain, and IgKappa in Fig. 1, which is seen split into two groups in Fig. 3. One metabolite signature correlates with CD19, CD79A and IgG heavy chain, markers of B cells; and the other metabolite signature correlates with SDF1, APRIL, CD138 and IgM heavy chain, markers related to plasma cell biology. Of interest BLyS, had a different metabolite profile than the rest of plasma cell

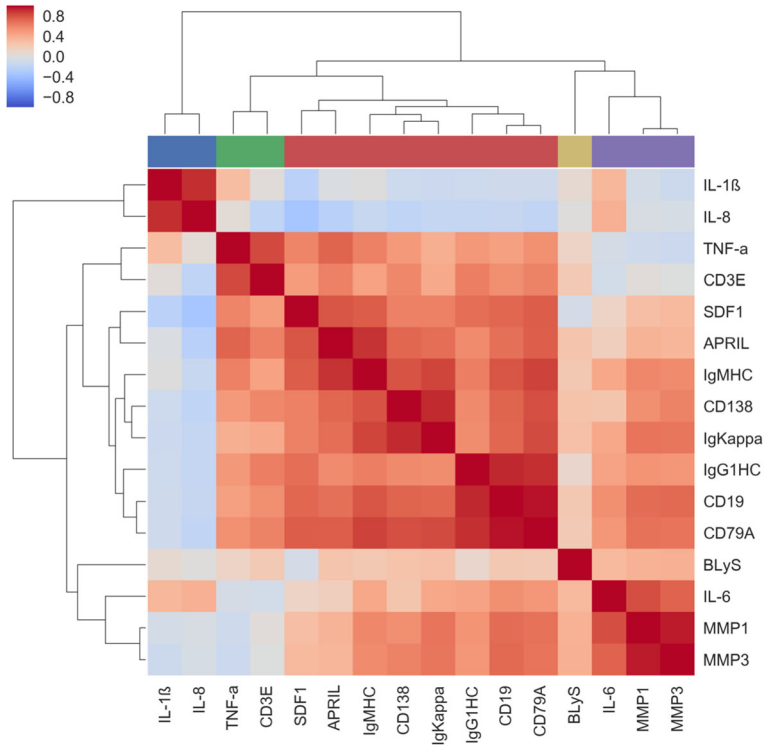


Fig. 1 Synovial markers clustering. Heat map and hierarchical cluster analysis indicates positive relationships between cytokines identified by quantitative PCR in synovial tissue from patients with rheumatoid arthritis. Pearson's correlation coefficients for each metabolite and hierarchical clustering with Euclidean distance metric are included. The color bar along the top indicates cytokine grouping based on hierarchical clustering. APRIL, a proliferation-inducing ligand; BLyS, lymphocyte stimulator; MMP, matrix metalloproteinase; SDF1, S cell-derived factor 1

biomarkers. Metabolite regression p values are displayed in Fig. 3b, where the row and column order are preserved from Fig. 3a and Additional file 2: Figure S1.

As observed in Fig. 3, metabolites can be grouped into five clusters (Fig. 4) that were further analyzed using the MetaboAnalyst [21, 22] web tool for functional enrichment of these groups of metabolites. Both pathway significance and pathway impact were assessed using this tool (Additional file 3: Figure S2).

We then determined the most strongly correlated or anti-correlated serum metabolites for each synovial marker, using linear regression, and controlling for both age and gender. We also included Benjamini-Hochberg false discovery rate (FDR)-adjusted p values to correct for multiple testing. As shown in Fig. 5, the synovial markers TNF-α and CD3E were negatively correlated with several metabolites in serum. The significant polar metabolites were mapped to known metabolic pathways using MetaboAnalyst 3.0 [22, 23]. and ranked by their overall p values (Fig. 5c). Additional file 4: Figure S3, Additional file 5: Figure S4, Additional file 6: Figure S5, and Additional file 7: Figure S6 show correlation between metabolites and the remaining synovial marker clusters.

Discriminant analysis

We then explored whether or not one or more metabolites in serum could discriminate between high or low levels of synovial marker gene expression. At present, no factors have been identified that fully explain or predict response to RA therapy [24], but pre-treatment differences at baseline between patient groups have been identified, including synovial tissue TNF expression and an increased number of synovial macrophages and T cells in patients who subsequently exhibited clinical improvement after initiation of anti-TNF therapy [25]. Therefore, we used stepwise discriminant function analyses to discriminate TNF-α or CD3E levels. Multivariate and cross-validation classification using the "leave-one--out" classification method was used for these calculations. We defined high or low marker levels according to their synovial marker gene expression mean. This stepwise discriminant analysis is presented in Fig. 6. For TNF-α discriminant analysis, three metabolites namely glutamine, TMA, and dimethylsulfone were sufficient to correctly classify 94.7% of TNF-α levels. There was canonical correlation of 0.821 and Wilks' lambda of 0.326 when these three variables were used, with high significance ($p < 0.001$; Fig. 6a). For CD3E discriminant

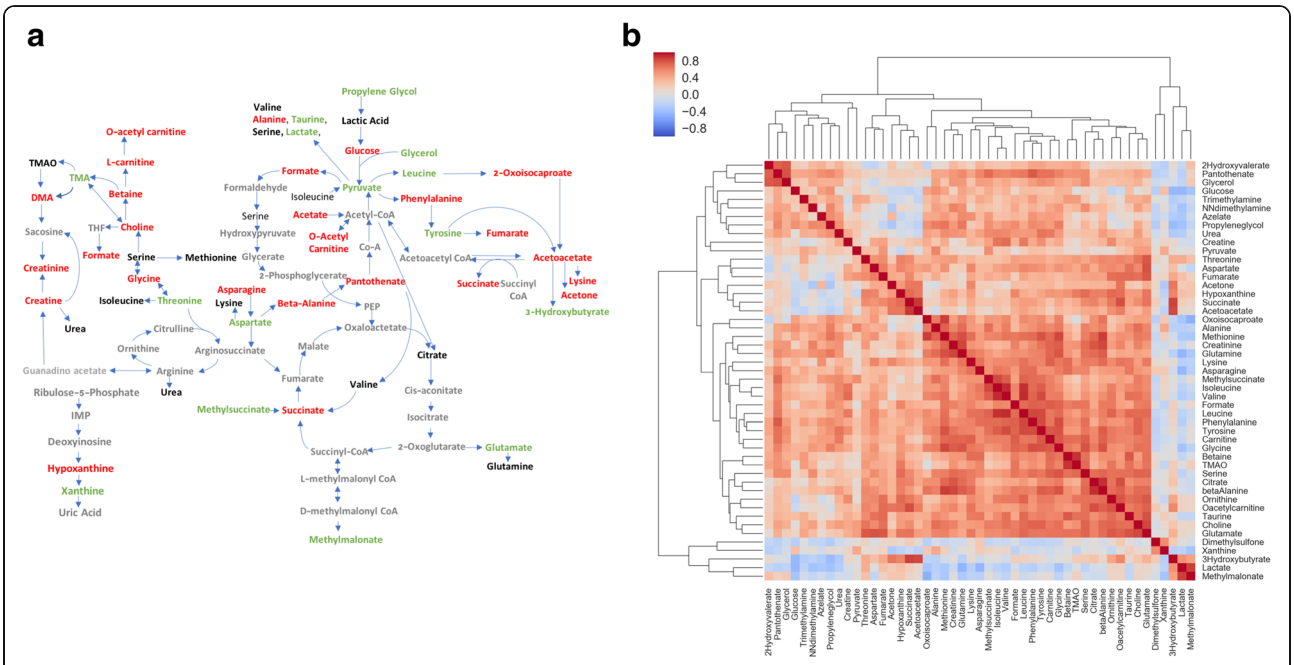


Fig. 2 Blood metabolite clustering. **a** Overview of the metabolites identified by ^{1}H-nuclear magnetic resonance (NMR) organized by metabolic pathway. Metabolites that were elevated by at least 20% compared to reference values are in green and metabolites that were decreased by more than 20% compared to reference values are in red.. Metabolites not identified by NMR are in gray. Abbreviations: TMA, trimethylamine; TMAO, trimethylamine N-oxide; DMA, NN-dimethylamine; THF, tetrahydrofolate; IMP, inosine monophosphate. **b** Heat map and hierarchical cluster analysis indicate positive relationships between polar metabolites identified by ^{1}H-NMR in serum from patients with rheumatoid arthritis before treatment with rituximab. Pearson's correlation coefficients for each metabolite and hierarchical clustering with Euclidean distance metric are shown

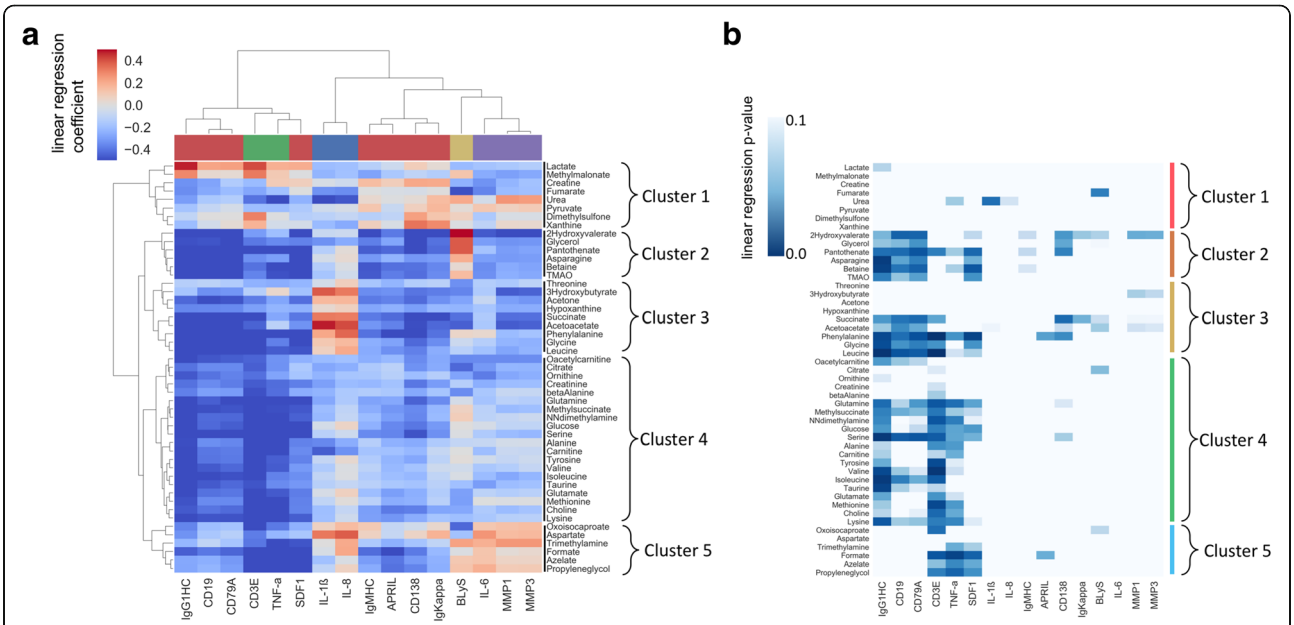


Fig. 3 Correlation of synovial markers with serum metabolites. **a** Linear regression was performed between each synovial marker–serum metabolite pair, controlling for age and gender. The regression coefficients for each pair were used to form a clustered heatmap, to lend insight into which groups of synovial markers were correlated with which groups of metabolites. The color bar along the top is preserved from Fig. 1, and indicates groups of similar cytokines. Row clusters have been identified by cophenetic cutting of the row dendrogram. **b** Metabolite regression *p* values are displayed in Fig. 3b, where the row and column order are preserved from Fig. 3a. APRIL, a proliferation-inducing ligand; BLyS, lymphocyte stimulator; MMP, matrix metalloproteinase; SDF1, S cell-derived factor 1

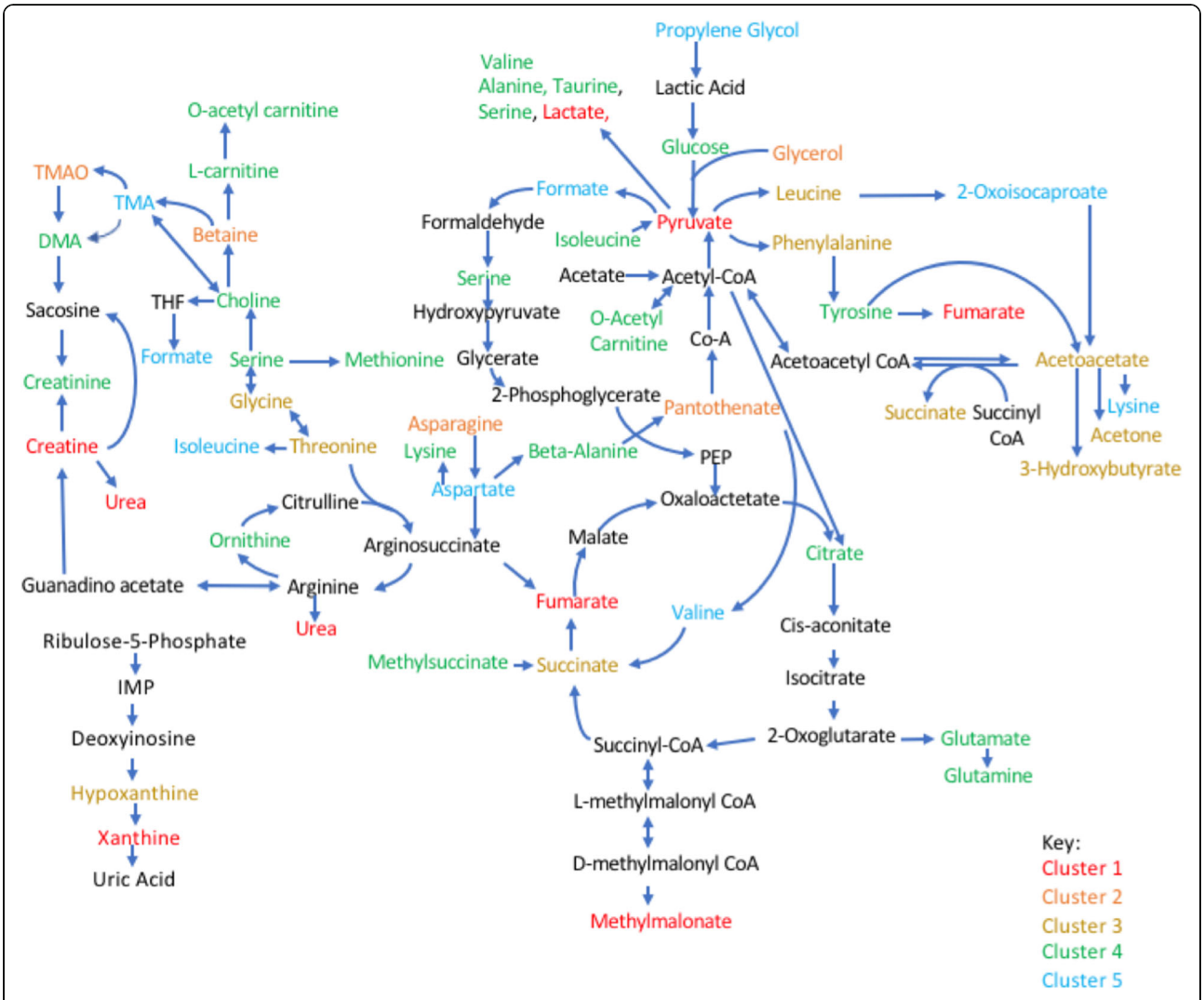


Fig. 4 Identified metabolites clusters. Overview of the metabolites identified by ^{1}H-nuclear magnetic resonance organized by metabolic pathway and colored by cluster. Abbreviations: TMA, trimethylamine; TMAO, trimethylamine N-oxide; DMA, NN-dimethylamine; THF, tetrahydrofolate; IMP, inosine monophosphate

analysis, two metabolites namely carnitine and methionine were sufficient to correctly classify 89.6% of CD3E levels. There was canonical correlation of 0.765 and Wilks' lambda of 0.414 when these three variables were used, with high significance ($p < 0.001$; Fig. 6b).

Discussion

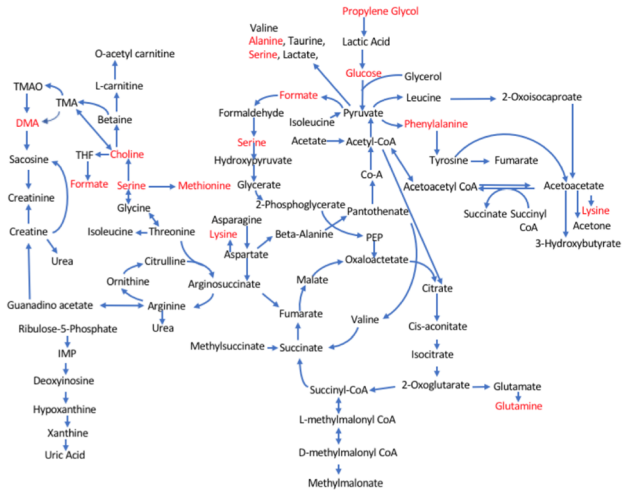
Our increasing understanding of the pathogenesis of RA has transformed the therapeutic options available for people with this disease. The introduction of newer agents and novel treatment strategies has resulted in improved outcomes for patients. However, these successes have raised the bar for the goals of therapy. At present, disease remission, or low disease activity at the very least, has become the new goal of treatment for all patients. Therefore, there is still an unmet need in RA. Biomarkers employed in "personalized" medicine might be useful in an attempt to match a patient with the most appropriate biologic therapy, and thereby optimize outcomes. The accessibility of a biological biomarker is an important factor in this approach [8]. Although sampling inflamed synovial tissue from joints might be critical to gain a better understanding of the pathogenic events of inflammatory arthritis, a biomarker that can be obtained in a minimally invasive manner is more attractive, particularly for patients in early stages of the disease, where mostly small joints are involved [8]. In this study, we attempt, for the first time, to find serum metabolomics profiles that correlate with synovial marker gene expression.

Recent studies have indicated that metabolic regulation and cell signaling are tightly and ubiquitously linked with immune responses. Metabolomics studies that aim

a

	TNF-a coef	p value	fdr_bh
Formate	-0.721795512	0.007615349	0.174737158
Propyleneglycol	-0.590410875	0.020238909	0.174737158
Glutamine	-0.573019026	0.023859919	0.174737158
Dimethylamine	-0.568085958	0.029498262	0.174737158
Lysine	-0.561321683	0.033189404	0.174737158
Azelate	-0.55830895	0.033986355	0.174737158
Phenylalanine	-0.545706629	0.038101508	0.174737158
Methionine	-0.528014471	0.040775615	0.174737158
Alanine	-0.557879697	0.041120035	0.174737158
Glucose	-0.530972967	0.045983406	0.174737158
Serine	-0.523916898	0.046117837	0.174737158
Choline	-0.512162739	0.048222123	0.174737158
	CD3E coef	**p value**	**fdr_bh**
Leucine	-0.745741039	0.001413016	0.041797774
Phenylalanine	-0.749050081	0.001706032	0.041797774
Valine	-0.703212891	0.003154410	0.051522025
Methionine	-0.639690429	0.009650316	0.083196136
Tyrosine	-0.640469250	0.010182686	0.083196136
Formate	-0.677584891	0.014503087	0.083196136
Dimethylamine	-0.624630481	0.014703349	0.083196136
Glutamine	-0.610377740	0.014876348	0.083196136
Isoleucine	-0.600778315	0.016781132	0.083196136
Serine	-0.609306927	0.016978803	0.083196136
Choline	-0.580601942	0.022115008	0.088324693
Oxoisocaproate	-0.607192712	0.024064158	0.088324693
Methylsuccinate	-0.569425349	0.027376216	0.088324693
Lysine	-0.573228375	0.029603555	0.088324693
Pantothenate	-0.581930411	0.029849975	0.088324693
Propyleneglycol	-0.559864214	0.030360237	0.088324693
Glucose	-0.569707225	0.030643261	0.088324693
Alanine	-0.570902608	0.036569913	0.094348881
Glycine	-0.549685228	0.038154935	0.094348881
Glutamate	-0.540404886	0.038509747	0.094348881
Succinate	-0.530451380	0.045877481	0.107047455

b



c

	Raw p	FDR	Impact
Tyrosine metabolism	0.0000012	8.30E-06	0.04724
Phenylalanine metabolism	0.0000012	8.30E-06	0
Thiamine metabolism	0.0000014	8.30E-06	0
Phenylalanine, tyrosine and tryptophan biosynthesis	0.0000015	8.30E-06	0.00738
Glycine, serine and threonine metabolism	0.0000022	8.30E-06	0.32378
Cyanoamino acid metabolism	0.0000022	8.30E-06	0
Methane metabolism	0.0000023	8.30E-06	0.16439
Aminoacyl-tRNA biosynthesis	0.0000662	0.00017495	0.16902

Fig. 5 Correlation between serum metabolites and synovial TNF-α and CD3E. **a** Correlation between serum metabolites and each synovial marker, using linear regression, controlling for both age and gender. We also included *p* values adjusted for Benjamini-Hochberg false discovery rate (fdr_bh) to correct for multiple testing. **b** Overview of the metabolites identified by ^{1}H-nuclear magnetic resonance organized by metabolic pathway. Metabolites that were negatively correlated with TNF-α and CD3 are shown in red. Abbreviations: TMA, trimethylamine; TMAO, trimethylamine N-oxide; DMA, NN-dimethylamine; THF, tetrahydrofolate; IMP, inosine monophosphate. **c** Pathway analysis of polar compounds by MetaboAnalyst. Pathway *p* values were calculated based on metabolites that were correlated with both TNF-α and CD3E. Coef, coefficient

to improve biological understanding through the analysis of metabolite profiles of the underlying biological pathways are certainly relevant and have been successful in other fields, especially oncology. Though the application of metabolomics to RA is still in its infancy, early studies have yielded promising results [19, 26–33]. A small number of metabolomics studies have focused on identifying metabolites associated with rheumatic diseases, primarily in the serum for diagnostic purposes [30–32], but none have attempted to predict synovial pathology.

We hypothesized that perturbations caused by inflamed synovial tissue will lead to changes that correlate with the concentrations of certain metabolites in the synovium that will be then reflected in blood serum. A recent publication on a study of metabolic profiling in the synovial tissue reported altered glucose and choline metabolism [34]. Both pathways have recently been involved in RA pathogenesis [27, 34, 35]. Choline levels in patients from our cohorts are decreased in blood compared to the normal range; this, along with an increased uptake in the joints on choline C-11 PET scanning in inflammatory arthritis [36] and high expression in fibrocyte-like synoviocytes (FLS) of choline like transporter (CTL)1 (high-affinity) and CTL2 (low-affinity) [37], suggest increased circulating choline uptake and consumption by the inflamed synovium. Glucose levels were decreased, and lactate levels increased in serum from our cohort. Glucose is consumed through upregulation of aerobic glycolysis and when metabolized, gives rise to production of copious amounts of lactate, which must be extruded from the cell to prevent lactic acidosis [38]. Several studies have highlighted the increase in glucose metabolism in the hypoxic joint [27, 35]. Thus, our results in serum seem to agree well with recently described synovial studies [34]. Of interest, both choline and glucose levels in the blood negatively correlated with TNF-α and CD3E gene expression in the synovium.

Literature in the field of oncology can help us to interpret some of our results. For instance, we observed an

a

Variables	Unstandardized coefficients	Standardized coefficients	Structure matrix	Centroids	Constant
Dimethylsulfone	-38.842	-.627	-.237	Low:-2.002 High:.924	-4.422
Glutamine	6.701	.748	.661		
Trimethylamine	1225.244	.689	.518		

b

Variables	Unstandardized coefficients	Standardized coefficients	Structure matrix	Centroids	Constant
Carnitine	-127.906	-1.178	.185	Low:-1.319 High: .959	-3.143
Methionine	338.898	1.681	.725		

Fig. 6 Discriminant analysis. Unstandardized and standardized discriminant function coefficients, structure matrix, centroids, and constant for direct discriminant function for TNF-α (**a**) and CD3E (**b**)

association of serine/glycine metabolism and aminoacyl-tRNA biosynthesis with TNF-α/CD3E and B/plasma cell signatures that suggest that lymphoid cells could be using these pathways after activation in the rheumatoid synovium. Although alterations in glucose and glutamine metabolism are central to metabolic transformation, recent studies have focused on the role of the nonessential amino acids serine and glycine in supporting tumor growth [39]. In addition to their role in protein synthesis, serine and glycine contribute to anabolic pathways important for the generation of glutathione, nucleotides, phospholipids, and other metabolites [40]. The requirement for intracellular serine and glycine for the support of cell growth and proliferation is clear. Other amino acids are also critical substrates that fuel mitochondrial metabolism and the biosynthesis of proteins, lipids, and other molecules. Of particular interest in cancer are key mitochondrial enzymes in the metabolism of glutamine, glutamate, proline, aspartate, and alanine [41]. The branched chain amino acids (BCAAs) valine, leucine, and isoleucine are also highly metabolized by transaminases. By coordinating cellular bioenergetics and biosynthesis through the tricarboxylic acid (TCA) cycle, amino acid metabolism could be critical not only in tumor cells but also in lymphoid cell proliferation and survival as described recently [42].

Another metabolite that correlates with several of our cytokine pathways is succinate. Succinate is an intermediate of the TCA cycle and plays a crucial role in adenosine triphosphate (ATP) generation in mitochondria. Recently, new roles for succinate outside metabolism have emerged. Succinate promotes expression of the pro-inflammatory cytokine IL-1β by inhibiting prolyl hydroxylases and stabilizing the transcription factor hypoxia-inducible factor-1α (HIF-1α) in activated macrophages, and stimulates dendritic cells via succinate receptor 1 [38, 43]. Furthermore, succinate has been shown to post-translationally modify proteins. Of interest, the succinate level in blood positively associated with synovial IL-1β gene expression although it did not reach statistical significance.

The cluster comprising MMP1/MMP3/IL-6, which could represent a fibroblast-driven phenotype, was negatively correlated with ketone bodies. Acetoacetate is the common precursor of the two other circulating ketone bodies, acetone and 3-hydroxybutyrate [44]. 3-hydroxybutyrate is the most abundant circulating ketone body and is less likely to degrade spontaneously into acetone than acetoacetate. One can speculate that rheumatoid fibroblasts require intracellular ketone bodies for the support of their invasive phenotype and that the increase in 3-hydroxybutyrate uptake and/or enzymes in this pathway could explain the negative correlation. Of note, the positive correlation between 3-hydroxybutyrate and IL-1β and IL-8 is also of interest, as 3-hydroxybutyrate, long viewed as a simple carrier of energy from the liver to peripheral tissues, also possesses signaling activities and is also an endogenous inhibitor of histone deacetylases (HDACs) [45]. Moreover, recent research has shown that 3-hydroxybutyrate can block the NOD-like receptor pyrin containing 3 (NLRP3) inflammasome [46]. Further studies are needed to understand the effect of these metabolites in the synovium in RA.

As mentioned above, metabolites can not only be biomarkers of perturbations caused by inflamed synovial tissue but also can have a pathogenic effect that would amplify synovial inflammation. Secondary roles have emerged

for glucose metabolites, metabolic enzymes, and TCA cycle intermediates outside of metabolism. Not only succinate but also other metabolites including α-ketoglutarate, fumarate, and acetyl-CoA might be expected to accumulate in macrophages and FLS under hypoxic conditions, and are involved in eliciting important epigenetic changes, with unexplored potential for driving chronic inflammation [47, 48]. Also, essential glycolytic enzymes have been shown to translocate to the nucleus or mitochondria where they function independently of their canonical metabolic roles in the regulation of cytokines and anti-apoptotic responses [49, 50]. Thus, metabolomics studies have also the potential of defining the elements of synovial metabolic pathobiology.

Although NMR spectroscopy has less sensitivity compared to mass spectrometry instrumentation, NMR requires minimal sample preparation, and is not only non-destructive, inherently untargeted, highly reproducible [51, 52], and intrinsically quantitative, but is also cheaper and more accessible than mass spectrometry [53–55]. Depending on the biological samples, NMR can identify and quantify more than 200 metabolites in an untargeted fashion and more than 100 metabolites are uniquely identified by NMR [56]. In this work, we also showed that the combination of only two or three metabolites identified in serum by NMR could discriminate between high or low levels of synovial TNF-α and CD3E gene expression. Studies in other cohorts of patients with active RA are needed to validate these results, yet the relationship between serum metabolic profiles and synovial biomarker profiling suggests that NMR may be a promising tool for predicting specific pathogenic pathways in the inflamed synovium in RA.

Although these findings are certainly promising, this study is not without limitations. Most importantly, we evaluated a small number of clinical samples. Despite similar clinical parameters for patient inclusion, large biological variance is expected in primary samples. In addition, patients had long-standing disease and were exposed to various therapies prior to the study, and were on methotrexate at the time of the study, which is reported to change several metabolic pathways including adenosine metabolism [57]. Confirmation of our results in a larger sample size from a cohort of patients with new onset inflammatory arthritis before treatment initiation, studied prospectively, is necessary to strengthen our conclusions. Comparison with other arthritides or other systemic inflammatory diseases to determine if these changes in metabolite levels come from the joints or from different sources is also critical to interpret our results. One other confounder is the microbiome, which is altered in RA and can potentially cause metabolic changes in both serum and synovial tissues [58–60]. In addition, further studies are needed to evaluate the relationship between circulating metabolites and synovial pathology. Metabolite profiles in blood, if they correlate with metabolic changes in synovial tissue, will certainly reveal more about RA etiology. We did not identify correlation between cytokine serum levels and cytokine synovial gene expression (Additional file 8: Figure S7), yet it remains unknown whether or not metabolic changes will display stronger correlation between blood and synovium.

Conclusions

The relationship between serum metabolite profiles and synovial biomarker profiling suggests that NMR may be a promising tool for predicting specific pathogenic pathways in the inflamed synovium of patients with RA. Further studies will help to better test the correlation and understand the metabolic profiles between cytokine and cell signatures, and address whether or not NMR metabolomics can be used to stratify patients with RA by predicting specific cellular infiltrates or other synovial biomarkers, and to identify specific responses to RA therapies.

Additional files

Additional file 1: Table S1. Baseline clinical characteristics of patients with rheumatoid arthritis. **Table S2.** Mean and standard deviation (SD) of synovial biomarker expression. **Table S3.** Mean and standard deviation (SD) of serum metabolites detected by ^{1}H-NMR (μM). Reference values are from the Human Metabolome Database (HMDB) and were collected via NMR, unless otherwise noted. ^{1}GC/MS; ^{2}HPLC; ^{3}HPLC-fluoroescence; ^{4}ion-exchange chromatography; ^{5}DFI/MS/MS ^{6}unknown. ND, no data available. Metabolites that were upregulated by at least 20% compared to reference values are in green. Metabolites that were downregulated by more than 20% compared to reference values are in red. (DOCX 26 kb)

Additional file 2: Figure S1. Correlation between synovial markers and serum metabolites. (TIFF 14826 kb)

Additional file 3: Figure S2. Pathway analysis of polar compounds by MetaboAnalyst. (TIFF 14826 kb)

Additional file 4: Figure S3. Correlation between serum metabolites and synovial CD19, CD79A, and IgGHC. (TIFF 14826 kb)

Additional file 5: Figure S4. Correlation between serum metabolites and synovial APRIL, CD138, SDF1, IgKappa, and IgMHC. (TIFF 14826 kb)

Additional file 6: Figure S5. Correlation between serum metabolites and synovial MMP1, MMP3, and IL-6. (TIFF 14826 kb)

Additional file 7: Figure S6. Correlation between serum metabolites and synovial IL-1β and IL-8. (TIFF 14826 kb)

Additional file 8: Figure S7. Correlation between serum cytokines and synovial cytokines and serum metabolites. (TIFF 14826 kb)

Abbreviations

APRIL: A proliferation-inducing ligand; ARISE: Assessment of rituximab's immunomodulatory synovial effects; ATP: Adenosine triphosphate; BLySB: Lymphocyte stimulator; CRP: C-reactive protein; CTL: Choline like transporter; DAS: Disease activity score; FLS: Fibroblast-like synoviocytes; HAQ: Health assessment questionnaire; HIF-1α: Hypoxia-inducible factor-1α; IL-1β: Interleukin; MMP: Matrix metalloproteinase; MTX: Methotrexate; NMR: ^{1}H-nuclear magnetic resonance; RA: Rheumatoid arthritis; RF: Rheumatoid factor; SDF1: S cell-derived factor 1; TCA: Tricarboxylic acid; TMA: Trimethylamine; TMAO: Trimethylamine N-oxide; TNF-α: Tumor necrosis factor

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
MG designed and supervised the overall project. DB and AK designed and conducted the ARISE trial. RN, RC, AK, SBR, and MG analyzed the data. ST, AL, and SS acquired and analyzed NMR data. SBR, AK, and MG wrote the manuscript. All authors read and approve the final manuscript.

Ethics approval and consent to participate
Patients were enrolled in the ARISE clinical trial following written informed consent. Ethical approval was granted by the Institutional Review Board (IRB) at UCSD.

Consent for publication
N/A

Competing interests
The authors declare that thet have no competing interest.

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4.2 Choline metabolite, trimethylamine N-oxide (TMAO), is associated with inflammation in psoriatic arthritis

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Choline metabolite, trimethylamine N-oxide (TMAO), is associated with inflammation in psoriatic arthritis

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ABSTRACT

Objective. *Dietary intake of choline has been linked to systemic inflammation through the microbial production of two metabolites, trimethylamine (TMA) and trimethylamine-N-oxide (TMAO). Herein we explore the association between choline metabolites and inflammation in psoriatic arthritis (PsA) patients.*

Methods. *Thirty-eight patients with PsA, all of whom satisfied the CASPAR classification criteria for PsA, were studied. Outcomes reflecting the activity of peripheral arthritis as well as skin psoriasis, Disease Activity Score (DAS)28, Clinical Disease Index (CDAI) and Body Surface Area (BSA) were assessed. Serum concentration of choline metabolites (choline, TMA, TMAO, betaine and carnitine) were determined by LC-MS, and metabolite levels associated with disease scores.*

Results. *Among the 38 PsA patients included, the mean DAS28PCR was 2.74±1.29. Twenty-seven patients had active skin disease, with an average BSA of 7.2±16.22. TMAO, but not TMA or choline, significantly correlated with measures of disease activity for both skin and peripheral joints.*

Conclusion. *In our cohort, only TMAO, but not TMA, choline, betaine or carnitine, was associated with inflammation in PsA patients, establishing a mechanistic link between TMAO and PsA phenotypes. Future studies will explore the modulation of TMAO and disease severity in PsA.*

Introduction

Choline metabolism has been recently strongly related to inflammation in relation to the pathogenesis of atherosclerosis (1). Choline is a semi-essential nutrient found in a variety of foods, but it is particularly abundant in egg yolk, meats, liver, fish, dairy products, nuts, and soybean (2). Choline is a component of phosphatidylcholine, and following oxidation, precursor to betaine (3). Choline and other trimethylamine (TMA) containing species like carnitine are metabolised to TMA by the gut microbiota (4). TMA is subsequently oxidised by at least one member of the flavin-containing monooxygenases, FMO3, forming trimethylamine-N-oxide (TMAO), which is then released into circulation (4). Several studies have shown that an elevated level of choline, betaine and TMAO increases the risk of atherosclerosis and subsequently of cardiovascular disease (CVD) (5). Also, a recent study revealed a link between the TMAO-producing enzyme FMO3 and obesity (6, 7).

Psoriatic arthritis (PsA) is an inflammatory disease affecting the joints and connective tissue and is associated with psoriasis of the skin and nails. Among the risk factors for psoriasis, evidence is accumulating that nutrition plays a major role in psoriasis pathogenesis. In particular, body weight, nutrition, and diet may exacerbate the clinical manifestations (8). Obesity is a known risk factor for developing PsA in patients with psoriasis and several studies have described that a successful weight loss might be associated with a higher rate of achievement of minimal disease activity in overweight/obese patients with PsA who started treatment with TNF-α blockers (9). Metabolic syndrome and high CVD risk are also more frequent in PsA than other rheumatic diseases such as rheumatoid arthritis (10). As choline metabolism is associated to all these comorbidities, we examined the relationship between circulating levels of choline, betaine, carnitine, TMA and TMAO, and skin and joint clinical scores in a cohort of PsA patients.

Methods

Patients

A cross-sectional cohort of 38 adult patients with PsA fulfilling the classification for PsA (CASPAR) criteria was recruited from the University of California San Diego (UCSD) Arthritis Clinics. The study was approved by the UCSD Institutional Review Board (no. 150272) and obtained the patient's written informed consent to publish the material. Clinical assessment included: evaluation of the number of tender (TJC) and swollen joints (SJC) (out of 28), the number of tender entheseal sites, the percentage of the body surface affected by psoriasis, functional status as assessed by Health Assessment Questionnaire (HAQ), and assessments of pain, fatigue, global disease severity

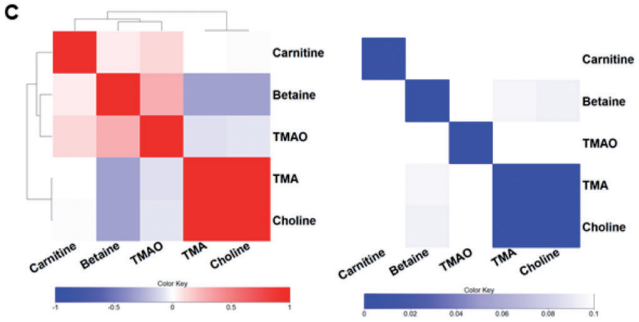
A

Characteristic	N/Mean (%/SD)
Male	22 (58%)
Age	50.8 (10.89)
Duration of PsA in years	10 (9.41)
Duration of psoriasis in years	16.9 (13.94)
Number of tender joints	1.9 (range: 0-10)
Number of swollen joints	2 (range: 0-10)
Pain	3.12 (2.84)
HAQ (Health Assessment Questionnaire)	0.47 (0.57)
DAS28CRP	2.74 (1.29)
CDAI	9.91 (10.04)
SDAI	11.93 (11.73)
Enthesitis	8 (21%)
BMI (Body Mass Index)	27.1 (5.42)
Body surface affected by psoriasis	7.2 (16.22)

B

Metabolite	Mean (SD)
Choline	47.45 (35.96)
TMA	45.08 (37.87)
TMAO	15.96 (15.51)
Betaine	792.83 (295.38)
Carnitine	163.06 (34.71)

C



D

Metabolite	Male Mean (SD)	Female Mean(SD)	p
Choline	43.5 (30.1)	52.89 (43.23)	0.46
TMA	41.9 (32.5)	49.35 (45.01)	0.58
TMAO	13.2 (9.7)	19.76 (20.84)	0.25
Betaine	887 (314.9)	663.3 (213.21)	0.01
Carnitine	172.1 (33.3)	150.63 (33.63)	0.06

Metabolite	Age ≤ 51 Mean (SD)	Age >51 Mean (SD)	p
Choline	41.4 (23.6)	53.4 (44.9)	0.31
TMA	39.2 (23.7)	50.9 (48)	0.35
TMAO	12.2 (8)	19.6 (20)	0.14
Betaine	900.7 (234.5)	684.8 (315.7)	0.02
Carnitine	167.2 (27.3)	158.8 (41)	0.46

Metabolite	BMI < 25 Mean (SD)	BMI > 25 Mean (SD)	p
Choline	55.4 (50.31)	45.3 (31.9)	0.71
TMA	53.8 (51.4)	42.7 (35.6)	0.65
TMAO	13.6 (15.4)	17.9 (17.3)	0.31
Betaine	697.1 (307.4)	826.6 (320.3)	0.48
Carnitine	143 (30.2)	164.6 (31.2)	0.12

Fig. 1. Cohort demographics and metabolites clustering.
A: Demographic and disease characteristics of the patients (n=38). **B**: The average value of the metabolites in our cohort of 38 patients is displayed in Fig. 1B.
C: Linear regression was performed between each metabolite – metabolite pair, controlling for age and gender.
Left: The regression coefficients for each pair were used to form a clustered heatmap. TMA: trimethylamine; TMAO: trimethylamine N-oxide.
Right: Metabolite regression *p*-values are displayed, where the row and column order are preserved from 1C left.
D: The average value of metabolites per age, gender and body max index (BMI) are displayed. T-test *p*-values are also displayed. Concentrations are provided as ng/ml.

by patients, and a global assessment of disease by physicians, using a Visual Analogue Scale ranging from 0 to 10. Composite measures of peripheral arthritis were calculated using the above measures: Disease Assessment Score using a 28 joint count and C reactive protein (DAS28-CRP), Clinical Disease Activity Index (CDAI) and Simple Disease Activity Index (SDAI). Blood samples were processed immediately, and sera aliquots were stored at -80°C until analysis.

Metabolite measure

Choline, TMA, TMAO, betaine and carnitine were assayed in serum samples using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as detailed in supplementary methods.

Data analysis

The data, consisting of 38 patient samples measured across 5 choline metabolites and 14 clinical outcomes were processed using R, v. 3.4.1. (www.r-project.org) as detailed in Supplementary methods.

Results

Cohort demographics and disease characteristics

Patient characteristics are summarised in Figure 1A. Of the 38 PsA patients included in this study, 58% were male and the mean age was 50.8 years ± 10.9 (range 23–75 years). The mean number of tender joints and swollen joints were 1.9±3 (range 0–10) and 2±3.2 (range 0–10) respectively. The average DAS28-CRP score was 2.74±1.29 (range 1–5.18). Twenty-five patients (71%) had active skin disease, with an average BSA of 7.22±16.22. Eight patients had enthesitis. 65% were receiving biological therapy (46% of them in association with a synthetic disease-modifying drug (sDMARD), mostly methotrexate. 14% received sDMARD as monotherapy, and 21% received no systemic treatment.

Choline metabolite profiling and clustering

Concentration of circulating betaine, carnitine and choline, TMA and TMAO are shown in Figure 1B. We also analysed choline metabolite clustering (Fig. 1C). Of interest, choline strongly correlated with its metabolite TMA, but did not correlate with TMAO. Betaine and carnitine did not correlate with any of the other metabolites. Concentration of betaine and carnitine were higher in males than in females (p=0.01 for betaine and p=0.06 for carnitine). Betaine was lower in patients older than 51 years old (p=0.02). There were no significant differences in the concentrations of choline and its metabolites between patient with normal BMI *versus* overweight and obese patients adjusted by age and gender (Fig. 1D).

Linear and logistic regression analysis between grouped serum choline metabolites and skin and joint clinical parameters.

We then analysed whether choline metabolites differentiate between high and low skin or joint disease activity scores. Figure 2A shows logistic regression analysis adjusted by age and gender by including these factors as covariates in the model, of each choline metabolite of patients classified between mild plus moderate (BSA ≤10) *versus* severe pso-

A

Metabolite	BSA ≤ 10 Mean (SD)	BSA > 10 Mean (SD)	p
Choline	49.3 (39)	40.4 (21.4)	0.59
TMA	46.9 (41.1)	38 (22.3)	0.63
TMAO	12.2 (10.3)	29.8 (23.4)	0.03
Betaine	772.3 (314.3)	869.6 (207.7)	0.19
Carnitine	160.7 (30.9)	171.8 (47.9)	0.19

B

Metabolite	DAS28CRP ≤ 2.67 Mean (SD)	DAS28CRP > 2.67 Mean (SD)	p
Choline	44.1 (31.6)	41.5 (32.9)	0.84
TMA	40.8 (32)	39.9 (34.2)	0.96
TMAO	10.9 (6)	19.7 (14.6)	0.11
Betaine	781.4 (284.5)	949 (273.7)	0.35
Carnitine	161.5 (27.6)	160 (44.3)	0.11

Metabolite	DAS28CRP ≤ 4.09 Mean (SD)	DAS28CRP > 4.09 Mean (SD)	p
Choline	44.4 (32.2)	28.5 (2.1)	0.49
TMA	41.6 (32.7)	23.7 (5.4)	0.47
TMAO	12.3 (8.6)	23.7 (17.3)	0.03
Betaine	800.9 (281.2)	1140.6 (225.7)	0.08
Carnitine	161.7 (32.3)	153.5 (19)	0.76

C

Metabolite	CDAI ≤ 10 Mean (SD)	CDAI >10 Mean (SD)	p
Choline	45.8 (32.7)	42 (29.6)	0.49
TMA	42.1 (33.3)	40.8 (30.3)	0.69
TMAO	11.4 (6.1)	24.3 (22.2)	0.05
Betaine	790.8 (298.6)	829.4 (281.3)	0.25
Carnitine	164.9 (27.2)	157.2 (44.9)	0.76

Metabolite	CDAI ≤ 22 Mean (SD)	CDAI > 22 Mean (SD)	p
Choline	42.8 (29.8)	52.8 (39.8)	0.53
TMA	39.7 (29.9)	51.5 (42)	0.46
TMAO	13.1 (11.4)	32.5 (24.2)	0.04
Betaine	776.6 (289.8)	954.1 (254.9)	0.04
Carnitine	160.8 (33.2)	168.3 (43.9)	0.36

D

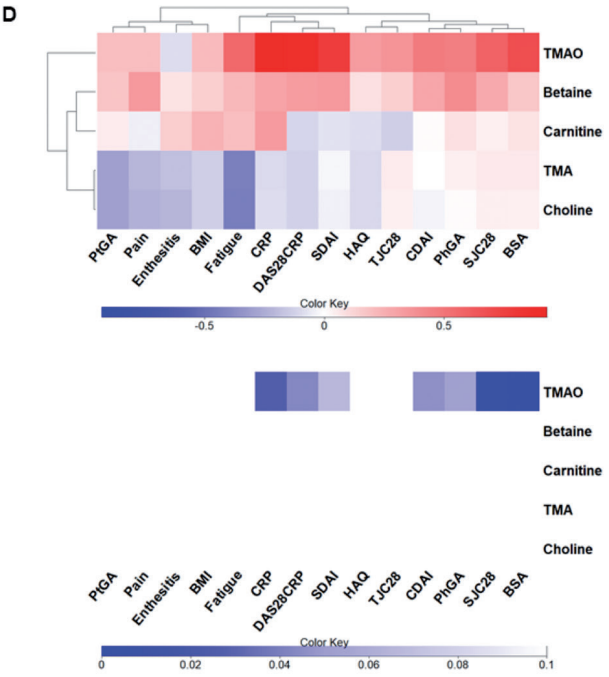


Fig. 2. Relation of choline metabolites with skin and joint disease activity.
A: Logistic regression was performed for each metabolite in patients with BSA ≤10 compared with patient with BSA >10, adjusting for age and gender.
B and **C**: Logistic regression was performed for each metabolite in patients with low disease activity (measured by either DAS28CRP ≤2.67 or CDAI ≤10) and patients with moderate and high disease activity (DAS28CRP >2.67 or CDAI >10), adjusting for age and gender. Logistic regression was also performed for each metabolite in patients with mild or moderate disease activity (measured by either DAS28CRP ≤4.09 or CDAI ≤22) and patients with high disease activity (DAS28CRP >4.09 or CDAI >22) adjusting for age and gender.
D: Linear regression was performed between each clinical score – metabolite pair, controlling for age and gender. In the left panel, the regression coefficients for each pair were used to form a clustered heatmap, to lend insight into which clinical scores were correlated with which metabolite. Row clusters have been identified by cophenetic cutting of the row dendrogram. In the right panel, metabolite regression *p*-values are displayed, where the row and column order are preserved from left panel.
TJC: tender joint count; SJC: swollen joint count; CDAI: Clinical Disease Activity Index; SDAI: Simple Disease Activity Index; HAQ: Health Assessment Questionnaire; PhGA: physician global assessment; PtGA: patient global assessment; BMI: body mass index; BSA: body surface area; DAS28CRP: disease assessment score 28. Concentrations are provided as ng/ml.

riasis (BSA >10). We also conduct this analysis of each choline metabolite of patients classified as low or high joint disease activity (Fig. 2B-C). TMAO was the only metabolite that significantly increased in patients with higher skin and joint scores. We also conducted linear regression that was performed between each choline metabolite-clinical parameter pair, controlling for age and gender. The regression coefficients for each choline metabolite-clinical parameter pair were used to form a clustered heatmap to lend insight into which groups of choline metabolites were correlated with which clinical parameters (Fig. 2D, left panel). Metabolite regression *p*-values are displayed in Figure 2D (right panel), where the row and column order are preserved from left panel. Interestingly, TMAO correlated with parameters of both skin (BSA) and joint disease activity (TJC, SJC, CDAI, SDAI, DAS28CRP). TMAO also correlated with HAQ and fatigue. Of note, two metabolites, choline and TMA, negatively correlated with fatigue.

Although some other studies have reported a relatively low correlation between items assessing joint symptoms with items assessing skin symptoms (11), in our series, these parameters showed high correlation between them (Fig. 3A-B). We then conducted linear regression between each choline metabolite-joint disease scores (TJC, SJC, DAS28, CDAI and SDAI) adjusted for BSA. We also conducted linear regression between each choline metabolite and BSA adjusted for CDAI. Figure 3C shows regression coefficients and Figure 3D metabolite regression *p*-values. Of note, TMAO still significantly correlates with both skin and joint clinical scores.

Discussion

In the present study, we show that circulating levels of TMAO correlates with skin and joint severity in a cohort of PsA patients. Previous studies showed that choline, betaine and TMAO were all associated with atherosclerosis and higher risk of CVD risk (5). Also, TMAO concentration was dependent on the dietary intake of choline (1), hence it was suggested that changes in diet could be a therapeutic approach for decreasing CVD risk. In our patients, however, there is no correlation between the concentration of choline and TMAO, and, furthermore, only TMAO and not choline and betaine correlated with skin and joint disease activity. This suggests that TMAO concentration does not depend on choline concentration in PsA patients, but more likely on the activity of FMO3,

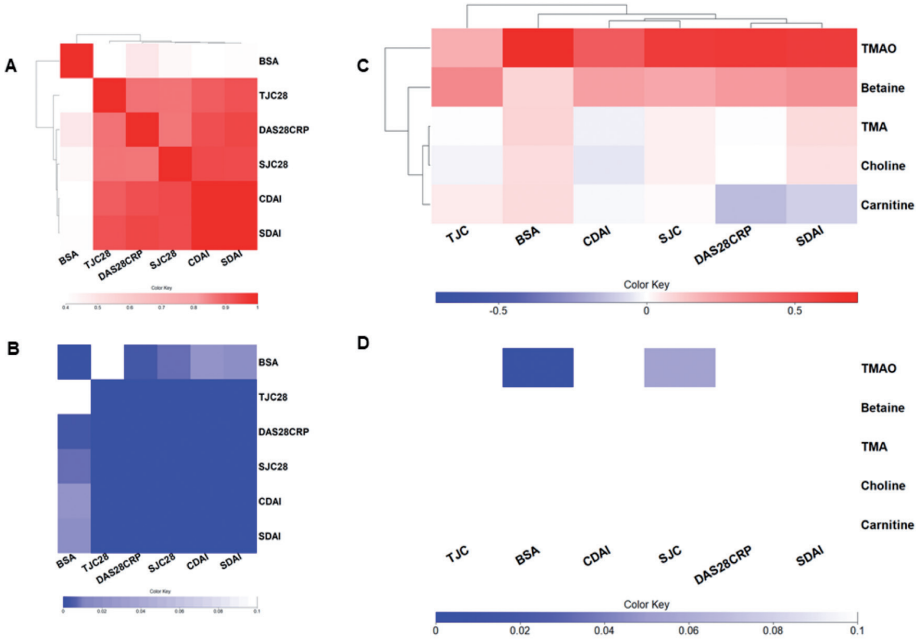


Fig. 3. Choline metabolite regression after BSA and CDAI.
A: The Pearson correlation coefficients for each pair of clinical scores were used to form a clustered heatmap, to evaluate the relationship between joint and skin disease scores.
B: The *p*-values are displayed in Fig. 3B where the row and columns are preserved from Fig. 3A.
C: Linear regression was performed between each clinical score – metabolite pair, controlling for age and gender. TJC, SJC, DAS28CRP, CDAI and SDAI were also adjusted by BSA, and BSA was also adjusted by CDAI. The regression coefficients for each pair were used to form a clustered heatmap.
D: Metabolite regression *p*-values are displayed in Figure 3D, where the row and column order are preserved from Fig. 3C.
TJC: tender joint count; SJC: swollen joint count; CDAI: clinical Disease Activity Index; SDAI: Simple Disease Activity Index; BSA: body surface area; DAS28CRP: disease assessment score 28.

which might be elevated in patients with inflammation and obesity.

A recent study showed that expression of FMO3 in adipose tissue in overweight patients positively correlated with BMI and waist-to-hip ratio, and negatively correlated with insulin sensitivity (6, 7), suggesting a link between TMAO-producing enzyme FMO3 and obesity. In our cohort, although TMAO levels did not reach significance in overweight patients, the ratio TMAO/TMA was higher in the obese group (0.3±0.2 in BMI ≤30 *vs*. 0.7±0.5 in BMI >30, p=0.08) suggesting a higher activity of FMO3 in our cohort as well. Given that there is solid epidemiologic evidence linking psoriasis and PsA to metabolic syndrome (12, 13) an increase of FMO3 activity could explain the elevated TMAO levels in PsA patients.

Very few studies have attempted to study whether or not elevated levels of choline metabolites have an impact on cell function *in vitro* or *in vivo*. Although previous studies have suggested that TMAO promotes vascular inflammation by activating several inflammatory pathways including the NLRP3 inflammasome (14), mitogen-activated protein kinase and nuclear factor-κB pathways (15), further studies are needed to explore the mechanistic links between TMAO and inflammation in PsA. In conclusion, in our cohort, only TMAO, but not TMA, choline, betaine or carnitine, was associated with inflammation in PsA patients. Future studies are needed to explore the mechanistic links between TMAO and skin and joint inflammation.

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4.3 Imbalance Between Omega-6- and Omega-3-Derived Bioactive Lipids in Arthritis in Older Adults

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Original Article

Imbalance Between Omega-6- and Omega-3-Derived Bioactive Lipids in Arthritis in Older Adults

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Abstract

Elderly-onset rheumatoid arthritis (EORA) and polymyalgia rheumatica (PMR) are common rheumatic diseases in older adults. Oxylipins are bioactive lipids derived from omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) that serve as activators or suppressors of systemic inflammation. We hypothesized that arthritis symptoms in older adults were related to oxylipin-related perturbations. Arthritis in older adults (ARTIEL) is an observational prospective cohort with 64 patients older than 60 years of age with newly diagnosed arthritis. Patients' blood samples at baseline and 3 months posttreatment were compared with 18 controls. A thorough clinical examination was conducted. Serum oxylipins were determined by mass spectrometry. Data processing and statistical analysis were performed in R. Forty-four patients were diagnosed with EORA and 20 with PMR. At diagnosis, EORA patients had a mean DAS28CRP (Disease Activity Score 28 using C-reactive protein) of 5.77 (*SD* 1.02). One hundred percent of PMR patients reported shoulder pain and 90% reported pelvic pain. Several n-6- and n-3-derived oxylipin species were significantly different between controls and arthritis patients. The ratio of n-3/n-6 PUFA was significantly downregulated in EORA but not in PMR patients as compared to controls. The top two candidates as biomarkers for differentiating PMR from EORA were 4-HDoHE, a hydroxydocosahexaenoic acid, and 8,15-dihydroxy-eicosatrienoic acid (8,15-diHETE). The levels of n-3-derived anti-inflammatory species increased in EORA after treatment. These results suggest that certain oxylipins may be key effectors in arthrtis in older adults and that the imbalance between n-6- and n-3-derived oxylipins might be related to pathobiology in this population.

Keywords: Oxylipins, Arthritis, Omega-3, Omega-6 polyunsaturated fatty acids

Elderly-onset rheumatoid arthritis (EORA) is defined as a de novo illness that usually develops after 65 years of age and has different characteristics compared to young-onset RA (YORA): more equal sex distribution, more frequent acute onset with constitutional symptoms (fever, weight loss, and asthenia), large joints more frequently involved, and a larger percentage of EORA subjects are negative for both rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibodies (1). This may lead to significant diagnostic difficulties at first presentation, as there are many similarities between seronegative EORA and other rheumatologic diseases, including polymyalgia rheumatica (PMR) (2,3). PMR is characterized by pain and stiffness in the shoulder and the hip girdle and elevated inflammatory markers, that occur in people older than 50 years of age (4). While a lot of research has focused on YORA, which is rapidly diag-

nosed and is generally treated adequately, EORA and PMR have received less attention. Thus, mechanistic studies aimed at identifying key pathobiological factors to assess the biological systems with a putative role in EORA and PMR are few in number, and mechanisms driving or maintaining arthritis in older adults are relatively less well known.

Oxylipins and related bioactive lipid mediators derived from polyunsaturated fatty acids (PUFAs) constitute a major bioactive lipid network, which is among the most complex and challenging pathways to map in a physiological context. PUFAs can be classified into n-3 fatty acids and n-6 fatty acids (5). Arachidonic acid (AA) is synthesized from the n-6 essential fatty acid linoleic acid (LA) which comes from diet (vegetable oils, meats, and eggs) and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are synthesized from the n-3 essential fatty acid α-linolenic acid (ALA), also from diet (green leafy vegetables, flax and chia seeds, canola, walnut, and soybean oils) with the participation of the same enzymes: Δ6 desaturase, the limiting step of the pathway, Δ5-desaturases and elongases (Supplementary Figure 1A). Synthesis of n-6 and n-3 PUFA-derived oxylipins is performed by a set of highly conserved enzymes: cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 enzymes (6–8). AA, the predominant n-6 PUFA, is the precursor of proinflammatory oxylipins, such as prostaglandins, thromboxanes, and leukotrienes, as well as anti-inflammatory lipoxins. EPA and DHA are the precursors of oxylipins that have a critical role in the resolution phase of inflammation, named specialized pro-resolving mediators (SPMs; resolvins, maresins, protectins) and antagonize the proinflammatory effects of n-6 fatty acids (Supplementary Figure 1B).

Oxylipins control many physiological and pathological processes, often in opposing directions. Each of these oxylipins serves a specific role as either activators or suppressors of systemic inflammation and collectively exert complex controls in regulating human physiology (6–8). Among the proinflammatory oxylipins, prostaglandin E2 (PGE2) is considered a key mediator of various aspects of inflammation, including swelling, fever, and inflammatory pain (9). Other oxylipins are involved in the resolution phase of inflammation, such as the cyclopentenone 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) and lipoxin A4 (LXA4), and have instead been shown to limit inflammatory processes. Moreover, an altered ratio of omega-6 to omega-3 PUFAs is involved in diseases including cancer, cardiovascular diseases, and inflammatory disease (10). In arthritis, phospholipase A2, the enzyme that releases fatty acids from membranes, was found to be overexpressed in synovial fluid from RA patients. COX2 is upregulated in synovial fibroblasts, mononuclear cells, and endothelial cells in the sublining and is also induced in vitro in RA cultured synovial fibroblasts stimulated with proinflammatory cytokines. PGE2 was also found to be increased in RA patients' synovial fluid. High levels of 5-LOX and leukotriene B4 were also found in RA patients' synovial fluid, serum, and synovium (11). However, while some information is available regarding the role of PG and leukotrienes in arthritis, very few studies have addressed the role of other oxylipins in this field.

Aging is accompanied by an increase in the circulating levels of saturated fatty acids and a decrease of the unsaturated ones (12). A previous publication found increased levels of proinflammatory oxylipins in healthy older adults compared to younger individuals (13), suggesting they could be one of the underlying mechanisms of the low-grade inflammation described in aging population (14,15). It could also offer an explanation for the higher prevalence of inflammatory conditions such as arthritis, diabetes, and obesity, with increasing age (16,17). We hypothesized that oxylipin-related perturbations may be related to arthritis in older adults, and that by defining this oxylipin profile, we might be able to define elements of inflammation pathobiology in this population. Here, we compared the oxylipin profile in older adults that developed arthritis with age-matched controls. We also compared the oxylipin profile of EORA patients with PMR patients.

Patients and Methods

Patient Selection and Assessment

This is an observational longitudinal prospective study (ARTIEL—Arthritis in Older Adults), which enrolled older adults with new-onset arthritis. The study was approved by the University Hospital Germans Trias i Pujol Institutional Review Board, and included patients older than 60 years with clinically newly diagnosed peripheral and/or rhizomelic arthritis. Patients with infections, neoplasias, dementia, and immunodeficiencies, or who had received glucocorticoids or any disease-modifying anti-rheumatic drugs (DMARDs) in the last 6 months were excluded. Patients were identified by a primary care physician and then referred to a rheumatologist who prescribed treatment according to the standard of care.

Clinical assessment included presence/absence of pelvic and shoulder pain, stiffness, edema, fatigue and loss of appetite, global pain using a Visual Analogue Scale (VAS: 0–10), evaluation of the number of tender (TJC) and swollen joints (SJC) (out of 28), functional status as assessed by Health Assessment Questionnaire (HAQ), and assessment of global disease severity by patients, and a global assessment of disease by physicians, using a VAS ranging from 0 to 10. Composite measures of peripheral arthritis were calculated using the above measures: Disease Activity Score using a 28-joint count and C-reactive protein (DAS28CRP), Clinical Disease Activity Index (CDAI), and Simple Disease Activity Index (SDAI). Blood samples were collected at baseline (first consult in rheumatology clinics) and 3 months posttreatment, processed immediately, and sera aliquots were stored at −80°C until analysis. Blood samples from 18 controls in the same age range were also collected.

Out of 64 patients that fulfilled the inclusion criteria, 44 were diagnosed with EORA according to the ACR/EULAR 2010 criteria (18) and 20 patients were diagnosed with PMR (2012 EULAR/ACR criteria (19)). Forty-three patients with EORA and 19 patients with PMR were also evaluated at 3 months posttreatment.

Lipid Extraction and LC-MS Measure of Oxylipin

All sera samples at baseline were stored at −80°C, thawed once, and immediately used for free fatty acid and oxylipin isolation as described (20). Briefly, 50 µL of sera was spiked with a cocktail of 26 deuterated internal standards that also included some selected PUFAs (individually purchased from Cayman Chemicals, Ann Arbor, MI) and brought to a volume of 1 mL with 10% methanol. The samples were then purified by solid phase extraction on Strata-X columns (Phenomenex, Torrance, CA), using an activation procedure consisting of consecutive washes with 3 mL of 100% methanol followed by 3 mL of water. The oxylipins were then eluted with 1 mL of 100% methanol, and the eluent was dried under vacuum, dissolved in 50 µL of buffer A (consisting of water–acetonitrile–acetic acid, 60:40:0.02 [v/v/v]), and immediately used for analysis.

Oxylipins in sera were analyzed and quantified by LC/MS/MS as previously described (20,21). Briefly, oxylipins were separated by reverse-phase chromatography using a 1.7 µm 2.1 × 100 mm BEH

Shield Column (Waters, Milford, MA) and an Acquity UPLC system (Waters). The column was equilibrated with buffer A, and 10 µL of sample was injected via the autosampler. Samples were eluted with a step gradient starting with 100% buffer A for 1 minute, then to 50% buffer B (consisting of 50% acetonitrile, 50% isopropanol, and 0.02% acetic acid) over a period of 3 minutes, and then to 100% buffer B over a period of 1 minute. The LC was interfaced with an IonDrive Turbo V ion source, and mass spectral analysis was performed on a triple quadrupole AB SCIEX 6500 QTrap mass spectrometer (AB SCIEX, Framingham, MA). Oxylipins were measured using electrospray ionization in negative ion mode and multiple reaction monitoring (MRM) using the most abundant and specific precursor ion/product ion transitions to build an acquisition method capable of detecting 158 analytes and 26 internal standards. The ionspray voltage was set at −4,500 V at a temperature of 550°C. Collisional activation of the oxylipin precursor ions was achieved with nitrogen as the collision gas with the declustering potential, entrance potential, and collision energy optimized for each metabolite. Oxylipins were identified by matching their MRM signal and chromatographic retention time with those of pure identical standards.

Oxylipins and free fatty acids were quantitated by the stable isotope dilution method. Briefly, identical amounts of deuterated internal standards were added to each sample and to all the primary standards used to generate standard curves. To calculate the amount of oxylipins and free fatty acids in a sample, ratios of peak areas between endogenous metabolite and matching deuterated internal standards were calculated. Ratios were converted to absolute amounts by linear regression analysis of standard curves generated under identical conditions. Oxylipin levels are expressed in picomol/milliliter (pmol/mL). To account for batch effects, quality control samples were run in each batch; the average coefficient of variance for the quantified oxylipins was 6% (*SD* 0.01)

The desaturase enzymes Δ6 and Δ5 are considered the limiting step in the conversion of ALA to EPA and DHA, as well as LA to dihomo-γ-linolenic acid (DGLA) and AA (Supplementary Figure 1A). Activity of desaturase enzymes can be inferred from product to precursor ratios (22). DGLA/LA, calculated as the sum of DGLA-derived oxylipins divided by the sum of LA-derived oxylipins, was used to estimate the Δ6 desaturase activity. Sum of EPA-derived oxylipins/sum of ALA-derived oxylipins and sum of DHA-derived oxylipins/sum of ALA-derived oxylipin ratios were used to estimate the activity of the Δ6 and Δ5 desaturases, since no oxylipins derived from eicosatrienoic acid were detected. The sum of AA-derived oxylipins/sum of DGLA-derived oxylipins ratio was used to estimate the activity of the Δ5 desaturase.

Data Analysis

The data were processed using R, version 3.5.1 (www.r-project.org). Continuous variables were expressed as mean ± *SD* and the categorical variables as percentage. Chi-squared test was used to compare categorical variables. Comparisons of oxylipins at baseline between the control group and the arthritis population, between EORA and PMR patients, as well as between baseline and 3 months posttreatment, were adjusted for confounders (age, sex, body mass index [BMI], non-steroidal anti-inflammatory drugs [NSAIDs] use, and DMARDs), by including as covariates in linear regression models. The Benjamini–Hochberg method was used to adjust for multiple comparisons. A partial least squares discriminant analysis (PLS-DA) was utilized to evaluate whether oxylipins could differentiate between arthritis and control subjects. In an effort to identify a minimal number of oxylipins that could distinguish between clinical phenotypes of EORA and PMR, a sparse PLS-DA was subsequently performed with the number of principal components restricted to two and the number of variables/oxylipins in each component restricted to two. MetaboAnalystR was utilized to perform the PLS-DA. The variables for the PLS-DA were normalized to the median, log transformed, and scaled using range scaling. Heatmaps were performed using the gplots package (heatmap.2 function) after data scaling and hierarchical clustering with euclidean distance metric. To identify the best combination of oxylipins that discriminates between EORA an PMR, stepwise discriminant analysis was employed, with the use of multivariate cross-validation and "leave-one-out" classification. Discriminant analyses were performed to determine coefficients for linear combinations of variables that assigned cluster membership to individual cases with the SPSS software version 25.0. The change in oxylipins from baseline to 3 months was assessed by subtracting the baseline concentration from the concentration at 3 months. The comparison in changes between EORA and PMR was also performed adjusting for confounders as stated above.

Ethics Approval and Consent to Participate

Patients were enrolled following written informed consent. Ethical approval was granted by the Institutional Review Board (IRB) at Hospital Universitari Germans Trias i Pujol (PI-13-001).

Results

Cohort Demographics and Disease Characteristics

Characteristics of control and patient population are summarized in Table 1. Eighteen controls (average age: 75.38, *SD* 6.04) and 64 patients (average age: 74.97, *SD* 7.03) were analyzed. Of these patients, 44 were diagnosed with RA and 20 with PMR. Sixteen EORA patients were seropositive (RF and/or CCP positive: EORA+), while 28 were seronegative (RF and CCP were negative: EORA−). EORA− patients were younger compared to EORA+ patients (Table 1).

At baseline, EORA patients had a mean DAS28CRP of 5.77 (*SD* 1.02) and a mean HAQ of 1.7 (*SD* 0.8). One hundred percent of PMR patients reported scapular pain and 90% reported pelvic pain. As expected, arthritic subjects, that is, those with EORA and PMR, presented with higher erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and platelet count, and lower hemoglobin (Hb) than controls with similar comorbidities (Table 1). Within the arthritic population, PMR patients presented with more pelvic pain and less peripheral arthritis than RA patients (Table 1). We did not observe any significant difference in ESR, CRP, and platelet counts between PMR and EORA patients. Comorbidities including diabetes mellitus (DM), high blood pressure (HBP), and dyslipidemia (DL) were also similar in both arthritic populations and controls. About 29.2% of the patients were on daily NSAIDs. At baseline (first visit in rheumatology clinics), none of them were on steroids, or on any synthetic or biological DMARDs.

Oxylipin Profiling and Clustering in Control Population

Eighty-five oxylipins, which are derived from AA, eicosapentaenoic acid (EPA), LA, DGLA, ALA, and docosahexaenoic acid (DHA), were identified by reverse-phase LC/MS in our cohort (Figure 1A; Supplementary Table 1). Forty of the detected oxylipins are in general considered proinflammatory and 45 anti-inflammatory species. Figure 1A shows the pro- (in red) and anti-inflammatory

Table 1. Baseline Characteristics of the Patients Included in the Analysis

				EORA (44)					
Characteristic	Controls (18)	Patients (64)	*p*	Total	EORA – (28)	EORA+ (16)	*p* EORA– vs EORA+	PMR (20)	*p* EORA vs PMR
Female (%)	61.11	59.38	1	47.73	42.85	56.25	.58	85	**.014**
Age (years), mean *SD*	75.38 (6.04)	74.97 (7.03)	.82	74.34 (7.76)	76.93 (6.98)	69.81 (7.11)	**.002**	76.4 (4.99)	.2
BMI (kg/m^2), mean *SD*	28.83 (5.56)	27.93 (4.61)	.49	28.23 (4.77)	28.52 (4.63)	27.73 (5.12)	.6	27.27 (4.32)	.44
DM (%)	16.67	35.94	.24	40.91	53.57	18.75	.052	25	.31
HBP (%)	61.11	71.88	.78	65.91	71.43	56.25	.48	85	.38
Dyslipidemia (%)	50	65.62	.58	68.18	67.86	68.75	1	60	.82
Hemoglobin (mg/dL)	13.72 (1.48)	12.43 (1.5)	**<.001**	12.68 (1.57)	12.53 (1.48)	12.94 (1.73)	.41	11.88 (1.2)	**.04**
Platelets (×10^9/L)	193.71 (32.13)	264.85 (78.04)	**.004**	261.85 (79.94)	274.42 (60.85)	251.87 (102.27)	.43	266.27 (78.06)	.83
ESR (mm/h)	17.92 (17.02)	55.59 (25.46)	**<.001**	54.59 (27.26)	53.29 (26.64)	56.88 (29.04)	.67	57.8 (21.5)	.64
CRP (mg/dL)	4.1 (6.78)	38.65 (48.84)	**<.001**	41.88 (55.76)	42.98 (51.58)	39.96 (64.16)	.86	31.56 (28.27)	.43
Fatigue (%)				68.18	71.43	62.5	.84	70	1
Loss of appetite (%)				45.45	53.57	31.25	.26	35	.6
Stiffness (%)				95.45	96.43	93.75	1	95	1
Edema (%)				45.45	57.14	25	.08	0	**.0008**
Patient general health score (1–100)				75 (18.08)	74.11 (17.27)	76.56 (20)	.66	81.25 (17.46)	.25
Physician general health score (1–100)				77.27 (16.89)	76.43 (16.82)	78.75 (17.46)	0.66	—	—
Shoulder pain (%)				77.27	78.57	75	1	100	.34
Pelvic pain (%)				45.45	46.43	43.75	1	90	**.002**
Tender joints count (0–28)				10.16 (6.03)	8.96 (5.9)	12.25 (6)	.08	2.7 (0.98)	**<.001**
Swollen joints count (0–28)				11.75 (5.62)	11.89 (6.06)	11.5 (5)	.82	0.25 (0.64)	**<.001**
HAQ				1.7 (0.8)	1.78 (0.79)	1.54 (1)	.34	1.5 (0.57)	.34
CDAI				37.14 (11.69)	35.91 (11.28)	39.28 (12.45)	.36	—	—
SDAI				41.32 (13.88)	40.21 (12.29)	43.27 (16.55)	.48	—	—
DAS28CRP				5.77 (1.02)	5.68 (0.96)	5.92 (1)	.46	—	—
NSAIDs (%)	0	29.2%		25	39	25	.52	34	.66

Notes: Demographics, comorbidities, and inflammatory parameters in controls and patients. Continuous variables are presented as means (*SD*) and categorical variables as percentage. BMI = body mass index; CDAI = Clinical Disease Activity Index; CRP = C-reactive protein; DAS28CRP = Disease Activity Score taking into account 28 joints and CRP; DM = diabetes mellitus; EORA = elderly-onset rheumatoid arthritis; ESR = erythrocyte sedimentation rate; HAQ = Health Assessment Questionnaire; HBP = high blood pressure; NSAIDs = non-steroidal anti-inflammatory drugs; PMR = polymyalgia rheumatica; SDAI = Simplified Disease Activity Index. The bold *p* values are considered statistically significant (for a level of significance $p < 0.05$).

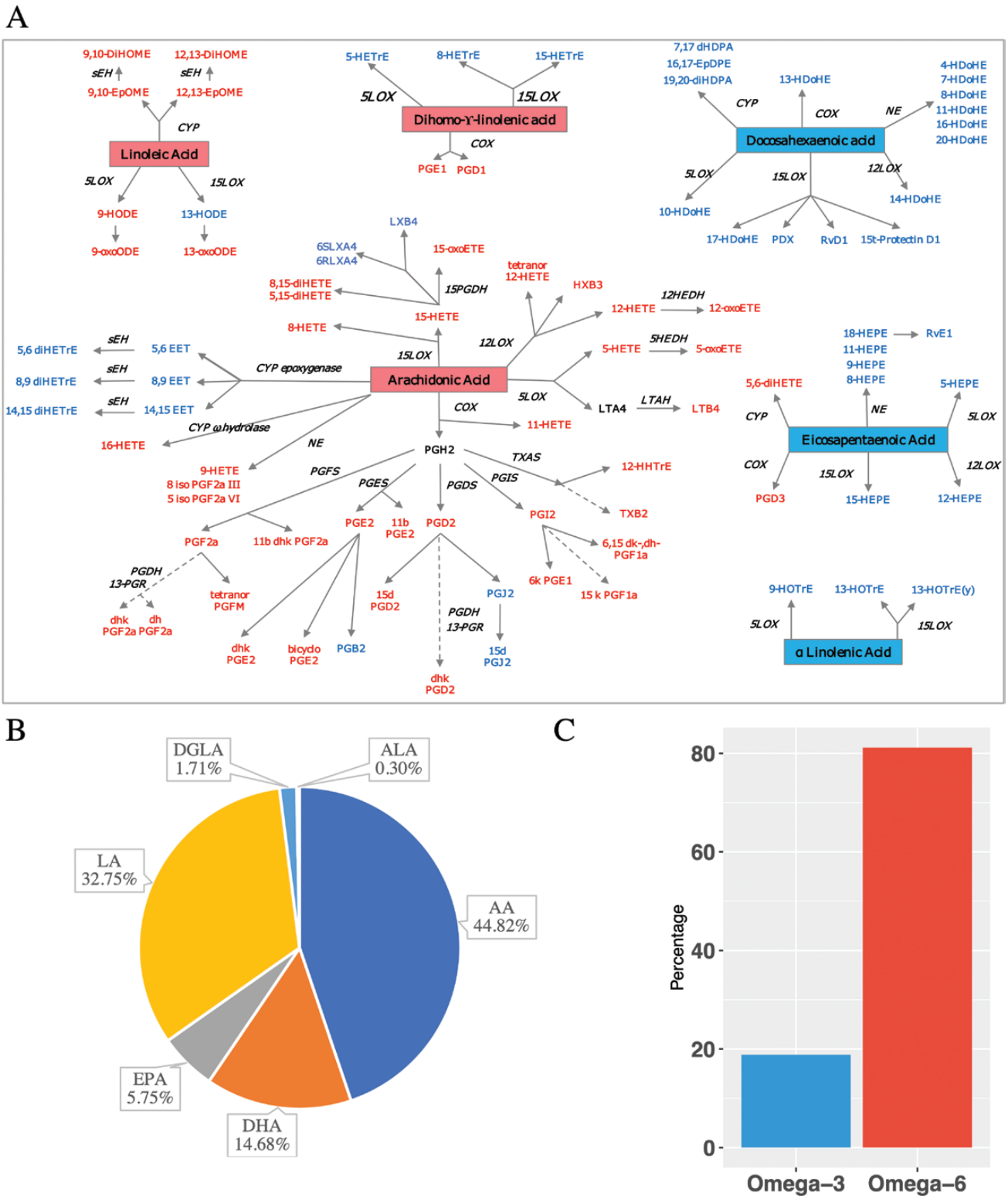


Figure 1. Oxylipin profiling in control population. (**A**) Oxylipins detected in our study by precursor and pathway are shown. Proinflammatory oxylipins are marked in red, while anti-inflammatory ones are marked in blue. The precursor n-3 polyunsaturated fatty acids (PUFAs) are marked in red, while the n-6 PUFAs are marked in blue, and the colors correspond to the barplot in Figure 1C. (**B**) Percentages of the different n-6 and n-3 PUFA-derived oxylipin mass in serum in the control population. (**C**) Total n-6 and n-3 PUFA mass in serum in control population. AA = arachidonic acid; aLA = alpha linolenic acid; COX = cyclooxygenase; CYP = cytochrome P450; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; HEDH = hydroxyeicosanoid dehydrogenase; LA = linoleic acid; LOX = lipoxygenase; LTAH = leukotriene A4 hydrolase; MDB = membrane dipeptidase; NE = non-enzymatic; PGDH = hydroxyprostaglandin dehydrogenase; PGDS = prostaglandin D synthase; PGES = prostaglandin E synthase; PGFS = prostaglandin F synthase; PGIS = prostaglandin I synthase; sEH = soluble epoxide hydrolase; TXAS = thromboxane A2 synthase; 13-PGR = 15-ketoprostaglandinΔ_{13} reductase.

(in blue) oxylipins and the different enzymatic pathways of the oxylipins detected in our samples. Among the detected oxylipins, 56 were derived from n-6 PUFAs, out of which 43 were derived from AA, 8 from LA, and 5 from DGLA. Out of the 29 n-3 PUFA-derived oxylipins, 3 were derived from ALA, 10 from EPA, and 16 from DHA (Figure 1A). Approximately, 80% of oxylipin mass in serum was derived from n-6 PUFA, particularly from AA (31%–63%), LA (18%–56%), and DGLA (1%–3%). n-3 PUFA-derived species were from DHA (8%–24%), EPA (1%–15%), and ALA (0.1%–0.5%; Figure 1B and C).

We then analyzed oxylipin clustering in the control population. We observed that anti-inflammatory oxylipins were grouped in two clusters, one comprised of DHA and EPA derivatives, and the other comprised of LA and ALA species (Supplementary Figure 2). Of interest, the first cluster negatively correlated with the second cluster. AA-derived species were distributed in two clusters, one comprised of most of the COX proinflammatory-derived oxylipin species, and the second comprised of most of the LOX proinflammatory-derived oxylipins.

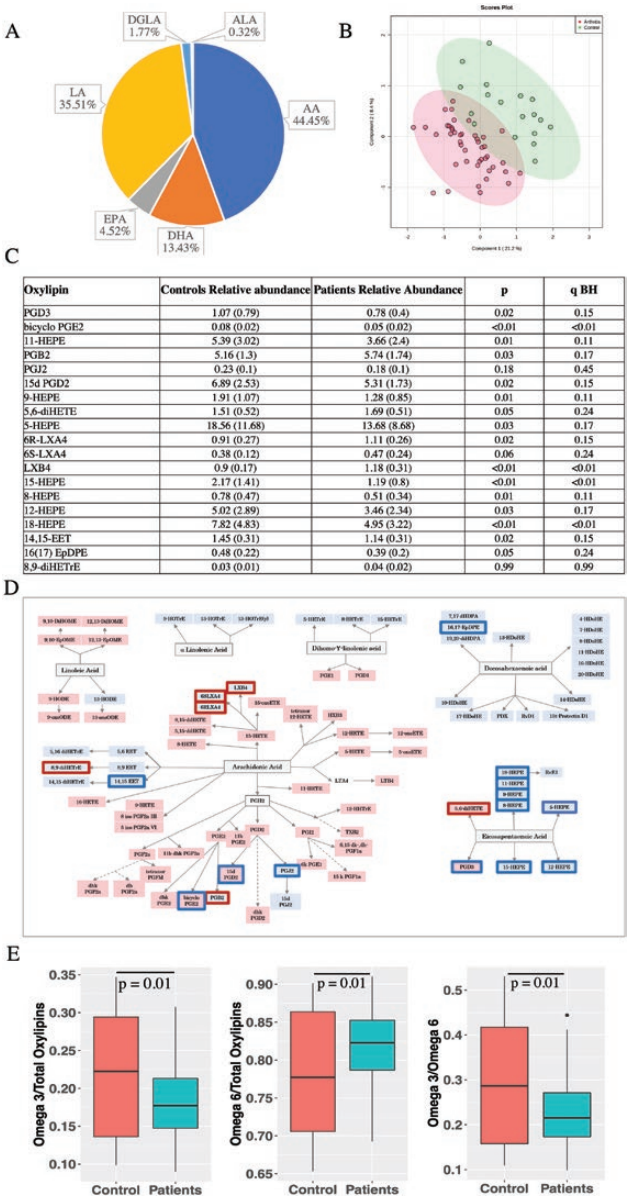
Oxylipin Profiling Differentiates Between Arthritic Patients and Controls

Similar oxylipin species were identified in the arthritic population. Approximately, 80% of oxylipin mass in serum was also derived from n-6 PUFA, particularly from AA (24%–60%), LA (19%–52%), and DGLA (0.7%–5%), and n-3 PUFAs-derived species were from DHA (7%–22%), EPA (1%–10%), and ALA (0.1%–1.4%) (Figure 2A). LA-derived oxylipins tended to be higher (p = .12), while EPA (p = .12) and DHA (p = .17) derived oxylipins tended to be lower in the arthritic patients compared to controls. Regression coefficients show positive relationships between identified oxylipin species in the arthritic population (Supplementary Figure 3). DHA-, EPA-, and ALA-derived anti-inflammatory oxylipins were also grouped in two different clusters as in the control group, yet, they positively correlated in the arthritic patients.

We then analyzed whether oxylipins could differentiate between arthritis and control subjects. Figure 2B shows that 21.2% of the variance can be explained by the first component and an additional 8.4% of the variance can be explained by the second component. This variance is sufficiently distinct to differentiate between control individuals and those with arthritis. In fact, several species were significantly different between control and patients after adjusting for age, sex, BMI, and NSAIDs (Figure 2C and D and Supplementary Table 2). A sparse PLS-DA (Supplementary Figure 3D) shows that tetranor-PGFM, 15-HEPE, 6R-LXA4, and LXB4 were the oxylipins that better distinguished between controls and arthritis patients. Of interest, most of the n-3 EPA and some DHA-derived oxylipin species were lower in patients compared to the control population (Figure 2D). Conversely, the n-3/n-6 PUFA ratio was lower in arthritis patients compared to control (p = .01; Figure 2E).

Baseline Oxylipin Profiling Differentiates Between EORA and PMR Patients

We further analyzed whether oxylipins were different between EORA and PMR. Oxylipin clustering shown in Supplementary Figures 4 and 5 differed in the EORA group compared to PMR patients. While oxylipin clustering in PMR population was similar to controls (Supplementary Figure 5), oxylipins clustered differently in EORA. The sparse PLS-DA between controls and EORA and controls and PMR was also different (Supplementary Figures 4D and 5D). While



Oxylipin	Controls Relative abundance	Patients Relative Abundance	p	q BH
PGD3	1.07 (0.79)	0.78 (0.4)	0.02	0.15
bicyclo PGE2	0.08 (0.02)	0.05 (0.02)	<0.01	<0.01
11-HEPE	5.39 (3.02)	3.66 (2.4)	0.01	0.11
PGB2	5.16 (1.3)	5.74 (1.74)	0.03	0.17
PGJ2	0.23 (0.1)	0.18 (0.1)	0.18	0.45
15d PGD2	6.89 (2.53)	5.31 (1.73)	0.02	0.15
9-HEPE	1.91 (1.07)	1.28 (0.85)	0.01	0.11
5,6-diHETE	1.51 (0.52)	1.69 (0.51)	0.05	0.24
5-HEPE	18.56 (11.68)	13.68 (8.68)	0.03	0.17
6R-LXA4	0.91 (0.27)	1.11 (0.26)	0.02	0.15
6S-LXA4	0.38 (0.12)	0.47 (0.24)	0.06	0.24
LXB4	0.9 (0.17)	1.18 (0.31)	<0.01	<0.01
15-HEPE	2.17 (1.41)	1.19 (0.8)	<0.01	<0.01
8-HEPE	0.78 (0.47)	0.51 (0.34)	0.01	0.11
12-HEPE	5.02 (2.89)	3.46 (2.34)	0.03	0.17
18-HEPE	7.82 (4.83)	4.95 (3.22)	<0.01	<0.01
14,15-EET	1.45 (0.31)	1.14 (0.31)	0.02	0.15
16(17) EpDPE	0.48 (0.22)	0.39 (0.2)	0.05	0.24
8,9-diHETrE	0.03 (0.01)	0.04 (0.02)	0.99	0.99

Figure 2. Oxylipins in patients compared to controls. (**A**) Percentages of n-6 and n-3 polyunsaturated fatty acid (PUFA)-derived oxylipin mass in serum in the arthritic population. (**B**) Partial least squares discriminant analysis (PLS-DA) between patients with arthritis and controls. The plot depicts the amount of variance between patients with arthritis and controls in the first two dimensions. Attributable variance is labeled on the axes. (**C**) The mean and *SD* of the relative abundance of the significantly different oxylipins between the two groups is presented with the correspondent *p*-values and *q*-values. (**D**) Upregulated (red squares) and downregulated (blue squares) proinflammatory (red background) and anti-inflammatory (blue background) oxylipins in patients compared to controls. (**E**) Total n-6 and n-3 PUFA mass, and n-3/n-6 ratio in control population compared to arthritis population. AA = arachidonic acid; aLA = alpha linolenic acid; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid. Expansion for the abbreviations in C and D can be found in Supplementary Table 1.

tetranor-PGFM, 6R-LXA4, and LXB4 were the oxylipins that better distinguished controls and EORA, tetranor-PGFM, bicyclo-PGE2, PGB2, and LTB4 were the oxylipins that better distinguished controls and PMR, suggesting a different pathobiology in this disease. When comparing EORA+ and EORA– patients, five metabolites

were found to be different (PGD1, 62.04 ± 31.44 in EORA seropositive vs 44.51 ± 17.49 in EORA seronegative, p = .02; dhk PGD2, 86.64 ± 48.32 in EORA+ vs 59.34 ± 27.10 in EORA–, p = .03; 11b dhk PGF2a, 4.25 ± 2.20 in EORA+ vs 2.74 ± 1.42 in EORA–, p = .02; 9,10 EpOME, 8.98 ± 3.99 in EORA+ vs 7.51 ± 2.50 in EORA–, p = .02; 9,10 diHOME, 11.96 ± 20.23 in EORA+ vs 4.85 ± 2.24 in EORA–, p = .01).

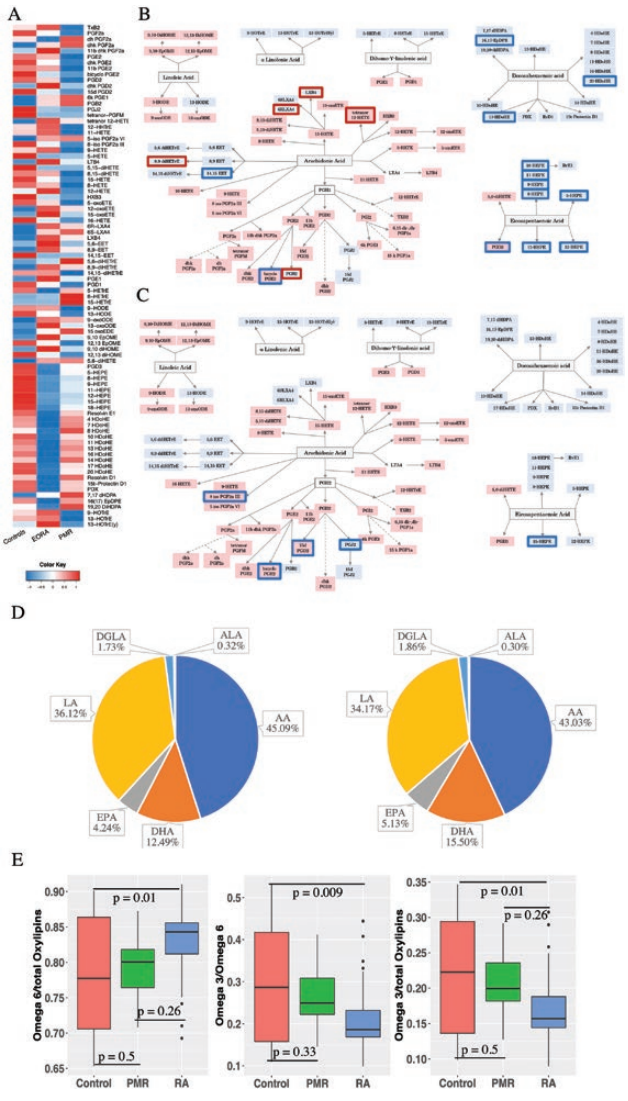


Figure 3. Oxylipins in elderly-onset rheumatoid arthritis (EORA) patients compared to polymyalgia rheumatica (PMR) patients. (**A**) Heatmap of relative abundance of oxylipins in EORA and PMR patients compared to controls. The relative abundances are scaled by row. (**B**) Upregulated (red squares) and downregulated (blue squares) proinflammatory (red background) and anti-inflammatory (blue background) oxylipins in EORA patients compared to controls. (**C**) Upregulated (red squares) and downregulated (blue squares) proinflammatory (red background) and anti-inflammatory (blue background) oxylipins in PMR patients compared to controls. (**D**) Percentages of n-6 and n-3 polyunsaturated fatty acid (PUFA)-derived oxylipin mass in serum in the EORA population. (**E**) Percentages of n-6 and n-3 PUFA-derived oxylipin mass in serum in the PMR population. (**F**) Total n-6 and n-3 PUFA mass, and n-3/n-6 ratio in EORA and PMR compared to control population. AA = arachidonic acid; aLA = alpha linolenic acid; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA - eicosapentaenoic acid; LA = linoleic acid. Expansion for the abbreviations in A, B and C can be found in Supplementary Table 1.

Figure 3A shows the heatmap of the relative abundance of oxylipins in EORA and PMR patients compared to controls. Several oxylipin species were significantly different between EORA, PMR patients, and the controls (Figure 3A and Supplementary Table 3). n-3 DHA- and EPA-derived oxylipins were significantly downregulated in EORA patients but not in PMR patients, while LA-derived oxylipins tended to be higher in both EORA and PMR patients compared to controls (Figure 3B and C). Conversely, the n-6 PUFA and n-3 PUFA mass, and the n-3/n-6 PUFA ratio was significantly different in EORA patients compared to controls, but not in the PMR group compared to controls (Figure 3D–F).

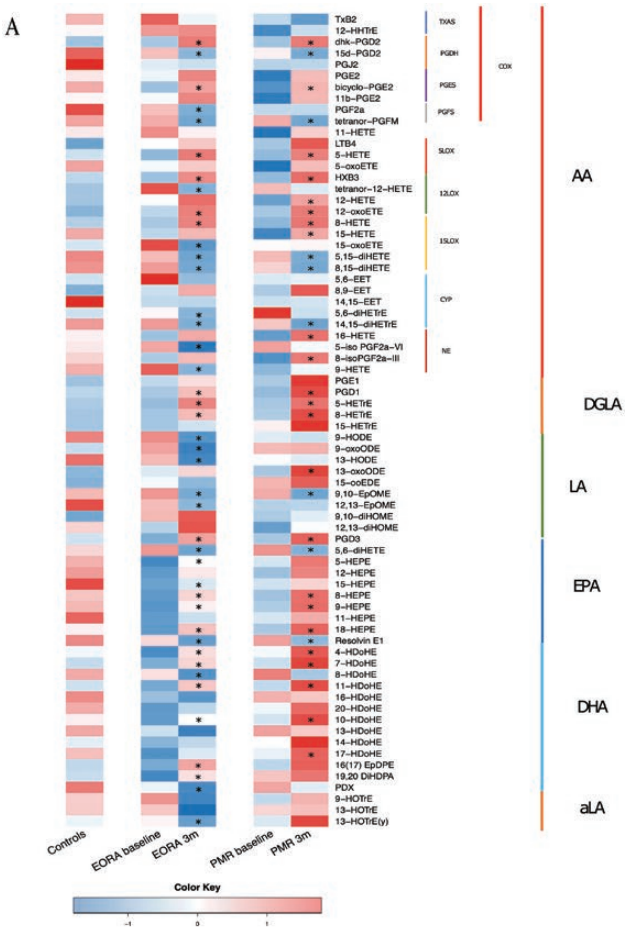
We then explored whether the oxylipins differentially expressed between controls and patients or between EORA and PMR were derived preferentially via either COX, LOX, or CYP pathways. As in Figures 2D and 3B and C, we did not observe a clear preference for COX-, LOX-, or CYP-derived oxylipins in arthritis patients. To determine whether the changes observed in EORA and PMR were related to changes in activity of the desaturase enzymes, the upstream step, we estimated their activity by studying the ratio between PUFA-derived oxylipins. The EPA/ALA ratio tended to be lower in EORA (17.27, p = .1) and PMR (18.21, p = .25) patients than in controls (25.72). The ratio of DHA/ALA (EORA 49.01, PMR 55.6, controls 64.5) tended to be lower in EORA than PMR patients and controls. DGLA/LA and AA/DGLA ratios were not different between the three studied groups (Supplementary Table 4).

We finally explored whether oxylipins in serum could discriminate between EORA and PMR. The stepwise discriminant analysis is presented in Supplementary Figure 6A. Two oxylipins, namely 4-HDoHE and 8,15-di dihydroxy-eicosatrienoic acid, were sufficient to correctly classify 70.3% of these patients. The canonical correlation of 0.821 and Wilks' lambda of 0.829 were found when these two variables were used, with high significance (p = .003; Supplementary Figure 6A). The top two candidates as biomarkers for differentiating PMR from RA, 4-HDoHE and 8,15-diHETE, had an area under the receiver operating characteristic curve (AUROC) of 0.76 (Supplementary Figure 6B). Yet, the disease phenotypes were unable to be fully distinguished using all the oxylipins as per a PLS-DA (Supplementary Figure 6C).

Ratio n-3/n-6 PUFA Increased in EORA Patients at 3 Months Posttreatment

At 3 months posttreatment, 39 out of the 44 patients diagnosed with EORA were on glucocorticoids (GCs, prednisolone) at an average dose of 5.51 mg/d (*SD* 3.27) (average dose after diagnosis was 8.83 mg/d, *SD* 3.21), and 32 received DMARD medication (methotrexate, leflunomide, or hydroxychloroquine). Thirty-three patients had a good and 8 patients a moderate response to treatment, according to the EULAR response criteria (23). Supplementary Table 5 shows the distribution of GCs and DMARDs across the response groups. At 3 months, all patients diagnosed with PMR were on GC at an average dose of 8 mg/d (*SD* 4.21) (average dose after diagnosis was 9.3 mg/d, *SD* 2.4). All PMR patients responded to treatment. Prednisolone dose was significantly higher (p = .01) in PMR patients compared to EORA patients at 3 months.

Seventy-two oxylipins were detected at 3 months posttreatment. Figure 4A shows a heatmap based on the concentrations of the oxylipins at baseline and 3 months posttreatment in EORA and PMR patients. The first column represents oxylipin concentrations in controls, which were quantified only at baseline. We compared the oxylipins at baseline and 3 months posttreatment. The significant



PUFA	Controls	EORA baseline	EORA 3m	p value	PMR baseline	PMR 3m	p value
AA (%)	44.67	44.317	49.033	0.07	43.049	45.413	0.683
DGLA (%)	1.76	1.828	3.04	< 0.001	1.99	3.089	0.019
LA (%)	32.34	37.189	28.608	0.001	35.083	30.242	0.063
EPA (%)	6.24	4.386	5.55	0.029	5.101	5.976	0.110
DHA (%)	14.68	11.964	13.536	0.005	14.486	15.002	0.337
aLA (%)	0.31	0.316	0.233	0.035	0.29	0.278	0.886

Figure 4. Oxylipins in controls compared to patients at baseline and 3 months. (**A**) Heatmap of relative abundance of oxylipins in elderly-onset rheumatoid arthritis (EORA) and polymyalgia rheumatica (PMR) patients at baseline and 3 months compared to controls. The relative abundances are scaled by row. The * represents a statistically significant difference (at a level of $p < 0.05$) between EORA at baseline and 3 months, and PMR at baseline versus 3 months post-treatment, respectively. (**B**) Total n-6 and n-3 polyunsaturated fatty acid (PUFA) mass, and n-3/n-6 ratio in EORA and PMR patients at baseline and 3 months compared to control population. (**C**) Percentage of the oxylipins for each PUFA precursor in the controls in the first column. The next columns show the percentage of oxylipins for each PUFA precursor in EORA and PMR patients at baseline and at 3 months posttreatment, along with the *p*-values. AA = arachidonic acid; aLA = alpha linolenic acid; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; PUFA = polyunsaturated fatty acid. Expansion for the abbreviations in A can be found in Supplementary Table 1.

changes are marked with asterisks. The n-3 EPA- and some of the DHA-derived anti-inflammatory oxylipins that were downregulated in EORA patients at baseline significantly increased in EORA compared to PMR patients (Figure 4A and Supplementary Table 6). Some of the EPA- and DHA- derived anti-inflammatory oxylipins also increased in PMR, although the total n-3 mass in PMR patients was similar between baseline and 3 months (Figure 4B and C and Supplementary Table 7). Both pro- and anti-inflammatory DGLA-derived oxylipins increased posttreatment in both disease groups. However, ALA-derived oxylipins decreased only in EORA ($p = .035$). Supplementary Table 8 shows the changes of oxylipins derived from each PUFA before and after treatment in both diseases. Interestingly, the desaturase activity changed significantly posttreatment in both EORA and PMR patients. The EPA/ALA and DHA/ALA, that tended to be lower in EORA compared to PMR and controls, increased in EORA patients (EPA/ALA: from 17.27 [11.21] at baseline to 27.9 [20.2], $p = .007$; DHA/ALA: from 49.01 [21.15] at baseline to 66.91 [30.31], $p < .001$). In Supplementary Table 9, it can also be observed that the ratios DHA/ALA and DGLA/LA significantly increased in both diseases posttreatment.

Discussion

Oxylipins comprise distinct classes of bioactive molecules with functions that are critical for joint disease (24). Evidence that the COX pathway might be involved in the pathogenesis of RA dates back to the 1970s, when elevated PG levels were reported in synovial fluid from patients with RA (25). Since then, studies in animals and patients have established a pivotal and complex role of PG in RA. High levels of phospholipase A2 (PLA_2), that catalyzes the release of AA and the production of its metabolites, such as PGs and leukotriene, are found in synovial tissue, inducing proliferative changes in synovial structures (26). However, few studies have addressed the role of other oxylipins in this field and none in the older adult population. The need for a more detailed understanding of how upstream oxylipin pathways influence disease risk is especially relevant to arthritis in older adults. Therefore, we conducted an extensive oxylipin profiling to comprehensively establish the association of circulating inflammatory oxylipins with arthritis in this population.

We hypothesized that oxylipin-related perturbations will be related to arthritic symptoms, and that by defining this oxylipin profile, we could define elements of inflammation pathobiology in this population. In this sense, new families of lipid mediators important in the resolution of inflammation have been discovered and are being investigated (27). The n-3 PUFA-derived oxylipins such as resolvins, protectins, and maresins have been identified in the resolving exudates of acute inflammation. EPA-derived E-series (RvE) and DHA-derived or D-series (RvD) resolvins display potent pro-resolving and immunoregulatory actions that include blocking the production of proinflammatory mediators. In addition to the D-series resolvins, DHA is also a precursor of other pro-resolving docosanoids named protectins (PDs) and maresins. These pro-resolving lipid mediators,

together with the above-mentioned resolvins, and the anti-inflammatory lipoxins, have been grouped together as SPMs (27). These are interesting compounds that constitute a novel topic of research, not only due to their bioactive role in the "return to homeostasis" process, but also in elucidating the physiological functions of other n-3 PUFAs (28).

Recent findings highlight that the omega-6 fatty acid AA appears increased, and omega-3 EPA and DHA decreased in most cancer tissues compared to normal ones. There is also compelling evidence that omega-3 PUFAs, particularly EPA and DHA, and an adequate balance of omega-6/omega-3 PUFAs play a determinant role in most physiological and biochemical processes occurring in cells and organisms, having great significance in decreasing the risk of many diseases or even resolving their inherent inflammation condition (28). In our study, we found a decreased n-3/n-6 PUFA-derived oxylipin ratio in patients compared to controls, with a stronger decrease in EORA compared to PMR patients. This profound decrease of n-3 PUFAs in EORA is not related to the degree of inflammation, as there were no differences in inflammatory biomarkers including CRP, ESR, or platelets count between PMR and EORA patients.

The DHA-derived downregulated oxylipins are the hydroxydocosahexaenoic acids (HDoHE—considered to have anti-inflammatory properties), among them 17-HDoHE, the precursor of resolvins, and 4-HDoHE, the precursor of maresins. The EPA-derived oxylipins that are downregulated are the hydroxy-eicosapentanoic acids (HEPE, also anti-inflammatory), 18-HEPE being another precursor of the resolvins. Total free DHA and EPA are also decreased in patients compared to controls, with a more prominent decrease in EORA compared to PMR patients, although without reaching a statistically significant difference. Interestingly, this was already observed in a previous study published by Rodríguez-Carrio and coworkers (29) who found decreased levels of palmitic, palmitoleic, oleic, arachidonic, total free EPA, and DHA in a Spanish cohort of YORA patients compared to healthy controls. Of interest, other anti-inflammatory oxylipins including lipoxins A4 and B4 that are derived from AA were upregulated in EORA, what would be expected to try to resolve a systemic inflammatory response.

We also explored whether the desaturase enzymes were inhibited in patients with arthritis. Δ5-desaturase and Δ6 desaturase exhibit affinity to metabolize n-3 over n-6 PUFA, provided that they exist in a ratio of 1:1–4. But, dietary intake of ALA is usually low compared to up to 30-fold higher intake of LA, which increases these enzymes' preference to metabolize n-6 PUFA, thus the conversion of ALA is poor in humans and only a small proportion is converted to EPA and DHA (30,31). Previous studies performed in RA patients suggest an increased activity of Δ6 desaturase, since they observed that treatment with TNF inhibitors decreased the activity of this enzyme (32) and higher levels of EPA were associated with a greater decrease of DAS28 in response to treatment with TNF inhibitors. In our study, the EPA/ALA ratio, but not the DGLA/LA and AA/DGLA ratios, surrogates for the activity of Δ5 and Δ6 desaturase, was lower in arthritis patients compared to controls (although it did not reach a statistical significance, likely due to a small sample size), and got back to control values after treatment. These data suggest a decreased activity of the desaturases only in the n-3 PUFA ALA pathway during inflammation. Steroids (dexamethasone, hydrocortisone, and triamcinolone) were shown to reduce the activity of the Δ5 and Δ6 desaturases (33) and are likely responsible of some of the changes observed at 3 months. In addition, we did not observe a clear preference for COX- or LOX-derived oxylipins in arthritis patients. However, these enzymes are active in the tissue and no studies have been performed to evaluate the relationship between tissue and circulating metabolites, hence, it makes the interpretation of our results difficult.

A larger percentage of EORA subjects are negative for both RF and anti-CCP antibodies (34–36). This may lead to significant diagnostic difficulties at first presentation, as there are many similarities between seronegative EORA and other rheumatologic diseases, including PMR. Moreover, an explosive onset of shoulder arthritis, resembling PMR, is observed in patients with early EORA (35). Conversely, peripheral arthritis has also been described in PMR patients (37). Imaging techniques such as FDG-PET/CT and ultrasound have shown some findings for differentiating PMR from EORA. In patients with PMR, abnormal FDG accumulation was observed at the entheses, suggesting the presence of enthesitis in addition to bursitis and synovitis (38). Ultrasound imaging showed that subdeltoid bursitis and biceps tendon sheath effusion were more frequent in patients with EORA, with a predominate symmetry and signs for massive inflammation (39). However, there is still an overlap of clinical and imaging features that would explain our oxylipin profiling results in these diseases. Although the n-3/n-6 ratio and some oxylipin levels were different in both diseases, the oxylipin profiling did not fully separate between RA and PMR. The stepwise discriminant analysis identified the combination of 4-HDoHE and 8,15-diHETE as sufficient to correctly classify 70% of the patients. 4-HDoHE is a metabolite of DHA via 5-LOX with anti-inflammatory properties and 8,15-diHETE is an AA-derived oxylipin via 5/15LOX. There is scarce data on both of these oxylipins. 4-HDoHE was shown to directly inhibit endothelial cell proliferation and angiogenesis via peroxisome proliferator-activated receptor γ (40), while 8,15-diHETE inhibits AA-induced autocrine neutrophil stimulation and LTB4-induced neutrophil chemotaxis, which also suggests an anti-inflammatory role (41). Since the biological relevance of these oxylipins in arthritis is unknown, more studies are required before deciding the adequacy of these metabolites as biomarkers of differential diagnosis between EORA and PMR.

Forty-one patients with EORA and 19 patients with PMR responded to treatment, which did not allow us to evaluate whether or not changes in the oxylipin profile were related to therapeutic response or could potentially predict response. However, we did observe that the ratio n-3/n-6 PUFA increased in EORA patients (Figure 4). In addition, most of these patients were receiving a low-dose prednisone. Although GCs have very well-known anti-inflammatory properties, there is no data about the effect of GC on systemic oxylipins, which could partially be responsible for the upregulation of most of the previously downregulated n-3 PUFA-derieved oxylipins. Although the effect of GC on COX enzymes is well known (42), little is published on its effect on LOX enzymes. Of note, a study showed that oral GC increased jejunal uptake of cholesterol and ileal uptake of lauric, palmitic, linoleic, and linolenic acid (43). Interestingly, the ALA-derived oxylipins 9-HOTrE, 13-HOTrE, and 13-HOTRE(γ) were still downregulated in EORA at 3 months.

Although these findings are certainly promising, this study is not without limitations. One of the limitations is the number of patients included in the study, but recruitment of patients with new-onset arthritis and DMARD naive is challenging. Confirmation of our results with a larger sample size from prospective cohorts of patients with new-onset inflammatory arthritis is necessary to strengthen our conclusions. Comparison with a YORA and other arthritides would help to determine if the described oxylipin changes are specific to rheumatoid arthritis/EORA/PMR or secondary to systemic inflammation. Yet, we believe that this work can improve our limited understanding of the role of oxylipins beyond AA metabolites and

leukotrienes in inflammatory arthritis and may lay the groundwork for a more targeted investigation of novel oxylipin- and lipidomics-based studies in arthritis.

Whether this abnormal n-3/n-6 ratio in serum reflects the tissue PUFA composition requires further studies. Oxylipin profiling of synovial or periarticular tissue could help to get more information about local and systemic oxylipin production or consumption. In addition, it is also unknown if altered patients' n-3/n-6 ratio at baseline could increase the risk of arthritis in older adults population. Research shows that the health-promoting effects of n-3 PUFAs are due to their competition with AA for the enzymatic metabolism, decreasing the formation of n-6-series lipid mediators that are predominately pro-angiogenic and proinflammatory and increasing n-3-series bioactive lipids with less detrimental and possibly beneficial effects (44–46). Further studies are needed to determine either a protective or therapeutic role of the n-3 PUFA diet in this population. Moreover, more studies are needed to evaluate the possible changes in the oxylipin profile related to corticosteroids and other treatments. In conclusion, we believe that this work can improve our limited understanding of the role of oxylipins beyond AA metabolites and leukotrienes in arthritis in older adults and may lay the groundwork for a more targeted investigation of novel oxylipin- and lipidomics-based studies in EORA and PMR.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

None reported.

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4.4 Profiling of Serum Oxylipins During the Earliest Stages of Rheumatoid Arthritis

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Profiling of Serum Oxylipins During the Earliest Stages of Rheumatoid Arthritis

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Objective. Eicosanoids modulate inflammation via complex networks involving different pathways and downstream mediators, including oxylipins. Although altered eicosanoids are linked to rheumatoid arthritis (RA), suggesting that metabolization is enhanced, the role of oxylipins in disease stratification remains unexplored. This study was undertaken to characterize oxylipin networks during the earliest stages of RA and evaluate their associations with clinical features and treatment outcomes.

Methods. In total, 60 patients with early RA (according to the American College of Rheumatology/European League Against Rheumatism 2010 criteria), 11 individuals with clinically suspect arthralgia (CSA), and 28 healthy control subjects were recruited. Serum samples were collected at the time of onset. In the early RA group, 50 patients who had not been exposed to disease-modifying antirheumatic drug (DMARD) or glucocorticoid treatment at the time of recruitment were prospectively followed up at 6 and 12 months after having received conventional synthetic DMARDs. A total of 75 oxylipins, mostly derived from arachidonic, eicosapentanoic, and linoleic acids, were identified in the serum by liquid chromatography tandem mass spectrometry.

Results. Univariate analyses demonstrated differences in expression patterns of 14 oxylipins across the RA, CSA, and healthy control groups, with each exhibiting a different trajectory. Network analyses revealed a strong grouping pattern of oxylipins in RA patients, whereas in individuals with CSA, a fuzzy network of oxylipins with higher degree and closeness was found. Partial least-squares discriminant analyses yielded variable important projection scores of >1 for 22 oxylipins, which allowed the identification of 2 clusters. Cluster usage differed among the groups ($P = 0.003$), and showed associations with disease severity and low rates of remission at 6 and 12 months in RA patients who were initially treatment-naive. Pathway enrichment analyses revealed different precursors and pathways between the groups, highlighting the relevance of the arachidonic acid pathway in individuals with CSA and the lipooxygenase pathway in patients with early RA. In applying distinct oxylipin signatures, subsets of seropositive and seronegative RA could be identified.

Conclusion. Oxylipin networks differ across stages during the earliest phases of RA. These distinct oxylipin networks could potentially elucidate pathways with clinical relevance for disease progression, clinical heterogeneity, and treatment response.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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INTRODUCTION

Rheumatoid arthritis (RA) is an immune-mediated rheumatic condition characterized by chronic inflammation and joint destruction (1). Early diagnosis and prompt treatment guided by treat-to-target goals is crucial to ensure long-term disease control (2). Interventions during the early phase of RA are associated with higher rates of remission, probably due to pathogenic mechanisms occurring at this point (3). Therefore, research into the early disease phase is of utmost scientific relevance. The development of RA is a multistep process, in which different phases can be distinguished (4). The recognition of the symptomatic phase preceding clinical arthritis, referred to as clinically suspect arthralgia (CSA), represents the first opportunity to identify patients at risk for progression to RA (5). This stage serves not only to open a window for characterization of the changes underlying the shift from systemic autoimmunity to overt joint synovitis, but also to delineate potential targets for prevention of disease progression (5,6). Although the cellular and proteomic characterization of these stages have been extensively pursued, the metabolomics, and mainly the lipidomics, have received less attention.

In the "omics" era, lipids are emerging as pivotal mediators for several biologic processes (7). Polyunsaturated fatty acids (PUFAs) and eicosanoids form one of the most complex networks in biology, controlling many physiologic and pathologic processes, often in opposing directions (8). The role of eicosanoids in RA dates back to the 1970s and mid-1980s, with the description of the cyclooxygenase (COX) and lipooxygenase (LOX) pathways (9). However, the presence of these enzymes could not be used to fully account for the underlying pathologic processes, and despite profound improvements in the clinical management of RA, these pathways were observed to remain active even after treatment (8). Consequently, a knowledge plateau was reached, in part due to technical limitations. It was not until recently that the CYP450-derived lipid species were described (10), although their role is still poorly characterized.

Lipidomics and high-throughput approaches have started a new investigative period aimed at attaining a better understanding of the control of local inflammation in RA and its progression to either resolution or chronification (11). A number of novel lipid mediators, such as oxylipins, are now recognized as active players in either controlling the resolution of the disease or fueling inflammation (11–13). Oxylipins have been reported to promote migration of polymorphonuclear cells, enhance vascular permeability, control cytokine production, and modulate oxidative stress species (11). Thus, oxylipins may be considered attractive candidate biomarkers for further clarifying the mechanisms of tissue injury and disease aggravation.

Previous results from our group revealed altered levels of different PUFAs in patients with RA (14), which may reflect an accelerated metabolization toward their downstream mediators, including oxylipins. It is plausible that applying new analytical technologies will allow us to answer the question as to whether PUFA-derived oxylipin networks are altered in RA, and help us to comprehensively analyze how disturbed oxylipin networks may be associated with the clinical phenotype of the earliest stages of the disease. Therefore, the aims of the present study were to 1) analyze oxylipin levels and networks during the earliest stages of RA, including the preclinical CSA stage, 2) evaluate whether oxylipin expression patterns may help identify patients with specific clinical features and treatment responses, and 3) evaluate whether profiling of oxylipins may identify pathways related to disease heterogeneity.

PATIENTS AND METHODS

Study participants. The study was approved by the local institutional review board (Comité de Ética de Investigación Clínica del Principado de Asturias) in compliance with the Declaration of Helsinki. All study subjects gave written informed consent.

Our study involved 60 patients with early RA, diagnosed according to the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria (15), from the early arthritic clinic in the Department of Rheumatology at Hospital Universitario Central de Asturias in Oviedo, Spain. Patients were recruited at the time of disease onset. A complete clinical examination (see Supplementary Methods, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract) was performed on all patients during the recruitment appointment. Composite measures of disease activity were calculated, including the Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR) (16) and the Simplified Disease Activity Index (SDAI) (17). Patients who had not been exposed to any disease-modifying antirheumatic drugs (DMARDs) or glucocorticoids at the time of recruitment were considered to be treatment-naive (n = 50), and this group was prospectively followed up with complete clinical examinations at 6 months (n = 46) and 12 months (n = 40) after having received treatment with conventional synthetic DMARDs (csDMARDs).

Clinical management was performed according to the EULAR recommendations for the management of RA with synthetic and biologic DMARDs (18). Clinical response was evaluated using the EULAR response criteria (19) at 6 and 12 months of follow-up, and patients exhibiting a good response were considered to be responders, whereas those with moderate or no response were classified as nonresponders. Patients switching to a different csDMARD during the first 12 months were also classified as nonresponders.

Individuals with CSA were recruited from the same clinic. These individuals were considered to have CSA if they met ≥4 of the criteria in the EULAR definition of arthralgia suspicious for progression to RA (5), which allows a sensitivity of 70% and specificity

of 93.6%. Subjects without arthritis were recruited as healthy controls among age- and sex-matched individuals from the same population of subjects who were without a diagnosis of inflammatory rheumatic disease.

Exclusion criteria applied to all of the groups included presence of a preexisting autoimmune or inflammatory condition, medical prescription for nonsteroidal antiinflammatory drugs or coxibs, usage of fish oil supplements, or history of cancer or recent infection (within 3 months of study recruitment). A fasting blood sample was collected from all individuals by venipuncture, and serum samples were transferred to the laboratory, processed under controlled, standardized procedures, and stored at –80°C within 2 hours of processing. Plasma samples from a subgroup of subjects (n = 6) were collected and processed in parallel.

Lipid extraction and liquid chromatography tandem mass spectrometry (LC-MS/MS) measurement of oxylipins. All serum samples obtained at baseline were thawed once and immediately used for isolation of free fatty acids and oxylipins as described previously (20,21). In brief, 50 μl of serum was spiked with a cocktail of 26 deuterated internal standards that also included some selected PUFAs, brought to a volume of 1 ml with 10% methanol. Thereafter, the samples were purified by solid-phase extraction on a Strata-X column (see Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract]).

Oxylipins in the sera were analyzed and quantified by LC-MS/MS as described previously (20,21) (see Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract]). Oxylipins and free fatty acids were quantitated using the stable isotope dilution method. Identical amounts of deuterated internal standards were added to each sample and to all of the primary standards used to generate standard curves (see Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract]). The levels of oxylipins are expressed in pmoles/ml.

Statistical analysis. Continuous variables are presented as the mean ± SD or median with interquartile range, whereas categorical variables are summarized as the number (percentage) of subjects. For each oxylipin, the fatty acid precursor and pathway (first enzyme acting on the precursors) were retrieved from the literature. If a nonenzymatic mechanism was described in the literature, regardless of the experimental setting, it was included as well, in a conservative approach.

Nonparametric tests were used to evaluate differences in oxylipin profiles across the groups, and the *P* values obtained were adjusted for multiple testing using the Benjamini-Hochberg procedure (22). Linear regression models were used to control comparisons for potential confounders. Oxylipin levels were log-transformed, normalized to the median value, and scaled using the range-scaling method (23).

Correlograms and network analyses were built to analyze the correlations among the oxylipins across conditions. Centrality measures were calculated for each metabolite and condition (24). Partial least-squares discriminant analysis (PLS-DA) was used to identify discriminant metabolites, with the analyses controlled for multicollinearity, and cross-validation accuracy and permutation model statistics were retrieved. Oxylipins contributing to group discrimination in the PLS-DA were selected on the basis of having a variable important projection (VIP) score of >1 (24). VIP-selected metabolites were used in unsupervised cluster analyses.

For comparisons between 2 groups, orthogonal PLS-DA (OPLS-DA) models were constructed to evaluate group discrimination. These models enhance the interpretation and discrimination based on intra- and interclass information, without a significant effect on prediction power (25). Cross-validation of the OPLS-DA models was performed by permutation analyses, and Q2 *P* values were computed. Correlation analyses against prespecified patterns were assessed, and correlation coefficients (determined using Spearman's rank correlation test), *P* values, and false discovery rates were computed.

Pathway enrichment analyses were performed using the KEGG human genome library, with a global test for pathway enrichment. Pathway topology analysis was used to assess betweenness centrality. Raw *P* values (adjusted), Holm's *P* values, and pathway impact were obtained for each pathway. Statistical analyses were carried out using R version 3.6.3 and MetaboAnalyst version 4.0.

RESULTS

Serum oxylipin levels during the earliest stages of RA. Serum oxylipins were measured in 60 patients with early RA (including 50 treatment-naive patients), 11 individuals with CSA, and 28 matched healthy controls (characteristics of the subjects are listed in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). A total of 74 oxylipins derived from arachidonic acid (AA) (n = 39), docosahexanoic acid (DHA) (n = 13), linoleic acid (LA) (n = 7), eicosapentanoic acid (EPA) (n = 7), dihomo-γ-linolenic acid (DHGLA) (n = 3), α-linolenic acid (ALA) (n = 2), and oleic acid (OA) (n = 2) were identified by LC-MS/MS in the serum from patients with early RA (a complete list of the identified oxylipins is shown in Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). In subgroup analyses, a good correlation was observed between the oxylipin levels measured in the serum and oxylipin levels measured in the plasma (median Spearman's R = 0.870, range 0.750–1.000).

Moreover, the peaks corresponding to leukotriene B_4 (LTB_4) and 5S,12S-dihydroxyeicosatetraenoic acid (5S,12S-

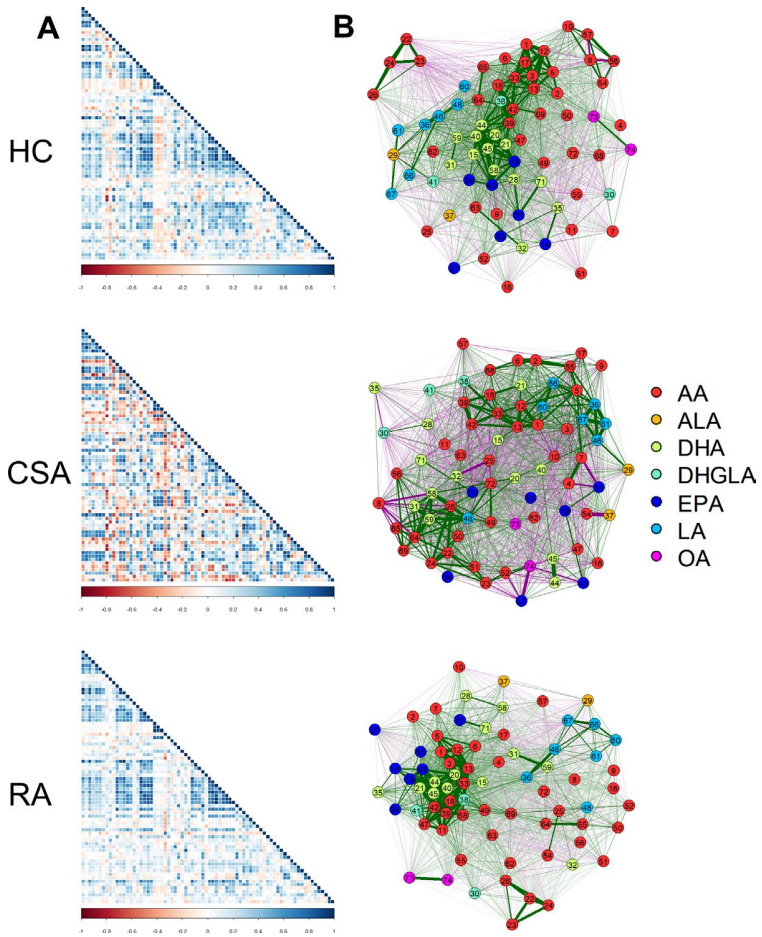


Figure 1. Analyses of correlations among oxylipins. **A**, Correlation matrices among oxylipins were plotted in correlograms by group (healthy controls [HC], individuals with clinically suspect arthralgia [CSA], and patients with rheumatoid arthritis [RA]). Tile colors represent the proportional strength of the correlation between each pair of oxylipins. **B**, Network analyses based on oxylipin concentrations were performed across study groups. Each node corresponds to a single oxylipin (numbered 1–74). Node colors represent each precursor, including arachidonic acid (AA), docosahexanoic acid (DHA), eicosapentanoic acid (EPA), linoleic acid (LA), dihomo-γ-linolenic acid (DHGLA), α-linolenic acid (ALA), and oleic acid (OA). Lines between nodes illustrate the strength (width) and type (positive [green] versus negative [red]) of the correlations between each pair of oxylipins. The relative position of the nodes parallels its correlation: nodes more closely correlated are located closer to each other. 1 = thromboxane B_2; 2 = prostaglandin $F_{2\alpha}$; 3 = prostaglandin E_2; 4 = prostaglandin D_2; 5 = thromboxane B_1; 6 = prostaglandin E_1; 7 = thromboxane B_3; 8 = prostaglandin E_3; 9 = 20-hydroxy-prostaglandin E_2; 10 = 13,14-dihydro-15-keto-prostaglandin E_2; 11 = tetranor 12-hydroxyeicosatetraenoic acid; 12 = 12-hydroxy-5,8,10-heptadecatrienoic acid; 13 = 11-hydroxyeicosatetraenoic acid; 14 = 11-hydroxyeicosa-pentaenoic acid; 15 = 13-hydroxy-docosahexaenoic acid; 16 = prostaglandin B_2; 17 = prostaglandin J_2; 18 = 9-hydroxyeicosatetraenoic acid; 19 = 9-hydroxyeicosa-pentaenoic acid; 20 = 16-hydroxy-docosahexaenoic acid; 21 = 20-hydroxy-docosahexaenoic acid; 22 = leukotriene B_4; 23 = 20-OH-leukotriene B_4; 24 = 12-oxo-leukotriene B_4; 25 = leukotriene E_4; 26 = 5-hydroxyeicosatetraenoic acid; 27 = 5- hydroxyeicosa-pentaenoic acid; 28 = 4-hydroxyoctadeca-4,7,10,12,16-pentaenoic acid; 29 = 9-hydroxy-10,12,15-octadecatrienoic acid; 30 = 5-hydroxyicosatrienoic acid; 31 = 7,17-dihydroxy-5,8,10,13,15,19-docosahexaenoic acid/resolvin D_5; 32 = 10,17-dihydroxydocosahexaenoic acid/neuroprotectin D_1; 33 = 15-hydroxyeicosatetraenoic acid; 34 = 15-hydroxy-5,8,11,13,17-eicosapentaenoic acid; 35 = 17-hydroxy-docosahexaenoic acid; 36 = 13-hydroxy-9,11-octadecadienoic acid; 37 = 13-hydroxy-10,12,15-octadecatrienoic acid; 38 = 15-hydroxyicosatrienoic acid ; 39 = 8-hydroxyeicosatetraenoic acid; 40 = 10-hydroxy-docosahexaenoic acid; 41 = 8-hydroxyicosatrienoic acid; 42 = 12-hydroxyeicosatetraenoic acid; 43 = 12-hydroxyeicosa-pentaenoic acid; 44 = 14-hydroxy-docosahexaenoic acid; 45 = 11-hydroxy-docosahexaenoic acid; 46 = 9-hydroxy-9,11-octadecadienoic acid; 47 = 12-oxoeicosa-5,8,10,14-tetraenoic acid; 48 = 9-oxooctadeca-10,12-dienoic acid; 49 = 20-hydroxyeicosatetraenoic acid; 50 = 18-hydroxyeicosatetraenoic acid; 51 = 17-hydroxyeicosatetraenoic acid; 52 = 16-hydroxyeicosatetraenoic acid; 53 = 18-hydroxyeicosa-pentaenoic acid; 54 = 5,6-epoxyeicosa-8,11,14-trienoic acid; 55 = 8,9-epoxyeicosa-8,11,14-trienoic acid; 56 = 11,12-epoxyeicosa-8,11,14-trienoic acid; 57 = 14,15-epoxyeicosa-8,11,14-trienoic acid; 58 = 16(17)-epoxy-4,7,10,13,19-docosapentaenoic acid; 59 = 19,20-dihydroxy-4,7,10,13,16-docosapentaenoic acid; 60 = 9,10-epoxyoctadec-12-enoic acid; 61 = 12,13-epoxyoctadec-12-enoic acid; 62 = 5,6-dihydroxyicosatrienoic acid; 63 = 8,9-dihydroxyicosatrienoic acid; 64 = 11,12-dihydroxyicosatrienoic acid; 65 = 14,15-dihydroxyicosatrienoic acid; 66 = 9,10-dihydroxy-12-octadecenoic acid; 67 = 12,13-dihydroxy-12-octadecenoic acid; 68 = arachidonic acid; 69 = adrenic acid; 70 = eicosapentanoic acid; 71 = docosahexanoic acid; 72 = 20-carboxy-arachidonic acid; 73 = 9-nitrooleate; 74 = 10-nitrooleate.

diHETE) were compared in the serum samples from patients with RA. Both metabolites showed similar fragmentation patterns by MS, but showed baseline separation under the LC conditions employed, thus excluding the possibility of a potential overlap in the chromatogram. Furthermore, a comparative analysis of both analytes allowed us to exclude the possibility of a significant platelet–neutrophil activation matrix during sample collection and preparation (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract), as 5S,12S-diHETE is considered a product of activated platelets and neutrophils.

A total of 14 oxylipins exhibited different serum levels across the groups (see Supplementary Table 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). Oxylipins exhibited different trajectories, with some showing peaking levels in patients with RA, whereas others showed peaking levels in individuals with CSA or healthy controls (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). The change was not always gradual across the groups, thus pointing to the existence of complex, individual patterns requiring a global approach. No associations with age, sex, body mass index, or traditional cardiovascular risk factors were observed in any of the study groups (all $P > 0.05$). Similarly, exclusion of patients who were receiving medications at the time of recruitment (n = 10) did not change these results. Oxylipin levels were not observed to parallel the extent of disease activity in RA patients (all $P > 0.05$). It must be noted that most of the patients had a status of high disease activity.

Correlograms showing the associations among the oxylipins (Figure 1A) provided evidence of more defined oxylipin groupings in healthy controls and patients with RA, in contrast to a more widespread pattern in individuals with CSA. Network graphs were generated to analyze the interactions among the oxylipins (Figure 1B). These analyses confirmed that the different clinical stages were hallmarked by distinct oxylipin profiles. Healthy controls exhibited a well-defined group of oxylipins that were closely associated with each other, comprising mostly AA-derived and DHA-derived oxylipins, the latter being in a central location. Overall, a relatively clear grouping pattern by precursors could be distinguished.

In patients with RA, a smaller group of oxylipins could be observed. These oxylipins were strongly correlated with each other, were located in an eccentric location, and included EPA-, DHA-, and AA-derived species, although a more diverse grouping was noted. Moreover, some nodes served as links between this group and smaller groups within the rest of the network.

Finally, the oxylipin network in individuals with CSA exhibited a fuzzy pattern, with less clear groupings, a more heterogeneous distribution of nodes, and a higher number of connections among them. Values for each centrality measure (degree, expected influence, betweenness, and closeness) supported these findings, since higher degree and closeness was observed in individuals with CSA, whereas specific compounds exhibited higher betweenness in patients with RA (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). These results confirm that quantitative and qualitative differences in oxylipin levels were present during the earliest phases of RA, even at the preclinical stage of arthralgia.

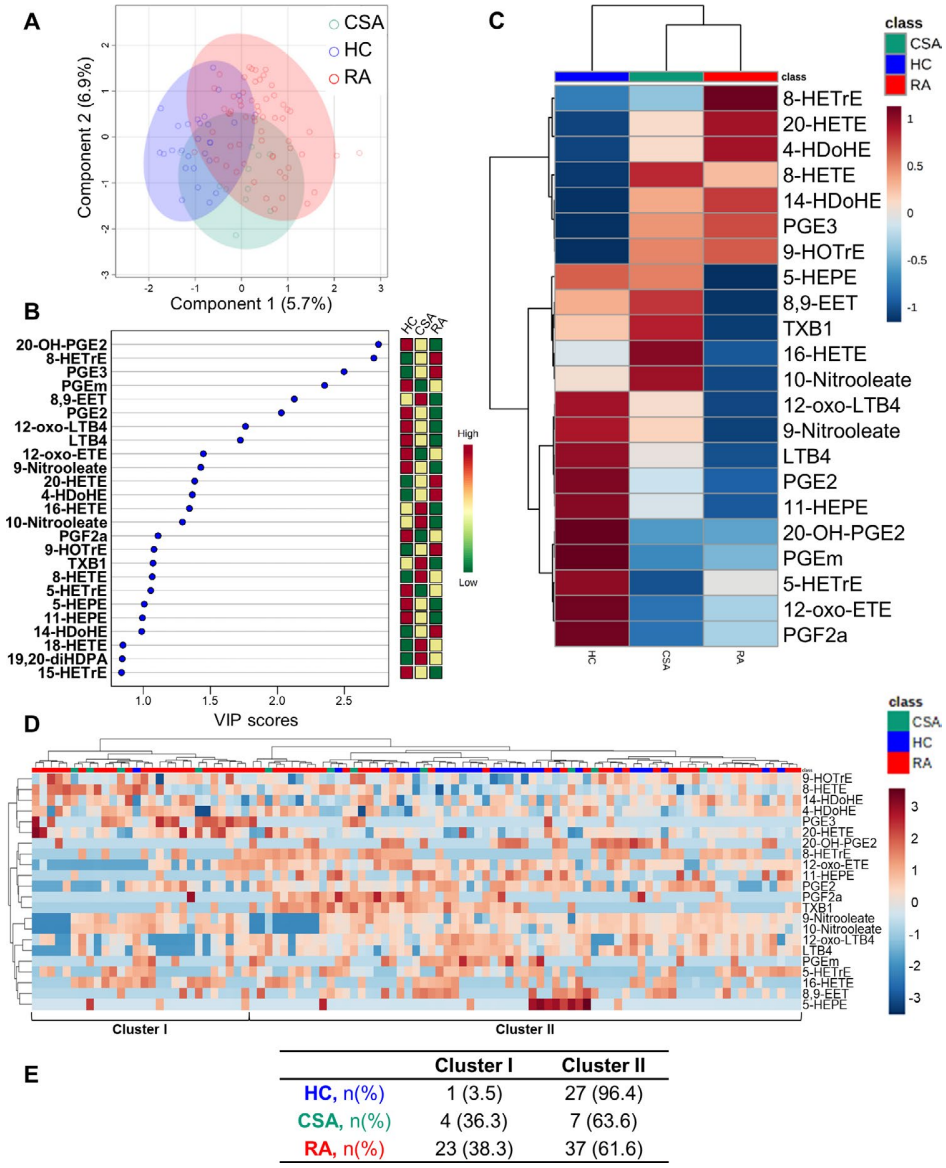
Identification of clinically relevant clusters by oxylipin profiling. Multivariate approaches were conducted to capture the global picture of oxylipin disturbances. A PLS-DA with all identified metabolites (12.1% of the total variance explained, $R^2 = 0.461$, 71.0% cross-validation accuracy, and empirical permutation $P = 5 \times 10^{-4}$) achieved a partial discrimination among the groups (Figure 2A), although a certain overlap existed. Interestingly, the PLS-DA findings in the CSA group revealed an intermediate oxylipin profile, falling between the profiles of the healthy control and RA groups. These findings indicate that oxylipin profiles may not be useful for accurate prediction of group classification, but do suggest that oxylipin-based group similarities may exist.

A total of 22 oxylipins had a VIP score of >1 (Figure 2B). This finding was used for heatmap visualization and cluster analysis (including all study subjects). A group-averaged heatmap (Figure 2C) confirmed the previous global differences in oxylipin levels, with an intermediate, "transitional" profile observed in individuals with CSA, although some CSA-specific disturbances were also noted. Interestingly, the RA and CSA groups showed a close similarity of profiles.

Results of the cluster analysis (Figure 2D) allowed the identification of 2 oxylipin clusters (cluster I and cluster II). Cluster usage differed among the groups ($P = 0.003$) (Figure 2E), thus confirming the differences observed in network analyses and the partial overlap observed in the PLS-DA model. These findings point to a potential clinical relevance of the oxylipin profiles.

Whereas the healthy controls mostly had groupings in cluster I, individuals with CSA and patients with RA were observed to have groupings in both cluster I and cluster II. Therefore, we analyzed whether the different oxylipin clusters were related to specific clinical features in RA. Patients with RA exhibiting cluster I had higher scores on visual analog scales (VAS) for patient global assessment ($P = 0.016$) and patient assessment of pain ($P = 0.003$) as compared to the scores from their cluster II counterparts, whereas no between-cluster differences in other RA features were noted (Table 1).

The clinical response to csDMARD treatment (low-dose glucocorticoids and methotrexate) was compared between clusters



	Cluster I	Cluster II
HC, n(%)	1 (3.5)	27 (96.4)
CSA, n(%)	4 (36.3)	7 (63.6)
RA, n(%)	23 (38.3)	37 (61.6)

Figure 2. Oxylipin profiling across study groups. **A**, Partial least-squares discriminant analysis (PLS-DA) was used to assess the discriminant capacity of all identified oxylipins, based on the amount of variance explained by the first 2 components. **B**, The top 25 oxylipins were ranked based on variable important projection (VIP) scores from the PLS-DA model (left). The heatmap indicates the concentration ranks across the different groups (right). **C**, A group-averaged heatmap was constructed based on the 22 oxylipins with a VIP score >1. **D**, A heatmap based on the 22 oxylipins with a VIP score >1 was used to identify 2 oxylipin clusters according to levels (ranging from high [shades of red] to low [shades of blue]). In **C** and **D**, the upper key indicates group classes. **E**, The number (%) of individuals in each oxylipin cluster is shown by study group. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract.

among the RA patients who were initially treatment-naive. Interestingly, at 6 months following csDMARD treatment, cluster I patients were less likely to achieve a EULAR good response compared to their cluster II counterparts (5 [31.2%] of 16 patients in cluster I versus 21 [70.0%] of 30 patients in cluster II classified as responders; $P = 0012$). Equivalent results were observed when the response at 6 months was assessed as achievement of DAS28 remission (4 of 16 patients in cluster I versus 20 of 30 patients in cluster II; $P = 0.007$) and SDAI remission (3 of 13 patients in cluster I versus 14 of 29 patients in cluster II; $P = 0.05$). Furthermore, differences in response between the 2 clusters were also seen at 12 months, according to the frequency of a EULAR good response (4 of 13 patients in cluster I versus 17 of 27 patients in cluster II; $P = 0.056$) and achievement of remission based on the DAS28 criteria (4 of 13 patients in cluster I versus 17 of 27 patients in cluster II; $P = 0.056$). Two patients in cluster I and 3 patients in cluster II were switched to a different csDMARD at 12 months. No patients were switched to a biologic DMARD.

Table 1. Clinical features of the rheumatoid arthritis patients at the time of recruitment, by oxylipin cluster*

	Cluster I (n = 23)	Cluster II (n = 37)
Clinical features		
Duration of symptoms, weeks	24.00 (11.50–37.00)	20.00 (8.50–30.00)
Morning stiffness, minutes	60.00 (15.00–90.00)	30.00 (12.50–120.00)
Tender joint count (of 28 joints)	9.00 (6.00–14.00)	8.00 (4.50–13.00)
Swollen joint count (of 28 joints)	6.00 (3.00–10.00)	5.00 (3.00–8.50)
ESR, mm/hour	19.00 (11.00–37.00)	20.00 (7.50–34.00)
CRP, mg/dl	0.80 (0.30–3.20)	0.60 (0.20–1.65)
Patient global assessment (0–100 VAS)	70.00 (60.00–90.00)	50.00 (40.00–72.50)†
Patient pain assessment (0–10 VAS)	8.00 (7.00–8.00)	6.00 (5.00–8.00)‡
DAS28 (scale 0–10)	5.66 (4.68–6.45)	5.05 (3.86–6.07)
SDAI (scale 0–86)	29.60 (23.12–39.37)	26.30 (18.15–35.05)
HAQ (scale 0–3)	1.50 (1.66–0.65)	1.10 (0.60–1.65)
Fatigue (0–10 VAS)	5.00 (0.00–8.00)	6.00 (1.50–8.00)
RF+, no. (%)	16 (69.5)	21 (56.7)
ACPA+, no. (%)	15 (65.2)	20 (54.0)
RF−/ACPA−, no. (%)	5 (21.7)	14 (37.8)
Traditional CV risk factors, no. (%)		
Hypertension	9 (39.1)	12 (32.4)
Diabetes	3 (13.0)	4 (10.8)
Dyslipidemia	8 (34.7)	11 (29.7)
Smoking	6 (26.0)	17 (45.9)
Treatments, no. (%)		
None§	18 (78.2)	32 (86.4)
Glucocorticoids	4 (17.3)	4 (10.8)
Methotrexate	2 (8.6)	3 (8.1)

* Except where indicated otherwise, values are the median (interquartile range). Differences were assessed by Mann-Whitney U or chi-square test (or Fisher's exact test, as appropriate), according to the distribution of the variables; other than those indicated by footnotes, all *P* values between cluster I and cluster II were nonsignificant at $P < 0.05$. ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analog scale; DAS28 = Disease Activity Score in 28 joints; SDAI = Simplified Disease Activity Index; HAQ = Health Assessment Questionnaire; RF+ = rheumatoid factor–positive; ACPA+ = anti–citrullinated protein antibody–positive; CV = cardiovascular.
† $P = 0.016$ versus cluster I.
‡ $P = 0.003$ versus cluster I.
§ Among the patients with early rheumatoid arthritis, 50 were recruited before being exposed to any treatment (designated treatment-naive).

All of these results suggest that oxylipin profiling can delineate disease clusters that could be differentiated across conditions, with CSA showing an intermediate profile. Moreover, these profiles are associated with clinical features and early treatment response in RA. Thus, our findings support the role of oxylipins in shaping the early RA clinical phenotype.

Identification of pathways with clinical relevance for arthritis using oxylipin signatures. We next aimed to identify whether oxylipins could delineate metabolic pathways related to disease progression or clinical heterogeneity at the onset of RA. First, an OPLS-DA method was carried out to evaluate whether oxylipins can discriminate between healthy controls and individuals with CSA (Figure 3A) (permutation empiric Q2 $P = 0.014$). Since a discrimination was achieved, a correlation analysis against the prespecified transition pattern of healthy control→CSA was performed to identify those oxylipins showing a linear increase in absolute levels in individuals with CSA relative to healthy controls (Figure 3B). A group of 8 oxylipins showing this pattern was identified, deriving from AA (4 oxylipins), LA (2 oxylipins), DHA (1 oxylipin), and DHGLA (1 oxylipin) precursors, and from the LOX (4 oxylipins), CYP (3 oxylipins), and COX (1 oxylipin) pathways (see Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract).

A similar discriminant analysis for the pattern of CSA→RA (permutation empiric Q2 $P = 0.054$) was carried out, resulting in identification of 5 species of significance in discriminating patients with early RA, derived from LA (2 oxylipins), EPA (1 oxylipin), AA (1 oxylipin), and DHGLA (1 oxylipin), with almost all (4 of the 5) originating from the LOX pathway (Figures 3C and D) (see also Supplementary Table 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract).

Furthermore, pathway enrichment analyses confirmed a higher impact of AA metabolism for the healthy control versus CSA comparison, whereas LA metabolism was ranked as the pathway with the highest impact for the CSA versus RA comparison (see Supplementary Tables 6 and 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). Interestingly, analysis of the healthy control→CSA→RA pattern identified a group of 18 species, mostly

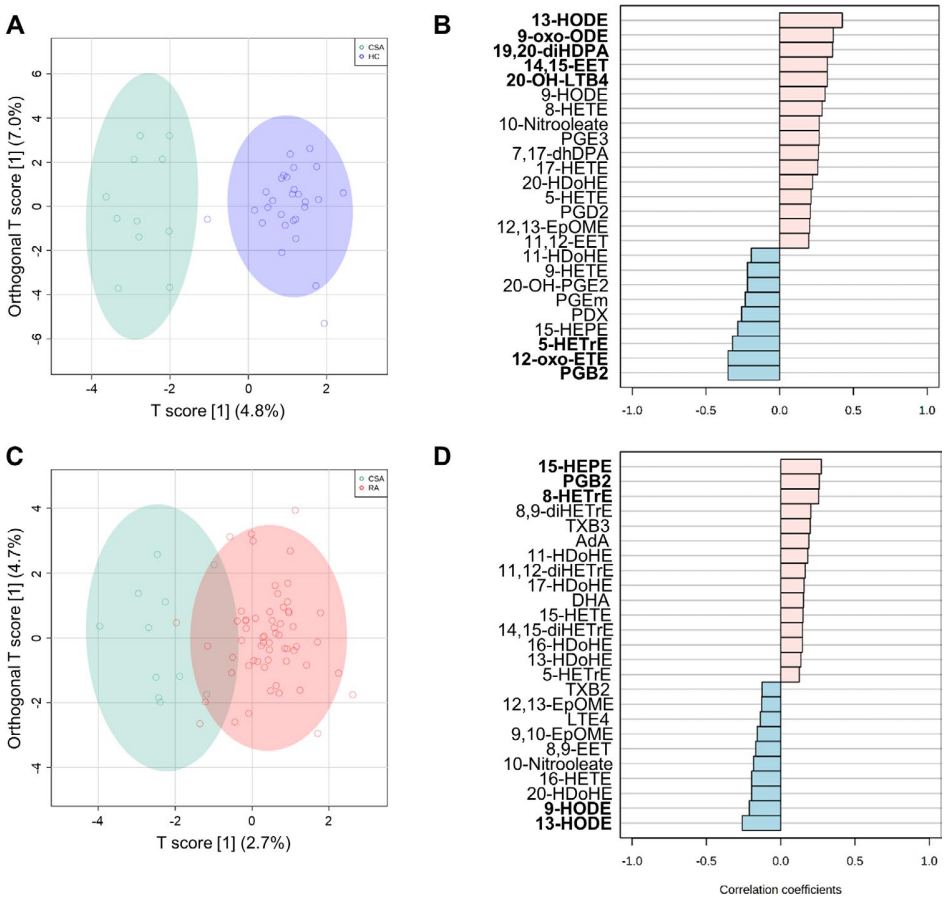


Figure 3. Oxylipin signatures associated with the earliest stages of RA. **A** and **C**, Orthogonal partial least-squares discriminant analysis models were constructed to evaluate the discriminant capacity of all oxylipins identified, showing comparisons between the healthy control and CSA groups (**A**) or the CSA and RA groups (**C**). **B** and **D**, Correlation patterns were determined for the top 25 oxylipins exhibiting the transition patterns healthy control→CSA (**B**) or CSA→RA (**D**), based on Spearman's rank correlation test as a distance measure. Oxylipins with statistically significant correlations are highlighted in boldface type. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract.

deriving from AA (12 species) and from the COX pathway (8 species) (see Supplementary Figures 4A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract).

In addition, we evaluated whether oxylipin expression patterns can reveal differences between the seropositive RA (rheumatoid factor [RF]–positive/anti–citrullinated protein antibody [ACPA]–positive) subset and the seronegative RA (RF-negative/ACPA-negative) subset. An OPLS-DA of the oxylipin profiles revealed a good discrimination between healthy controls and patients with seronegative RA (permutation empiric Q2 $P = 0.05$) (Figure 4A). Correlation analyses identified a group of 7 oxylipins that were differentially expressed (Figure 4B), mostly deriving from AA (3 oxylipins) and OA (2 oxylipins), with the COX pathway (3 oxylipins) and nitration pathway (2 oxylipins) being the most important (see Supplementary Table 8, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). An OPLS-DA of the oxylipin profiles also revealed that healthy controls could be discriminated from patients with seropositive RA (permutation Q2 empiric $P = 0.054$), although a partial overlap was noted (Figure 4C). In this case, a higher number of oxylipins (13 oxylipins) was found to be significant in the correlation analysis (Figure 4D), with AA (9 oxylipins) and 5-LOX (6 oxylipins) being the most common precursors and pathways retrieved, respectively (see Supplementary Table 9, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract). Accordingly, pathway enrichment analyses revealed a higher relevance of the AA metabolism pathway in the seropositive RA subset than in the seronegative RA subset (see Supplementary Tables 10 and 11, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract).

Thus, oxylipin profiling may help define the relevant pathways related to disease heterogeneity in patients with RA. We found that

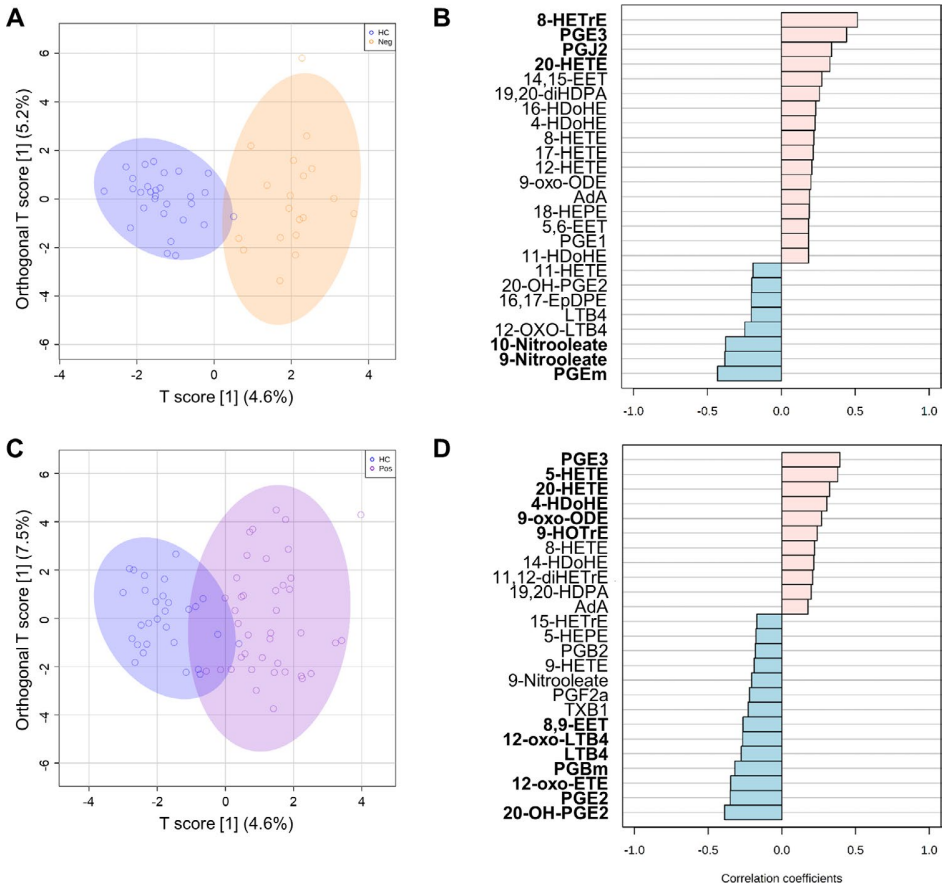


Figure 4. Oxylipin signatures associated with seropositivity in early RA. **A** and **C**, Orthagonal partial least-squares discriminant analysis models were constructed to evaluate the discriminant capacity of all oxylipins identified, showing comparisons between the healthy control and seronegative RA (rheumatoid factor–negative/anti–citrullinated protein antibody–negative) groups (**A**) or the healthy control and seropositive RA groups (**C**). **B** and **D**, Correlation patterns were determined for the top 25 oxylipins exhibiting the transition patterns healthy control→seronegative RA (**B**) or healthy control→seropositive RA (**D**), based on Spearman's rank correlation test as a distance measure. Oxylipins with statistically significant correlations are highlighted in boldface type. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract.

different species were related to the different disease stages underlying the early phase of RA. Similarly, the oxylipin profiles revealed divergent pathways between seronegative and seropositive RA.

DISCUSSION

A growing body of studies supports the use of lipidomics to gain a greater understanding of complex diseases and unveil pathogenic circuits linked to disease progression and new targets. The results herein support, for the first time, the occurrence of alterations in PUFA-derived oxylipins during the earliest phases of arthritis. Oxylipin profiling can identify subsets of patients with different clinical features and treatment responses, and may elucidate specific metabolic pathways that are differentially expressed between disease subsets. Based on our findings, oxylipin profiling could be considered an attractive source of biomarkers and potential targets for the early phase of inflammatory arthritis.

A remarkable finding from our study was the noticeable disturbance in oxylipin networks across the groups, which delineated distinct global patterns among the healthy controls, individuals with CSA, and patients with RA. Importantly, these patterns could not be attributed to class-specific (ω-3– or ω-6–derived) general impairments, but rather, might be attributed to distinct patterns for each individual compound. This notion has already been previously documented by our group (14) and by others (26) at the level of fatty acid precursors, thus underlining the need for complex, global approaches to account for the heterogeneity of these species and for a simultaneous assessment of the main pathways involved. Equivalent results were obtained in synovial fluid (27), thereby supporting this notion. Moreover, oxylipin levels were not related to demographic features or traditional cardiovascular risk factors in any of the study groups, consistent with previously reported findings. Furthermore, our findings in the cohort of treatment-naive patients with early RA

rule out the possibility of the influence of disease duration and treatment exposure, thus pointing to oxylipin networks as playing an active role in the disease, as opposed to being innocent bystanders.

The most interesting result from our study was the identification of alterations in oxylipins during the earliest stages of RA, including early RA as well as CSA. Preventive interventions at the CSA stage (28) are limited because of the poor characterization of the underlying pathogenic mechanisms. Although previous studies have identified some metabolites (29), the lipid compartment has been largely neglected. Our results support the role of oxylipins as potential factors in this setting. These findings are in line with studies showing lower ω-3–derived PUFA levels in individuals at high risk of developing RA (30), and demonstrating changes in gene expression related to lipid metabolism at this stage (31). Moreover, we have recently reported that reduced circulating DHA, EPA, and AA levels can be found in patients with RA at the time of disease onset (14), suggesting that lipid metabolism is potentially disturbed in RA. The results herein reinforce this hypothesis, and go further by identifying the actual species altered in the arthralgia stage downstream of the main PUFA precursors.

Individuals with CSA exhibited a genuine widespread oxylipin network with higher degree and betweenness. This may be the result of a transitional status in which a high number of metabolic interactions are operating, or also a consequence of group heterogeneity. CSA itself is a very heterogeneous stage of arthritis, with a number of possible clinical outcomes reported (32), from resolution to chronification. The fact that individuals with CSA were equally represented in the 2 oxylipin clusters identified and that there was overlap in the PLS-DA findings between the healthy control and RA groups support this idea. More importantly, absolute levels of oxylipins also revealed specific alterations in the CSA group that were not present in patients with RA or healthy controls. Furthermore, an important number of oxylipins exhibited decreased levels in individuals with CSA, with a certain degree of recovery observed in patients with RA, although other trajectories were also noted (see Supplementary Figure 2, [http://onlinelibrary.wiley.com/doi/10.1002/art.41537/abstract]), and therefore further research is warranted.

Correlation analyses comparing healthy controls to individuals with CSA or individuals with CSA to patients with RA led to insights into the potential changes occurring in the multistep development of RA. First, the analysis comparing healthy controls to individuals with CSA identified 8 differentially expressed species originating from 4 different precursors and major pathways, whereas the comparison of individuals with CSA to patients with RA yielded fewer differentially expressed species, mostly derived from LAs and the LOX pathway. Pathway analyses supported these differences. Overall, these findings suggest that distinct oxylipin alterations are associated with the different stages along the course of RA. A more diverse picture is evident in the earlier preclinical arthralgia stage, which is consistent with the different risk factors and mechanisms associated with the first events in the triggering of RA (33), while a more convergent effect is evident when comparing individuals with CSA to patients with RA, in which mechanisms are thought to be shared among disease subsets (34,35). Importantly, these discrepancies may be the result of the natural regulation of eicosanoid pathways, hallmarked by an initial production of proinflammatory species (mostly AA-derived) that prompt a class-switch to an anti-inflammatory, homeostatic response (36,37). However, antiinflammatory and proresolving functions are not equivalent (38), and it is plausible that a stronger shift toward proresolution may be needed to control the phase of CSA transitioning to RA.

Oxylipins showing a significant difference between individuals with CSA and patients with RA may be conceived as potential therapeutic targets to prevent disease progression. Actually, the LOX pathway has been recently described to be up-regulated at the synovial level in patients with RA in comparison to patients with osteoarthritis, while other enzymatic pathways remain unchanged (27). Due to the relevance of LOX-derived species in this setting, LOX inhibition may be an attractive therapeutic candidate. In fact, zileuton-mediated LOX inhibition has already been studied in patients with RA, although no clinical efficacy was demonstrated in those with established disease (39). However, in light of our results, further research on the effect of LOX inhibition on disease progression, rather than on management of the disease, must be considered. This is supported by studies in animal models in which treatment was administered very early (40). The fact that LOX expression is persistent along the disease course, remaining unchanged by conventional treatments (41), emphasizes the need to initiate earlier intervention. Alternatively, and due to the synergistic effects among the oxylipins, dual COX/LOX inhibitors may also be considered (42).

Importantly, differences were noted between the 2-step analyses (healthy controls versus individuals with CSA and individuals with CSA versus patients with RA) and the global analyses (healthy controls versus individuals with CSA versus patients with RA). Although the latter global approach mostly supported the role of AA and LOX as a whole, a compartmentalization was noted in the 2-step process, which aligns with the different oxylipin trajectories and allows for the identification of potential targets for tailored strategies, the main goal of personalized medicine (43), thus supporting the rationale of our analyses.

Our results shed new light on the potential role of oxylipins in early RA. Decreased EPA and DHA levels, which were linked to the altered levels of their derived species, were observed at the time of disease onset, thereby strengthening our previous findings (14). Cluster analyses revealed that 2 oxylipin profiles could be distinguished among RA patients. One of the clusters was predominantly present in healthy controls, which may be a more homeostatic profile, whereas the other cluster identified a group of RA patients with more severe clinical features, including higher VAS pain scores. This is aligned with previous evidence from clinical trials assessing fish oils and omega-3 supplements,

in which a protective effect on pain was demonstrated in patients with RA (44,45). Importantly, eicosanoid metabolites are known to activate nociceptive pathways (46), but the actual mediators are unknown. Moreover, this cluster was also associated with csDMARD treatment outcomes. Since early remission is an important aim in treat-to-target strategies (47,48), oxylipin networks should be further studied either for their role as biomarkers or for their actionable mechanisms, to facilitate clinical management.

Finally, oxylipin profiling led us to identify differences between seronegative and seropositive RA. Although previous metabolomics studies have shown distinct metabolomic signatures in seronegative RA patients, the exact compounds have not been elucidated (49). Our results confirm that whereas both subsets could be distinguished from controls based on oxylipin signatures, the precursors and pathways greatly differed between them. These results underscore the differences between these 2 RA subsets and add another layer of complexity by identifying oxylipin networks as potential contributors. Whereas AA metabolism clearly dominated the oxylipin signature in seropositive RA patients, less impact was observed in seronegative RA patients, as demonstrated in the correlation and pathway analyses. Importantly, OA-derived nitrooleates, which are strong antiinflammatory lipids (50), and DHA- and EPA-derived species, in addition to COX products, were associated with seronegative RA. Due to the complexity of the seronegative subset of the disease, these findings warrant further research into these pathways.

In summary, the results of this study demonstrate that serum oxylipin levels were altered during the earliest stages of RA, and specific alterations were found even at the arthralgia stage, which may reflect an altered PUFA metabolism. Oxylipin networks at the time of onset of RA were related to the clinical phenotype of the disease and can be predictive of the response to treatment. More importantly, oxylipin profiling helped to identify metabolic pathways relevant to the heterogeneity of the disease.

Our study has key strengths, such as the comprehensive recruitment of the study subjects as well as characterization of the subjects from a clinical point of view, the use of a robust targeted metabolomics platform, and the use of a robust and well-adjusted multiparametric statistical approach. Yet, this study has some limitations that must be noted, including the cross-sectional design, which did not allow for prospective follow-up of the subjects with CSA, and lack of information on other lipid species. Although our approach included cross-validation and permutation tests, an important limitation of our findings is the lack of an external validation cohort. Under these circumstances, it is unclear whether the results could be generalized beyond the patient cohort recruited.

The potential effect of diet may also be a factor that should be considered, although our previous results failed to demonstrate a significant effect of diet on PUFA levels (51,52). Moreover, the effect of the sample type (serum versus plasma) must be taken into account to ensure comparability with other studies.

Finally, pathway assignment was based on information from the existing literature. Whether a promiscuous oxylipin production by other enzymes or by nonenzymatic reactions exists in pathologic conditions cannot be totally ruled out. However, data on the involvement of nonenzymatic pathways were extracted from the broad literature, and it has not been proven that these pathways were the main mechanisms in the setting of RA; therefore, its relevance needs to be evaluated with caution. Nevertheless, the use of pathway enrichment analyses and a well-recognized genome library confers some degree of validation to our results.

Taken together, these findings should notably improve our understanding of the eicosanoid networks in very early RA and should pave the ground for future, larger, multicentric and prospective studies to address this topic. However, studies in larger external cohorts of patients are needed to ensure the generalizability of our findings. In addition, to assess underlying metabolic changes in the inflamed joint, future research should also focus on the synovial membrane, as has been the focus in studies by other groups (27), including analysis of paired serum and synovial samples from RA patients. Expanding the lipid spectra to be investigated in patients with RA and conducting a global integration of the lipid layer with the rest of the clinical and biologic data are key steps that should be implemented as part of the research agenda.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Rodríguez-Carrio had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Rodríguez-Carrio, Coras, Guma, Suárez.
Acquisition of data. Rodríguez-Carrio, Coras, Alperi-López, López, Ulloa, Ballina-García, Armando, Quehenberger, Guma, Suárez.
Analysis and interpretation of data. Rodríguez-Carrio, Coras, Guma, Suárez.

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Erratum

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In the article by FitzGerald et al in the June 2020 issue of *Arthritis & Rheumatology* (2020 American College of Rheumatology Guideline for the Management of Gout [pages 879–895]), under the section "High-fructose corn syrup" on page 889, the following sentence was incorrect: "In the National Health and Nutrition Examination Survey, artificially sweetened carbonated beverage consumption was associated with higher SU levels (101)." The correct sentence should be "In the National Health and Nutrition Examination Survey, sugar-sweetened carbonated beverage consumption was associated with higher SU levels (101)."

We regret the error.

4.5 Liquid biopsies to guide therapeutic decisions in rheumatoid arthritis

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Liquid biopsies to guide therapeutic decisions in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a systemic, immune-mediated inflammatory disease that has transitioned from a debilitating disease to a chronic, controllable disease. This has been possible due to the introduction of new treatment strategies like "treat-to-target," in which the clinician treats the patient aggressively enough to reach low disease activity or remission, and the introduction of new therapeutic agents, such as biological therapies, which can lead to the prevention of damage by early diagnosis and initiation of treatment. Attention is now being directed toward identifying the optimal treatment for each patient, one that will be the most efficient and have the least number of side effects. Much work has been done to find serologic and synovial biomarkers of response to various RA treatments. Proteomics, genomics and, in the past few years, metabolomics, have all been used in the quest of identifying these biomarkers. Blood-based liquid biopsies provide a minimally invasive alternative to synovial biopsies to identify cellular and molecular signatures that can be used to longitudinally monitor response and allow for personalized medicine approach. Liquid biopsies are comprised of cell-free DNA, immune circulating cells, and extracellular vesicles, and are being increasingly and successfully used in the field of oncology for diagnosis, progression, prognosis, and prediction of response to treatment. Recently, researchers have also begun investigating the usefulness of liquid biopsies in the field of rheumatology; in this review, we will focus on the potential of liquid biopsy blood samples as biomarkers of response to treatment in patients with RA. (Translational Research 2018; 201:1–12)

Abbreviations: cfDNA = circulating cell-free DNA; CIC = circulating immune cells; DMARDs = disease-modifying disease drugs; ExV = extracellular vesicles; FLS = fibroblast-like synoviocytes; IFN = interferon; IL = interleukin; NSAIDs = nonsteroidal anti-inflammatory drugs; RA = rheumatoid arthritis; TNF = tumor necrosis factor

INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory arthritis, affecting between 0.5% and 1% of the population worldwide.[1] Though it was originally considered benign, it has since been proven to be debilitative, and patients with RA have an increased death rate when compared to the general population.[2] Since 1898, when aspirin was introduced as a treatment for rheumatic fever, there have been great changes in RA treatment due to a better understanding of its pathogenesis.[3]

In the first half of the 20th century, a pyramidal model was used to treat RA: in the first stage, only symptomatic treatments were used; these included salicylates, from which nonsteroidal anti-inflammatory drugs (NSAIDs) are derived, and analgesics. This stage was associated with bed rest, splinting, physical therapy, heat therapy, and occupational therapy. The second stage saw the addition of disease-modifying disease drugs (DMARDs) such as gold salts, methotrexate, and penicillamine (Fig 1). At the time, this

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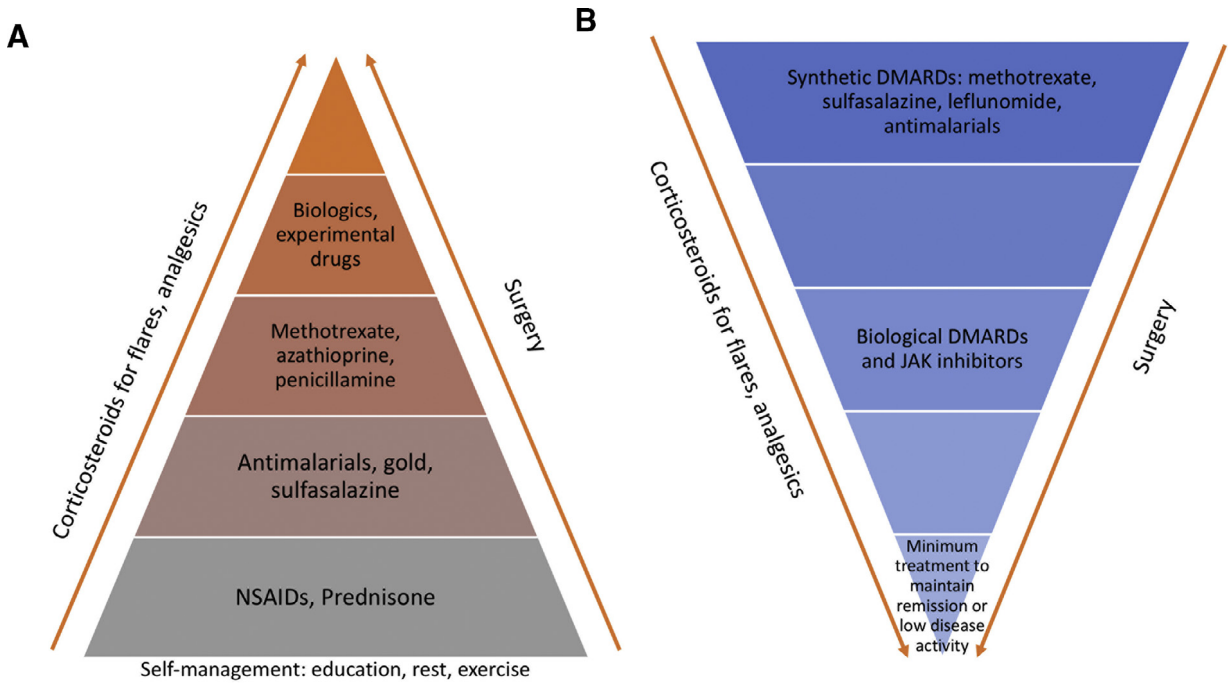

Fig 1. Historic pyramidal treatment approach and its current inverted aspect**.** (**A**) Previously, NSAIDs made up the base of RA treatment followed by DMARDs and experimental therapies. DMARDs were added later in the course of disease, as they were considered more toxic than NSAIDs or prednisone. Surgery and physical therapy were also important, and intra-articular corticosteroids were administered during flares. (**B**) Over time, there has been an inversion of the original pyramid, and DMARDs are now introduced early in the course of disease followed by biological therapies if control of the disease is not reached. The goal is to maintain a minimum disease activity, or remission if possible, on the least number of medications. Corticosteroids are still being used for arthritic flares, but surgery is rarely necessary because damage is generally prevented by early treatment. DMARDs, disease-modifying disease drugs; NSAIDs, nonsteroidal anti-inflammatory drugs (NSAIDs).

method of treatment was justified based on the assumption that DMARDs were too toxic for routine use.[4]

A few significant developments in the understanding of RA contributed to a shift in treatment toward DMARDs. First, RA became widely acknowledged as a seriously debilitating disease; Wolfe and Sharpe clearly demonstrated that patients with untreated RA developed severe joint damage due to increasing deformities, progression of erosions, and joint space narrowing.[8] Second, it was shown that NSAIDs were not benign, as had been previously thought, and had associated gastrointestinal complications.[9-12] Often, patients who were hospitalized or died due to NSAID complications tended to be older, had longer disease duration, higher disability scores, and higher incidence of comorbidities.[10] Third, there was accumulating evidence that DMARDs were efficient in controlling symptoms, interfering with joint destruction, and decreasing inflammation in RA. Consequently, DMARDs were shown to provide better control in disease progression, improvement in pain symptoms, and less disability than other treatments.[13,14] There was also growing evidence that DMARDs are no more toxic than NSAIDs, making them an increasingly practical option for RA treatment.[13] Finally, a number of new therapeutic agents were made available to the medical community, including pharmacologic and biologic-(b)DMARDs.[15-17]

These developments have allowed for new approaches to RA treatment (Fig 1). DMARDs and bDMARDs are being used earlier in treatment and are continued throughout the course of the disease with the aim of maintaining the patient's level of disability close to normal.[5,6] Since the initial approval of tumor necrosis factor (TNF)-neutralizing therapies, other biologic agents have been approved, such as the anti-T-cell (and/or dendritic cell) therapy, the B-cell-depleting antibody, an interleukin (IL)-6 receptor antagonist, and drugs targeting IL-23 and IL-17 cytokines. In addition, a janus-kinase inhibitor has recently been added into the RA therapeutic armamentarium.[7] Because choosing and initiating the right treatment earlier in the course of disease could help the patient reach remission, considerable efforts are being made to develop the tools necessary to employ a ''personalized'' medicine approach. This approach can potentially allow physicians to match patients with the most appropriate therapy option for their disease subtype.

Identification of biomarkers for diagnosis, prognosis, monitoring of therapeutic responses, and selection of patients for specific DMARDs therapies is urgently required, and is therefore an area of intensive research. For this purpose, researchers have used synovial tissue, obtained from the joints by arthroscopy, surgery or ultrasound-guided biopsy, synovial fluid obtained during surgery, or more commonly by arthrocentesis, and peripheral blood. Obtaining synovial tissue or synovial fluid is inherently more risky to the patient than taking a blood sample. In addition, taking further repetitive synovial tissue of fluid samples during treatment is difficult. More easily accessible samples, such as blood, are preferred for researchers, as they involve less risk for patients and less hassle for scientists. Blood samples allow quantification of different types of biomarkers using proteomics, lipidomics, transcriptomics, or RNA sequencing, and the processing of samples for these methods is relatively easy.[8] Thus, blood-based liquid biopsies provide a minimally invasive alternative to standard synovial biopsies in order to identify cellular and molecular signatures that can be used to longitudinally monitor response and allow for a personalized medicine approach. In this review, we will focus on the potential of using the blood-based liquid biopsy to study biomarkers of response to treatment in patients with RA.

SYNOVIAL BIOPSY

Liquid biopsies intend to get information about the main tissues involved in a specific disease pathogenesis.[9] Therefore, we will start with a brief review of the advances in synovial biopsy studies for a personalized medicine approach in RA.[10] Yet, although the synovial tissue is the main target of inflammation in RA,[11] recent work has shown that systemic autoimmunity precedes synovial inflammation in RA, with several altered lymphoid cell subsets in lymph node biopsies obtained during earlier phases of RA.[12-14] Furthermore, animal models have suggested that lymph node changes may precede inflammation in the synovial tissue.[15]

The rheumatoid synovium presents with a hyperplasic intimal lining layer and with accumulation of recruited inflammatory cells in the sublining.[16] An increasing number of studies have focused on studying synovial tissue in order to increase knowledge of disease pathogenesis, explore early diagnosis and biomarkers for disease activity, and evaluate response to treatment.[17-22] Advances in synovial biopsies such as arthroscopy and ultrasound-guided biopsy have increased accessibility of synovial samples, allowing for such research.[23] While a number of studies focus on identifying features of the inflamed synovium to allow for early diagnosis of the disease, a greater number are focused on finding markers that can predict response to different types of treatment, allowing the clinician to orient his practice towards personalized medicine. Many of the biomarkers discovered for this purpose are cellular markers identified by immunohistochemistry, synovial cytokines, chemokines, and gene-expression profiles.

Lymphocyte aggregates. Though these are only found in about 30% of RA patients and there is no correlation with disease activity,[24,25] one study found that the number of lymphocyte aggregates was predictive of clinical response to infliximab.[26]

Lymphocytes. It has long been shown that RA presents with an increase in the number of cells infiltrating the synovium[27,28]. Cells in the synovium are studied by immunostaining and cell counting. Numerous studies have described a decrease in the number of T cells after treatment with prednisolone (CD4, CD5, and CD38 plasma cells),[29] conventional synthetic disease-modifying drugs like methotrexate (CD3, CD8, and CD38 plasma cells)[30] and bDMARDs like infliximab ($CD3^+$ cells[31] and $CD22^+$ cells[32]) and rituximab (B cells, T cells, and macrophages).[33-36]

Macrophages. Macrophages (CD68+ cells) appear to be the most convincing cellular biomarker of response to treatment. This has been demonstrated by several studies and is independent of treatment type.[36,37] Studies have shown that prednisolone,[29] methotrexate,[38] leflunomide,[39] and infliximab[32,40] produced a decrease in the number of macrophages in the synovial sublining. Anakinra,[41] rituximab, and methotrexate reduced the number of macrophages in the intimal layer, and gold salts did so in all synovial layers. Importantly, the decrease in the number of CD68+ cells has been shown to correlate with clinical improvement.[36,37] For this reason, CD68+ has been proposed as a biomarker of response for new therapies.[36]

Fibroblast-like synoviocytes. Fibroblast-like synoviocytes (FLS) are synovial resident cells that express characteristic markers on their surface including intercellular adhesion molecule 1, podoplanin, vascular adhesion molecule 1, and CD55; these markers are used for FLS identification via immunohistochemistry.[42] Few papers have studied FLS in the synovial membrane, their phenotyping, and their response after treatment. However, a recent paper gave comprehensive epigenomic descriptions of RA FLS,[43] and another has identified different FLS subpopulations by RNA-seq analysis,[44] with different synovial localization and gene expression signatures. Still,

further experimentation is needed to correlate these new findings with patient stratification and therapeutic response.

Cytokines and chemokines. Several synovial proinflammatory cytokines have been studied as potential biomarkers, including TNF α, IL-6, and IL-1β. Although the pretreatment levels of these cytokines correlates with disease activity,[45] there is scarce and contradictory information on their value as possible biomarkers of response to targeted therapy.[30,39,46] Two groups found that RA patients who responded to TNF inhibitors had higher TNF-α levels in the synovial tissue (determined by immunostaining).[40,45] One group found that synovial phosphorylated STAT is decreased after treatment with Tofacitinib, indicating that cytokine-related signaling could also potentially be used to predict response to treatment.[47] Finally, patients who responded to infliximab treatment also showed decreased monocyte chemotactic protein 1 and IL-8.[32]

Gene expression studies. In a small study of 18 RA patients with active disease, 1 group found differences in the gene expression profiles between responders vs nonresponders to infliximab. Interestingly, the genes related to inflammation were upregulated in responders.[48] Another group found that RA patients who had good response to adalimumab also had different gene expression profiles than nonresponders, showing an overexpression of genes related to regulation of immunity and cell division. Patients that were poor responders to treatment had higher baseline levels of IL-18, IL-18 receptor accessory, IL-7 receptor α chain, and proliferation marker Ki-67.[49] Promising results were obtained by Dennis[50] et al that described 4 groups of synovial subtypes using array analysis: myeloid, lymphoid, low inflammation, and fibroid subtypes. They showed that patients with an overexpression of the myeloid signature and TNF-related genes have a better response to TNF inhibitor; these results were confirmed in another independent study.[51] Parting from the 4 subtypes of synovial signature, the authors went further to find possible serum biomarkers that could be representative of different synovial subtypes. They found that a combination of 2 biomarkers could predict response to tocilizumab and adalimumab: soluble intercellular adhesion molecule and C-X-C motif chemokine 13.

LIQUID BIOPSY

While several authors continued their work to identify biomarkers that predict response to therapy in synovial tissue, other groups have considered the possibility that liquid biopsies, which are already being used in oncology, could lead to more predictive biomarkers. The liquid biopsy is an emerging and promising detection tool that is both noninvasive and convenient.[52] Liquid biopsies can be used to analyze tissue-derived information, including circulating cell-free DNA (cfDNA), circulating tissue-derived cells, and exosomes in the blood or other bodily fluids.[53] Several studies suggest that liquid biopsies are useful to guide therapeutic decisions in cancer,[54-56] although less information is available on synovial-derived information and the role of liquid biopsies in autoimmune diseases including RA (Fig 2).

Cell-free DNA. cfDNA was first detected in human blood in 1948[57]; in 1977, it was discovered that patients with cancer had higher levels of cfDNA[58] than the general population. Moreover, patients with metastasis had higher levels of cfDNA than patients without, and these levels decreased with therapy.[58] Thus, cfDNA detection is mainly used in cancer patients for disease monitoring and response to treatment. Of interest, high levels of cfDNA were also found in several other conditions such as trauma, infections, exercise, transplantation, cerebral infarctions, and inflammation.[59] It is thought that cfDNA is released from cells by at least 2 nonexclusive mechanisms: a passive one and an active one.

The passive mechanism is the process by which apoptotic and necrotic cells release nuclear and mitochondrial DNA when they die. In physiological conditions, macrophages eliminate the fragments that result from cell death, but in cancer and inflammation there are alterations to this process, increasing the amount cfDNA in the plasma.[60,61] The active mechanism involves the spontaneous release of DNA fragments by cells in circulation, which might also increase due to inflammation, and has been observed in cultured cells of different origins.[62-64] However, the exact mechanism of cfDNA release remains elusive. Other sources could include release of DNA to form neutrophil extracellular traps[65]; this could be another source of cfDNA in plasma of RA patients, as RA-derived neutrophils are more prone to NETosis.[66,67]

In contrast to the growing amount of information on cfDNA in cancer, literature on this topic in autoimmune disease is scarce. However, the literature that can be found is promising, and cfDNA appears to be a good candidate as a biomarker of early diagnosis of RA,[68] for disease monitoring [69] and prediction of response to treatment.[70] In the 1970s, it was shown that cfDNA levels were higher in the plasma of RA patients with less than 10 years of disease evolution in comparison to healthy controls, especially

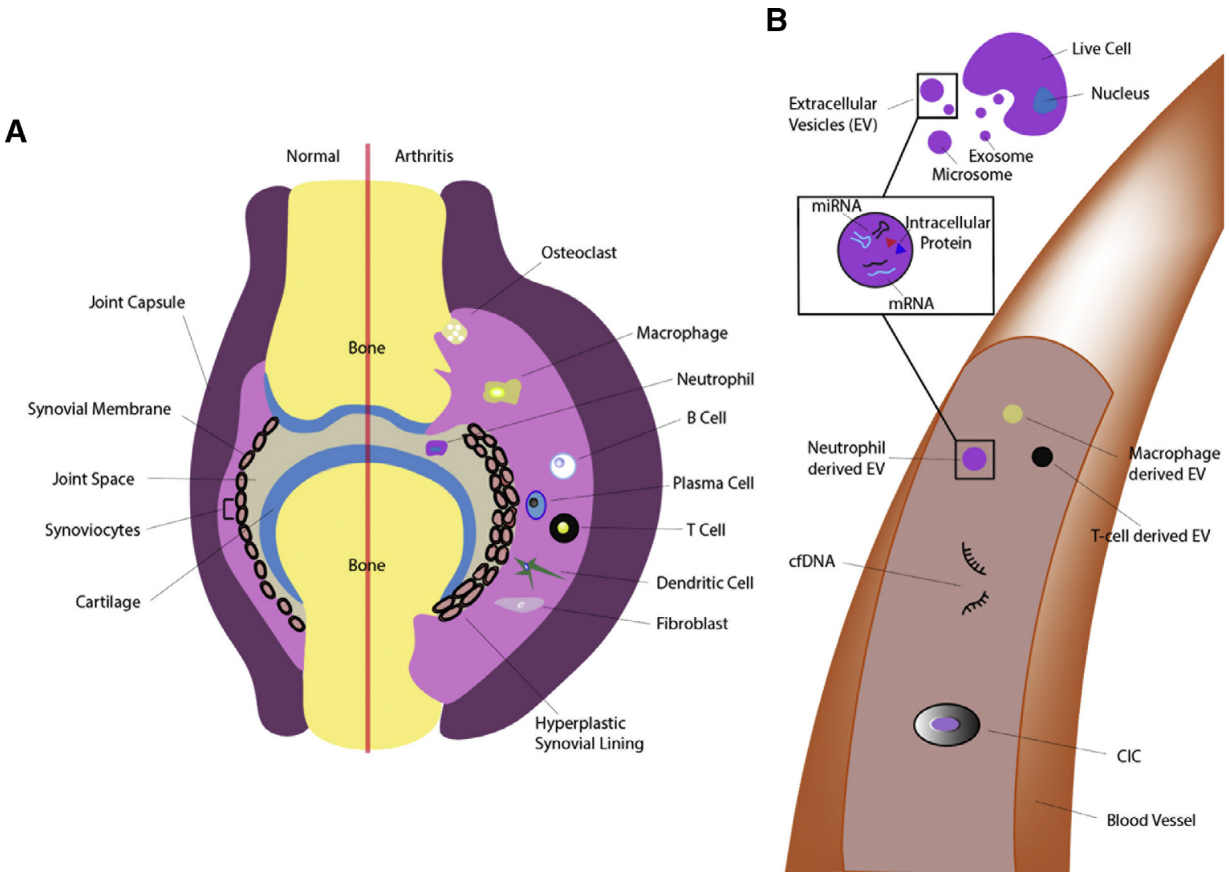

Fig 2. Cells involved in RA pathogenesis and components of liquid biopsies (**A**) This figure shows a synovial joint which is normal on the left and affected by rheumatoid arthritis on the right. The left side depicts a normal synovial lining while the right side displays a hyperplastic synovial lining. At the same time, an erosion is also present on the affected side of the joint. The diseased side of the joint shows an accumulation of various types of cell that are involved in the pathogenesis of RA including fibroblast-like synoviocytes, B and T cells, macrophages, neutrophils, plasma cells, and dendritic cells. (**B**) Circulating free DNA (cfDNA), circulating immune cells (CIC), and exosomes containing for instance microRNA can be used to analyze synovium-derived information. RA, rheumatoid arthritis.

in seronegative patients with more severe disease.[71,72] Other studies corroborated this finding.[69,70,73] Of note, cfDNA levels were in a high range, similar to ones reported in neoplasia.[74] Studies also evaluated the effect of different therapies on cfDNA levels. CfDNA concentrations in plasma and serum did not vary with the administration of classical DMARDs such as methotrexate, hydroxychloroquine, or sulfasalazine, or in-between doses of TNF inhibitors. However, after the infusion of anti-TNF monoclonal antibody, cfDNA significantly changed in 70% of the patients, suggesting that DNA release could be a TNF-related mechanism.[73]

Another study evaluated not only nuclear but also mitochondrial DNA, both cell-free and cell-surface bound (csb).[69] In this study, only csb mitochondrial DNA was found to be a good discriminant between RA patients and controls. The authors showed that cfDNA concentrations were higher in RA patients with higher disease activity (disease activity score >5.1) in comparison to patients with lower disease activity and healthy controls. This study also included a group of 14 patients that received rituximab and achieved European League Against Rheumatism moderate and/or good response at week 24. They found that csb mitochondrial DNA levels tended to be lower in patients treated with rituximab in comparison to the group that received only methotrexate.[69] Another study[70] suggests that levels of cfDNA might decrease after long periods of low disease activity or with disease remission. Of note, an increase in cfDNA was observed 8 weeks post-treatment initiation, which correlated with the improvement of the Simple Clinical Disease Activity Index, which could be related to the apoptosis in the synovium observed after anti-TNF therapy. Finally, Kawane et al observed that DNAse II knockout mice developed symmetrical polyarthritis, and suggested

that cfDNA[75] might be directly implicated in the etiology of RA. Unfortunately, these studies did not explore whether a lack of DNase II in mice is associated with higher levels of cfDNA.

Recently, 2 groups noticed that epigenomic (and thus cell-type specific) information can be detected by examining cfDNA.[76,77] These studies are based on the rationale that cfDNA contains an epigenomic footprint that can be used to identify the origin of a dying tissue. Genomic DNA is fragmented by enzymes that prefer to cut in unexposed sections that are not protected by nucleosomes. Therefore, the cfDNA fragments released are those protected by nucleosomes and transcription factors. Based on this rationale, 1 group examined the nucleosome positioning of cfDNA.[76] By identifying known nucleosome positioning profiles, these patterns can be used to identify cell type of origin. Another group investigated whether or not cfDNA methylation could also be a marker of tissue type.[77] The team profiled a number of tissues using a methylation array to find tissue-specific CpGs (5′—C—phosphate—G—3′). They then examined cfDNA in the blood and identified tissue-specific patterns of methylation. Future studies will be needed to find out whether or not this information can be helpful to match cfDNA to specific synovial tissue.

Circulating immune cell profiling in RA. RA is a systemic autoimmune disease, and previous work has suggested that lymph node changes may precede inflammation in the synovial tissue.[12-14] Importantly, several studies have also shown expanded peripheral cell subsets that are recruited into the RA joint,[78] suggesting that peripheral blood is a good source for immune cell profiling. On the other hand, there is no evidence that resident synoviocytes (FLS and macrophages-like synoviocytes) can migrate outside the joint and circulate peripherally, despite the FLS well-known ability to invade and migrate in vitro,[79,80] and animal models suggesting the possibility of circulating FLS.[81] In this review, we will therefore consider circulating immune cells as liquid biopsies, as they might originate in other pathogenic organs in RA patients, such as the lymph nodes, and have the potential to give information on the patient's disease.

In RA, the cells that drive immune responses are also druggable targets (B and T cell, macrophage, FLS), making them promising biomarker candidates. Moreover, immune cells might not only provide information about the *status quo* of an immune response, but also about their histories (for example, through measurement of the frequency and specificity of memory T cells) and potentially about their futures (for example, by measuring cellular responses to *in vitro* stimulation). Several assays, including flow cytometry, mass cytometry, gene expression profiling by RNA-sequencing, and cellular function assays, have been shown to be helpful in the analysis of circulating cells in RA patients.[82]

Gene expression of immune circulating cells. Gene expression profiling is used to analyze expression of thousands of genes in order to create a global picture of biological functions for the population of interest.[83] Gene expression profiling is at the forefront of personalized medicine, especially in oncology.[84] Because inflammation is a biological process that involves several cell types and produces many inflammatory factors, changes in gene expression could provide information about activated signatures or activated cell populations at different stages of RA.

Although many groups have studied gene expression in immune cells as biomarkers, the results have not been very encouraging. The only exception is the prediction of response to rituximab.[85,86] Two groups showed that a type-I interferon (IFN) signature in whole blood was a predicting biomarker of response. Good responders had a low or absent IFN response activity at baseline, whereas nonresponders displayed an activated type-I IFN system before the start of treatment. A set of 305 IFN type-I genes were validated as a predictor of non-response to rituximab. These results suggest that RA patients with a high IFN signature represent a different pathogenic subset of patients. Another study[87] observed an increase in IFN response activity in patients who responded to rituximab therapy, while the IFN signature in nonresponders stayed the same. In responders, the IFN signature score returned to baseline values 6 months after the start of treatment. Future studies are necessary to determine whether this phenomenon shows that an increase in IFN response activity during rituximab treatment is necessary for good response or is merely a noncausal correlation.

Phenotypic characterization of immune cell subsets. Cellular immunophenotyping by flow cytometry (which can detect as many as 20 parameters per cell) and mass cytometry (which can measure more than 50 parameters per cell) allows for analysis of cell surface markers and analysis of cytoplasmic and nuclear proteins, including cytokines, cell proliferation markers, cell signaling responses, and transcription factors. In RA, several studies have analyzed immune cell subsets in peripheral blood and showed expansion of several subsets including IL-17-producing helper T cells relative to regulatory T cells,[88,89] terminally differentiated, cytotoxic $CD4^{+}CD28^{-}$ T cells,[90] $CD4^{+}$ T cells that were positive for activation-induced surface markers,[91] immature cells characterized as CD45RB (bright)CD45RA(+)CD62L(−), a large population that

coexpresses CD45RA and CD45RO,[92] and recently, PD-1^{hi}CXCR5^{-} 'peripheral helper' T (T_{PH}) cells that also express factors enabling B-cell help, including IL-21, C-X-C motif chemokine 13, ICOS, and MAF.[78]

Still, only a small number of studies have reported an association of immune cell populations to response to certain treatment. For instance, high frequency of CD4^{+}CD28^{-} T cells was associated with a poor clinical response prior to initiation of anti-TNF therapy.[93] An interesting dichotomy was described with cCD27^{+} memory B cells. While low numbers of cCD27^{+} memory B cells and CD27^{hi}CD38^{hi} preplasma circulating cells at baseline were associated with a better clinical response to rituximab,[94,95] a high frequency of CD27^{+} memory B cells was correlated with a favorable response to TNF inhibition.[96] Finally, the analysis of CD19^{+} cells in the peripheral blood from RA patients who are treated with B-cell therapy (rituximab) is the only routine application of flow cytometry in current rheumatology practice and is used to monitor B cells. Some studies suggest that rituximab dosage should be adjusted based on the number of residual circulating B cells after rituximab therapy.[97]

In vitro assays of immune circulating cells. Several in vitro assays can be used to assess immune cell function (cell proliferation, changes in gene or protein expression, or phagocytosis). Still, functional cellular assays are not present as routine clinical laboratory assays. A possible immunophenotyping approach would be to trigger polyclonal rheumatic populations with different ligands and then measure the secretion of several analytes to assess induced innate or adaptive immune responses.[98] This approach could potentially identify dominant pathways in individual patients and provide guidance for therapeutic decisions in RA.

Extracellular vesicles. Extracellular vesicles (ExV) are particles released from cells that are found in all types of bodily fluids. They can originate in endosomes (exosomes) or bud directly from the cell membrane (microvesicles). In this review, we will be referring to them as ExV. These vesicles contain or expose a variety of molecules on their surfaces, ranging from lipids, RNA, proteins, and DNA; these molecules can be useful for exosome identification. It was shown that ExV have different content amongst them, depending on their parental cells, and have a role in cell to cell communication. They can cause changes in gene transcription, including genes involved in inflammation[99,100] and cell proliferation.[101-103] In cancer, they can contribute to disease progression by participating in the crosstalk between tumor cells and stromal cells and can aid in metastasis by carrying behavior-changing information to cells located at a distance from the primary tumor.[104]

Disease conditions change the specific content and membrane composition of ExV. Thus, these EVs might serve as biomarkers of disease. In RA, the ExV originate from a multitude of cells that infiltrate the synovial membrane (Fig 2). It was demonstrated that in patients with RA, the total number of ExV was significantly higher in comparison to healthy controls.[105] In addition to qualitative differences, quantitative differences were also found between RA patients and healthy controls. One study found 10 proteins specific for ExV in RA patients. Interestingly, more than half of the proteins were citrullinated.[106]

Changes in ExV content (lipids, proteins, DNA, and miRNA) could be used as potential biomarkers for different stages of disease.[107-110] MiRNAs are fragments of single stranded, noncoding RNA, comprised of 19−25 nucleotides.[111] There is strong accumulating evidence for the potential use of miRNAs as biomarkers of diagnosis, disease progression, and prediction of response to treatment, resulting in increased exploration of miRNAs in various diseases. Although most of these studies do not specify the location of miRNAs, it was shown that most plasma miRNA is contained in ExVs.[112]

In rheumatology, an increasing number of studies are being done on both the role of miRNA in the pathogenesis of RA and on its potential as a biomarker. Several studies have shown that the expression of miRNAs is altered in immune and resident cells that are involved in the pathogenesis of RA, and contributes to the typical features of RA[113]; these studies have also shown that miRNAs could potentially be therapeutic predictive biomarkers. For instance, higher pretreatment levels of miR-16 in the sera of treatment naïve patients with early RA was predictive of better improvement in disease activity during the first 3 months of follow-up, after therapy was initiated with conventional DMARDs.[114] In a study by Castro-Villega et al,[115] investigators measured plasma miRNA in 95 patients with active RA before treatment and 6 months after initiation of a combined therapy with anti-TNF and/or DMARDs. They found 75 miRNAs that were upregulated and 9 that were downregulated after treatment. In general, the miRNAs were downregulated in patients that were nonresponders to treatment vs responders. Furthermore, a negative correlation was found between the changes in the expression levels of almost all of the validated miRNAs and the changes in various clinical and inflammatory parameters. Receiver operating characteristic analyses demonstrated that high levels of hsa-miR-23-3p and hsa-miR-223-3p might act as predictors of response to therapy.

Another study,[116] a placebo-controlled, double-blind, prospective study of patients with early RA found an association between whole blood miR-22

Fig 3. Serum metabolites correlation with synovial CD3E and CD19. Nineteen active seropositive RA patients on concomitant methotrexate were studied. One of the involved joints was a knee or a wrist appropriate for arthroscopy. A Bruker Avance 700 MHz spectrometer was used to acquire NMR spectra of serum samples. Gene expression in synovial tissue obtained by arthroscopy was analyzed by real-time PCR. Correlation of serum metabolites for CD3E and CD19 using linear regression, controlling for both age and gender was conducted. Overview of the metabolites identified by NMR was organized by metabolic pathway. Metabolites that were negatively correlated with CD3E and CD19 are shown in red. NMR, nuclear magnetic resonance; RA, rheumatoid arthritis; TMA, trimethylamine; TMAO, trimethylamine N-oxide; DMA, NN-dimethylamine; THF, tetrahydrofolate; IMP, inosine monophosphate.

and miR-886.3p, and response to adalimumab. Specifically, the probability for achieving a good response to adalimumab (in combination with methotrexate) increased from 65% to 95% in patients with high expression of miR-886.3p and low expression of miR-22. Cuppen et al[117] tried to reproduce the results of some of the previous studies using a discovery cohort and then a validation cohort. Higher circulating values of miR23a were found to be predictive of response to TNF inhibitors and showed similar results to a study by Krintel et al.[116] However, some of their results were contradictory to previous reports. A recently published study by Sode et al,[118] on a cohort of patients with early RA treated with a combination of adalimumab, methotrexate, and glucocorticoids found that a higher pretreatment level of miR-27a-3p and a decrease in miR-27a-3p level during the first 3 months was associated with ACR/European League Against Rheumatism Boolean remission at 12 months. Finally, one study[119] showed that miR-125b was higher in RA patients in comparison to healthy controls and that its baseline level was negatively correlated with disease activity. The levels increased after 3 months of therapy and the higher baseline cellular levels were predictive of a better response to DMARDs. Higher levels of miR-125 b were also found to be predictive of better response to rituximab.[120]

CONCLUSIONS AND FUTURE DIRECTIONS

Despite a great number of studies in the fields of epigenetics, genetics, and proteomics, no biomarker for prediction of response to RA treatment has yet been identified. Reasons for this include a lack of standardized protocols for the identification of different candidate biomarkers and, sadly, the lack of validation of existing results. Over the last few years investigators have started to look into other options for biomarker exploration including metabolomics, synovial biopsies, and liquid biopsies; these have potential to guide therapeutic decisions in RA. Although few metabolomic studies have been conducted so far, our group showed that a serum metabolomic profile identified by H-NMR and UPLC−MS/MS could be predictive of response to rituximab.[121] Of note, our unpublished results also suggest that serum metabolomics profiles might predict synovial gene expression, (Fig 3) suggesting that metabolomics may be a promising tool for predicting specific pathogenic pathways in the inflamed synovium of RA patients. With the advent of ultrasound-guided synovial biopsy, examination of pathobiological specimens from the target organ, the arthritic synovium, might become a part of standard clinical care and help with management decisions, much like other branches of medicine including gastroenterology, dermatology and nephrology. Finally, the practice of liquid biopsies has revolutionized the care of patients with cancer. In RA, though liquid biopsies are still in their infancy, technical advances and some encouraging clinical studies suggest that they hold great promise in the near future.

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5 Global Summary of Results

The objective of this thesis was to evaluate the potential use of circulating metabolites profile for the characterization of inflammatory arthritis pathogenesis and the discovery of biomarkers of disease and response to treatment.

5.1 The role of metabolomics in identifying biomarkers of synovial pathology

In our first study we evaluated the role of metabolites in the characterization of synovial pathology. We first analyzed the expression of synovial inflammatory markers and found that IL-6, MMP1, and MMP3 highly correlated among themselves but inversely correlated with TNF-α, which interestingly, highly correlated with CD3E. MMP1 and MMP3 also inversely correlated with another cluster that included IL-1β and IL-8. In addition, there was a big cluster comprised of B and plasma cell markers, and growth factors, including SDF1, APRIL, CD138, CD19, CD79A, IgG and IgM heavy chains, and IgKappa.

We also characterized the blood metabolites. Most of the metabolites were downregulated compared to reference values suggesting that these metabolites might be consumed by the inflamed synovium due to an increase in its metabolic demand. A few metabolites were upregulated compared to reference values, including glycolytic metabolites such as lactate and pyruvate. Interestingly TMA was also elevated in our RA patients. In addition, 3-hydroxybutyrate, a ketone body, and select amino acids, such as leucine, threonine, tyrosine, and aspartate were upregulated in active RA patients. We also analyzed whether blood metabolites could be clustered into group according to their biological functions and chemical classification. As expected, the group of metabolites that were elevated in patients, namely lactate, methylmalonate, xanthine and 3-hydroxybutyrate, were inversely correlated with most of the metabolites.

We next examined the relation between circulating metabolites and the synovial expression of inflammatory markers. We observed significant clustering structures in the data. Interestingly, the clusters of synovial cytokines almost corresponded to the clusters observed within synovial cytokines, suggesting that cytokine clusters in the synovial tissue have a similar metabolite signature in blood. The most striking difference was seen in the cluster comprising SDF1, APRIL, CD138, CD19, CD79A, and IgG and IgM heavy chain and IgKappa, which was split into two groups. One metabolite signature correlated with CD19, CD79A and IgG heavy chain, markers of B cells; and the other metabolite signature correlated with SDF1, APRIL, CD138 and IgM heavy chain, markers related to plasma cell biology. Of interest, BLyS had a different metabolite profile than the rest of plasma cell biomarkers.

We then determined the most highly correlated or anti-correlated serum metabolites for each synovial cytokine, using linear regression, and controlling for both age and gender. The synovial cytokines TNF-α and CD3E negatively correlated with several metabolites in serum. TNF-α negatively correlated with formate, propyleneglycol, glutamine, alanine, glucose, serine and choline among others. CD3E negatively correlated with phenylalanine, methionine, formate, glutamine, serine, choline, lysine and glucose, among others.

We then explored whether or not one or more metabolites in serum could discriminate between high or low levels of synovial cytokine gene expression. We used stepwise discriminant function analyses to discriminate TNF-α or CD3E levels. Multivariate and cross-validation using "leave-one-out" classification method was used for these calculations. We defined high or low cytokine levels according to their synovial cytokine gene expression mean. For TNF-α discriminant analysis, three metabolites, namely glutamine, TMA, and dimethylsulfone were sufficient to correctly classify 94.7% of TNF-α levels. The canonical correlation of 0.821 and Wilks' lambda of 0.326 were found when these three variables were used, with high significance ($P < 0.001$;). For CD3E discriminant analysis, two metabolites, namely carnitine and methionine were sufficient to correctly classify 89.6% of CD3E levels. The canonical correlation of 0.765 and Wilks' lambda of 0.414 were found when these three variables were used, with high significance ($P < 0.001$).

5.2 The role of metabolomics in identifying disease pathogenesis biomarkers

Our hypothesis implies that circulating metabolites would be biomarkers of the underlying mediators of synovial inflammation and we were able to explore this in our studies in RA and PsA patients.

We observed a different metabolomic profile in patients with inflammatory arthritis compared to controls, suggesting a dis-regulation of several metabolic pathways may be marker of disease in these patients.

In one of our studies we characterized the oxylipin profile in elderly patients with RA and PMR compared to control. Eighteen controls (average age: 75.38, SD 6.04) and 64 patients (average age: 74.97, SD 7.03) were recruited for this study. Of these patients, 44 were diagnosed with RA and 20 with PMR. Sixteen EORA patients were seropositive (RF and/or CCP positive: EORA+), while 28 were seronegative (RF and CCP were negative: EORA). EORA patients were younger compared to EORA+ patients.

At baseline, EORA patients had a mean DAS28CRP of 5.77 (SD 1.02) and a mean HAQ of 1.7 (SD 0.8). One hundred percent of PMR patients reported scapular pain and 90% reported pelvic pain. As expected, arthritic subjects, that is, those with EORA and PMR, presented with higher erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and platelet count, and lower hemoglobin (Hb) than controls with similar comorbidities. Within the arthritic population, PMR patients presented with more pelvic pain and less peripheral arthritis than RA patients. We did not observe any significant difference in ESR, CRP, and platelet counts between PMR and EORA patients. Comorbidities including diabetes mellitus (DM), high blood pressure (HBP), and dyslipidemia (DL) were also similar in both arthritic populations and controls. About 29.2% of the patients were on daily NSAIDs. At baseline (first visit in rheumatology clinics), none of them were on steroids, or on any synthetic or biological DMARDs.

Eighty-five oxylipins, which are derived from AA, eicosapentaenoic acid (EPA), LA, DGLA, ALA, and docosahexaenoic acid (DHA), were identified by reverse-phase LC/MS in our cohort. Forty of the detected oxylipins are in general considered proinflammatory and 45 anti-inflammatory species. Approximately 80% of oxylipin mass in serum was derived from n-6 PUFA, particularly from AA (31%–63%), LA (18%–56%), and DGLA (1%–3%). n-3

PUFA-derived species were from DHA (8%–24%), EPA (1%–15%), and ALA (0.1%–0.5%).

Cluster analysis of the oxylipins in the control population showed that anti-inflammatory oxylipins were grouped in two clusters, one comprised of DHA and EPA derivatives, and the other comprised of LA and ALA species. Of interest, the first cluster negatively correlated with the second cluster. AA-derived species were distributed in two clusters, one comprised of most of the COX proinflammatory-derived oxylipin species, and the second comprised of most of the LOX proinflammatory-derived oxylipins.

Similar oxylipin species were identified in the arthritic population. Approximately 80% of oxylipin mass in serum was also derived from n-6 PUFA, particularly from AA (24%–60%), LA (19%–52%), and DGLA (0.7%–5%), and n-3 PUFAs-derived species were from DHA (7%–22%), EPA (1%–10%), and ALA (0.1%–1.4%). LA-derived oxylipins tended to be higher ($p = .12$), while EPA ($p = .12$) and DHA ($p = .17$) derived oxylipins tended to be lower in the arthritic patients compared to controls. DHA-, EPA-, and ALA-derived anti-inflammatory oxylipins were also grouped in two different clusters as in the control group, yet, they positively correlated in the arthritic patients.

We then analyzed whether oxylipins could differentiate between arthritis and control subjects. Several species were significantly different between controls and patients after adjusting for age, sex, BMI, and NSAIDs. A sparse PLS-DA showed that tetranor-PGFM, 15-HEPE, 6R-LXA4, and LXB4 were the oxylipins that better distinguished between controls and arthritis patients. Of interest, most of the n-3 EPA and some DHA-derived oxylipin species were lower in patients compared to the control population. Conversely, the n-3/n-6 PUFA ratio was lower in arthritis patients compared to control ($p = .01$).

We then explored whether the oxylipins differentially expressed between controls and patients or between EORA and PMR were derived preferentially via either COX, LOX, or CYP pathways. We did not observe a clear preference for COX-, LOX-, or CYP-derived oxylipins in arthritis patients. To determine whether the changes observed in EORA and PMR were related to changes in activity of the desaturase enzymes, the upstream step, we estimated their activity by studying the ratio between PUFA-derived oxylipins. The EPA/ALA ratio tended to be lower in EORA (17.27, $p = .1$) and PMR (18.21, $p = .25$) patients than in controls (25.72). The ratio of DHA/ALA (EORA 49.01, PMR 55.6, controls 64.5) tended to be lower in EORA than PMR patients and controls. DGLA/LA and AA/DGLA ratios were not different between the three studied groups.

We finally explored whether oxylipins in serum could discriminate between EORA and PMR. Two oxylipins, namely 4-HDoHE and 8,15-di dihydroxy-eicosatrienoic acid, were sufficient to correctly classify 70.3% of these patients. The canonical correlation of 0.821 and Wilks' lambda of 0.829 were found when these two variables were used, with high significance (p = .003). The top two candidates as biomarkers for differentiating PMR from RA, 4-HDoHE and 8,15-diHETE, had an area under the receiver operating characteristic curve (AUROC) of 0.76. Yet, the disease phenotypes were unable to be fully distinguished using all the oxylipins as per a PLS-DA.

We next compared the oxylipin profile in a cohort of patients with early RA, patients with clinically suspect arthralgia (CSA) and controls. We found an alteration of the oxylipin profiles across all the groups, which delineated distinct global patterns among the healthy controls, individuals with CSA, and patients with RA. Serum oxylipins were measured in 60 patients with early RA (including 50 treatment-naive patients), 11 individuals with CSA, and 28 matched healthy controls. A total of 74 oxylipins derived from arachidonic acid (AA) (n = 39), docosahexanoic acid (DHA) (n = 13), linoleic acid (LA) (n = 7), eicosapentanoic acid (EPA) (n = 7), dihomo-gamma-linolenic acid (DHGLA) (n = 3), alpha-linolenic acid (ALA) (n = 2), and oleic acid (OA) (n = 2) were identified by LC-MS/MS in the serum from patients with early RA. In subgroup analyses, a good correlation was observed between the oxylipin levels measured in the serum and oxylipin levels measured in the plasma (median Spearman's R = 0.870, range 0.750–1.000).

A total of 14 oxylipins exhibited different serum levels across the groups. Oxylipins exhibited different trajectories, with some showing peaking levels in patients with RA, whereas others showed peaking levels in individuals with CSA or healthy controls. The change was not always gradual across the groups, thus pointing to the existence of complex, individual patterns requiring a global approach. No associations with age, sex, body mass index, or traditional cardiovascular risk factors were observed in any of the study groups (all $P > 0.05$). Similarly, exclusion of patients who were receiving medications at the time of recruitment (n = 10) did not change these results. Oxylipin levels were not observed to parallel the extent of disease activity in RA patients (all $P > 0.05$). It must be noted that most of the patients had a status of high disease activity.

Correlograms showing the associations among the oxylipins provided evidence of more defined oxylipin groupings in healthy controls and patients with RA, in contrast to a more

widespread pattern in individuals with CSA. Network graphs were generated to analyze the interactions among the oxylipins. These analyses confirmed that the different clinical stages were hallmarked by distinct oxylipin profiles. Healthy controls exhibited a well-defined group of oxylipins that were closely associated with each other, comprising mostly AA-derived and DHA-derived oxylipins, the latter being in a central location. Overall, a relatively clear grouping pattern by precursors could be distinguished.

In patients with RA, a smaller group of oxylipins could be observed. These oxylipins were strongly correlated with each other, were located in an eccentric location, and included EPA-, DHA-, and AA-derived species, although a more diverse grouping was noted. Moreover, some nodes served as links between this group and smaller groups within the rest of the network.

Finally, the oxylipin network in individuals with CSA exhibited a fuzzy pattern, with less clear groupings, a more heterogeneous distribution of nodes, and a higher number of connections among them. Values for each centrality measure (degree, expected influence, betweenness, and closeness) supported these findings, since higher degree and closeness was observed in individuals with CSA, whereas specific compounds exhibited higher betweenness in patients with RA. These results confirm that quantitative and qualitative differences in oxylipin levels were present during the earliest phases of RA, even at the preclinical stage of arthralgia.

Multivariate approaches were conducted to capture the global picture of oxylipin disturbances. A PLS-DA with all identified metabolites (12.1% of the total variance explained, R2 = 0.461, 71.0% cross-validation accuracy, and empirical permutation P = 5 × 104) achieved a partial discrimination among the groups, although a certain overlap existed. Interestingly, the PLS-DA findings in the CSA group revealed an intermediate oxylipin profile, falling between the profiles of the healthy control and RA groups. These findings indicate that oxylipin profiles may not be useful for accurate prediction of group classification, but do suggest that oxylipin-based group similarities may exist.

A total of 22 oxylipins had a VIP score of >1. This finding was used for heatmap visualization and cluster analysis (including all study subjects). A group-averaged heatmap confirmed the previous global differences in oxylipin levels, with an intermediate, “transitional” profile observed in individuals with CSA, although some CSA-specific disturbances were also noted. Interestingly, the RA and CSA groups showed a close similarity of profiles.

Results of the cluster analysis allowed the identification of 2 oxylipin clusters (cluster I and cluster II). Cluster usage differed among the groups (P = 0.003) thus confirming the differences observed in network analyses and the partial overlap observed in the PLS-DA model. Whereas the healthy controls mostly had groupings in cluster I, individuals with CSA and patients with RA were observed to have groupings in both cluster I and cluster II. Therefore, we analyzed whether the different oxylipin clusters were related to specific clinical features in RA. Patients with RA exhibiting cluster I had higher scores on visual analog scales (VAS) for patient global assessment (P = 0.016) and patient assessment of pain (P = 0.003) as compared to the scores from their cluster II counterparts, whereas no between-cluster differences in other RA features were noted.

We next aimed to identify whether oxylipins could delineate metabolic pathways related to disease progression or clinical heterogeneity at the onset of RA. First, an OPLS-DA method was carried out to evaluate whether oxylipins can discriminate between healthy controls and individuals with CSA (permutation empiric Q2 P = 0.014). Since a discrimination was achieved, a correlation analysis against the prespecified transition pattern of healthy control→CSA was performed to identify those oxylipins showing a linear increase in absolute levels in individuals with CSA relative to healthy controls. A group of 8 oxylipins showing this pattern was identified, deriving from AA (4 oxylipins), LA (2 oxylipins), DHA (1 oxylipin), and DHGLA (1 oxylipin) precursors, and from the LOX (4 oxylipins), CYP (3 oxylipins), and COX (1 oxylipin) pathways.

A similar discriminant analysis for the pattern of CSA→RA (permutation empiric Q2 P = 0.054) was carried out, resulting in identification of 5 species of significance in discriminating patients with early RA, derived from LA (2 oxylipins), EPA (1 oxylipin), AA (1 oxylipin), and DHGLA (1 oxylipin), with almost all (4 of the 5) originating from the LOX pathway.

Furthermore, pathway enrichment analyses confirmed a higher impact of AA metabolism for the healthy control versus CSA comparison, whereas LA metabolism was ranked as the pathway with the highest impact for the CSA versus RA comparison. Interestingly, analysis of the healthy control→CSA→RA pattern identified a group of 18 species, mostly deriving from AA (12 species) and from the COX pathway (8 species).

In addition, we evaluated whether oxylipin expression patterns can reveal differences

between the seropositive RA (rheumatoid factor [RF]–positive/anti–citrullinated protein antibody [ACPA]–positive) subset and the seronegative RA (RF-negative/ACPA-negative) subset. An OPLS-DA of the oxylipin profiles revealed a good discrimination between healthy controls and patients with seronegative RA (permutation empiric Q2 P = 0.05). Correlation analyses identified a group of 7 oxylipins that were differentially expressed, mostly deriving from AA (3 oxylipins) and OA (2 oxylipins), with the COX pathway (3 oxylipins) and nitration pathway (2 oxylipins) being the most important. An OPLS-DA of the oxylipin profiles also revealed that healthy controls could be discriminated from patients with seropositive RA (permutation Q2 empiric P = 0.054), although a partial overlap was noted. In this case, a higher number of oxylipins (13 oxylipins) was found to be significant in the correlation analysis, with AA (9 oxylipins) and 5-LOX (6 oxylipins) being the most common precursors and pathways retrieved, respectively. Accordingly, pathway enrichment analyses revealed a higher relevance of the AA metabolism pathway in the seropositive RA subset than in the seronegative RA subset.

5.3 The role of metabolomics in identifying biomarkers of disease activity

Assessment of disease activity in inflammatory arthritis is critical for the management of the disease, allowing adequate treatment choice to avoid progression and disability. Our studies in RA patients included patients with high disease activity, which did not allow the study of metabolites in relation with degree of inflammation in these patients. However, our studies in PsA patients allowed the identification of biomarkers of disease activity.

We started our study exploring the metabolism of choline and its derivates in PsA. Of the 38 PsA patients included in this study, 58% were male and the mean age was 50.8 years ± 10.9 (range 23 to 75 years). The mean number of tender joints and swollen joints were 1.9 ± 3 (range 0 to 10) and 2 ± 3.2 (range 0 to 10) respectively. The average DAS28-CRP score was 2.74 ± 1.29 (range 1–5.18). Twenty-seven patients (71%) had active skin disease, with an average BSA of 4.73 ± 14.5. Eight patients had enthesitis. 65% were receiving biological therapy (46% of them in association with a synthetic disease modifying drug (DMARD), mostly methotrexate. 14% received sDMARD as monotherapy, and 21% received no systemic treatment.

We analyzed choline metabolites clustering and found that choline strongly correlated with its metabolite TMA, but did not correlate with TMAO. Betaine and carnitine did not correlate with any of the other metabolites. Concentration of betaine and carnitine were higher in males than in females (p = 0.01 for betaine and p = 0.06 for carnitine). Betaine was lower in patients older than 51 years old (p = 0.02). There were no significant differences in the concentrations of choline and its metabolites between patient with normal BMI versus overweight and obese patients adjusted by age and gender.

We then analyzed whether choline metabolites differentiate between high and low skin or joint disease activity scores. TMAO was the only metabolite that significantly increased in patients with higher skin and joint scores. Interestingly, TMAO correlated with parameters of both skin (BSA) and joint disease activity (TJC, SJC, CDAI, SDAI, DAS28CRP). TMAO also correlated with HAQ and fatigue. Of note, two metabolites, choline and TMA, negatively correlated with fatigue.

Both studies on RA patients included patients with high disease activity, which did not allow the study of metabolites in relation with degree of inflammation in these patients.

5.4 The role of metabolomics in identifying biomarkers of response to treatment

In our first work on the relation between circulating metabolites and synovial inflammatory markers we found that the combination of only two or three metabolites identified in serum by NMR could discriminate between high or low levels of synovial TNF- α (decreased dimethysulfone, glutamine and trimethylamine in high TNF levels) and CD3E (carnitine and methionine) gene expression. The patients included in the ARISE study received treatment with rituximab after a prior failure of at at least one TNF inhibitor, and no correlations were found between synovial TNF-α expression and response [252]. However, our group previously identified serum metabolites related to response in these patients: 7 polar metabolites (phenylalanine, 2-hydroxyvalerate, succinate, choline, glycine, acetoacetate and tyrosine) [253].

At 3 months posttreatment, 39 out of the 44 patients diagnosed with EORA were on glucocorticoids (GCs, prednisolone) at an average dose of 5.51 mg/d (SD 3.27) (average dose

after diagnosis was 8.83 mg/d, SD 3.21), and 32 received DMARD medication (methotrexate, leflunomide, or hydroxychloroquine). Thirty-three patients had a good and 8 patients a moderate response to treatment, according to the EULAR response criteria. At 3 months, all patients diagnosed with PMR were on GC at an average dose of 8 mg/d (SD 4.21) (average dose after diagnosis was 9.3 mg/d, SD 2.4). All PMR patients responded to treatment. Prednisolone dose was significantly higher (p = .01) in PMR patients compared to EORA patients at 3 months.

Seventy-two oxylipins were detected at 3 months posttreatment. We compared the oxylipins at baseline and 3 months posttreatment. The n-3 EPA- and some of the DHA-derived anti-inflammatory oxylipins that were downregulated in EORA patients at baseline significantly increased in EORA compared to PMR patients. Some of the EPA- and DHA- derived anti-inflammatory oxylipins also increased in PMR, although the total n-3 mass in PMR patients was similar between baseline and 3 months. Both pro- and anti-inflammatory DGLA-derived oxylipins increased posttreatment in both disease groups. However, ALA-derived oxylipins decreased only in EORA (p = .035). Interestingly, the desaturase activity changed significantly posttreatment in both EORA and PMR patients. The EPA/ALA and DHA/ALA, that tended to be lower in EORA compared to PMR and controls, increased in EORA patients (EPA/ALA: from 17.27 [11.21] at baseline to 27.9 [20.2], p = .007; DHA/ALA: from 49.01 [21.15] at baseline to 66.91 [30.31], p < .001). The ratios DHA/ALA and DGLA/LA significantly increased in both diseases posttreatment.

In the YORA cohort we found correlations of certain oxylipins with response to DMARDs in patients with early RA. The clinical response to csDMARD treatment (low-dose glucocorticoids and methotrexate) was compared between clusters among the RA patients who were initially treatment-naive. Interestingly, at 6 months following csDMARD treatment, cluster I patients were less likely to achieve a EULAR good response compared to their cluster II counterparts (5 [31.2%] of 16 patients in cluster I versus 21 [70.0%] of 30 patients in cluster II classified as responders; P = 0012). Equivalent results were observed when the response at 6 months was assessed as achievement of DAS28 remission (4 of 16 patients in cluster I versus 20 of 30 patients in cluster II; P = 0.007) and SDAI remission (3 of 13 patients in cluster I versus 14 of 29 patients in cluster II; P = 0.05). Furthermore, differences in response between the 2 clusters were also seen at 12 months, according to the frequency of a EULAR good response (4 of 13 patients in cluster I versus 17 of 27 patients in cluster II; P = 0.056)

and achievement of remission based on the DAS28 criteria (4 of 13 patients in cluster I versus 17 of 27 patients in cluster II; P = 0.056). Two patients in cluster I and 3 patients in cluster II were switched to a different csDMARD at 12 months. No patients were switched to a biologic DMARD.

6 Global Summary of Discussion

Metabolomics is a tool recently incorporated in the study of disease pathogenesis and exploration of biomarkers. This thesis employed metabolomics to demonstrate a potential relation between circulating metabolites and synovial inflammation, as evaluated by gene expression of inflammatory markers. At the same time, this work explored the use of lipidomics, a branch of metabolomics, to characterize the oxylipin profile in patients with elderly onset RA, as well as very early stages of RA.

6.1 The role of metabolomics in identifying biomarkers of synovial pathology

RA is a chronic inflammatory disease where the main pathological processes occur in the synovial membrane. In spite of the availability of a relative high number of DMARDs, and of our increased understanding of the disease pathogenesis, around 30% of patients do not present a good therapeutic response. Predictive biomarkers would be helpful in identifying the optimum therapy to reach disease remission or, at the very least, low disease activity. Although during recent years sampling synovial tissue has become fundamental in the process of understanding the mechanisms underlying inflammation [47, 48, 45, 46], it is still an invasive technique not widely available for clinicians. However, a minimally invasive sample, such as blood, is more attractive for biomarker development. This is especially useful for patients with mostly small joint involvement, from which very small amounts of tissue can be obtained even by biopsy. In our first study study, we explored, for the first time, the relation between serum metabolomics profiles and synovial marker gene expression [254].

The study of synovial pathology has flourished in the last years, due to a few groups who have managed to set up synovial biopsy programs with minimally invasive procedures. As mentioned in the Introduction, these groups have characterized synovial pathotypes which

correlate with disease severity and response to disease modifying drugs. In terms of metabolomics, the majority of the studies performed so far have focused on serum and for diagnostic purposes, but none have attempted to predict synovial pathology until recently. In a recent paper published in *Nature Communications*, Gomez et al found an upregulation of pro-resolving mediators, including 15R-LXA4 and MCTR2 (13R-cysteinylglycinyl, 14S-hydroxy-4Z, 7Z, 9E, 11E, 13R, 14S, 16Z, 19Z - docosahexaenoic acid), in peripheral blood from patients with a pauci-immune-fibroid pathotype (characterized by histologic analysis of synovial tissue form biopsies). Moreover, an upregulation of pro-inflammatory and immunosuppressive mediators including PGD2 and TxB2 (stable metabolite of TxA2) was also found in plasma of these patients [255]. The same paper also found different lipid profiles were associated to response to DMARDs: DHA-derived RvD4 (4S,5R,17S-trihydroxy-6E,8E,10Z,13Z,15E,19Z-docosahexaenoic acid) and 10S, 17S-diHDPA (10S,17S-dihydroxy-7Z,11E,13Z,15E,19Z-docosapentaenoic acid) were the most important mediators in predicting treatment responsiveness, with 15R-LXA4 (5S,6R,15R-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid), 5S, 12S-diHETE (5S,12S-dihydroxy-6E,8Z,10E,14Z-eicosatetraenoic acid), 4S, 14S-diHDHA (4S,14S-dihydroxy-5E,7Z,10Z,12E, 16Z, 19Z-docosahexaenoic acid) and n-3 DPA-derived Maresin 1 (MaR1n-3 DPA) (7R,14S-dihydroxy-8E,10E,12Z,16Z,19Z-docosapentaenoic acid) also displaying a marked contribution, although to a lesser extent than the RvD4 and 10S, 17S-diHDPA. Moreover, plasma 5-HETE, 5-HEPE, 7-HDPA and 7-HDHA, markers of LOX5 activity, were upregulated in DMARD non-responders when compared with responders. An upregulation of ALOX12 markers (14-HDPA and 14-HDHA) and ALOX15 markers (17-HDPA, 17-HDHA, 15-HEPE and 15-HETE) was also observed in non-responders.

Of interest, choline and glucose metabolism were shown to be altered in the synovial tissue in a prior study of synovial metabolomics [242]. In our cohort, choline levels were decreased in serum compared to the normal range; this, along with an increased uptake in the joints on choline C-11 PET scanning in inflammatory arthritis [256] and high expression in fibroblast-like synoviocytes (FLS) of choline like transporter (CTL)1 (high-affinity) and CTL2 (low-affinity) [257], suggest increased circulating choline uptake and consumption by the inflamed synovium. Glucose levels were decreased, while lactate levels were increased in serum from our cohort. Glucose is consumed through upregulation of aerobic glycolysis and when metabolized, gives rise to production of copious amounts of lactate, which must be extruded from the cell to prevent lactic acidosis [258]. Several studies have highlighted

the increase in glucose metabolism in the hypoxic joint [259, 95]. Thus, our results in serum seem to agree well with recently described synovial studies [242].

We also identified alterations (downregulation) of several aminoacids in the serum of our patients. Serine/ glycine metabolism, which is associated to aminoacyl-tRNA biosynthesis, negatively correlated with TNF-α/CD3E and B/plasma cell signatures, which suggests that lymphoid cells could be using these pathways after activation in the rheumatoid synovium. These nonessential amino acids, serine and glycine, support tumor growth [260] and, in addition to their role in protein synthesis, they contribute to anabolic pathways important for the generation of glutathione, nucleotides, phospholipids, and other metabolites [261]. Other amino acids are also critical substrates that fuel mitochondrial metabolism and the biosynthesis of proteins, lipids, and other molecules. Of particular interest in cancer are key mitochondrial enzymes in the metabolism of glutamine, glutamate, proline, aspartate, and alanine [262]. The branched chain amino acids (BCAAs) valine, leucine, and isoleucine are also highly metabolized by transaminases. By coordinating cellular bioenergetics and biosynthesis through the tricarboxylic acid (TCA) cycle, amino acid metabolism could be critical not only in tumor cells but also in lymphoid cell proliferation and survival as described recently [263].

Serum succinate is another metabolite that is decreased in our patients and correlates with several of the cytokine pathways, such as a negative correlation with TNF-α and CD3E. It is an intermediate of the TCA cycle with a crucial role in adenosine triphosphate (ATP) generation in mitochondria with new roles that have been described in recent years. Succinate promotes expression of the pro-inflammatory cytokine IL-1 by inhibiting prolyl hydroxylases and stabilizing the transcription factor hypoxia-inducible factor-1 (HIF-1) in activated macrophages, and stimulates dendritic cells via succinate receptor 1 [258]. Furthermore, succinate has been shown to post-translationally modify proteins.

Since synovial biopsies are still relatively invasive procedures and they are not routinely used in the clinical setting, further studies are needed to better understand the relationship of systemic metabolic profiling and synovial inflammation.

6.2 The role of metabolomics in identifying disease pathogenesis biomarkers

Our hypothesis implies that circulating metabolites would be biomarkers of the underlying mediators of synovial inflammation and we were able to explore this in our studies in RA and PsA patients. According to this theory, increased levels of circulating metabolites could imply a higher production of these in the joint, which are then released in the circulation, reflecting the upregulated metabolic processes in the joint. On the contrary, decreased levels may imply a local consumption or synovial uptake, as energy sources required for synovial cells changes under activation. For instance, FLS switch to an aggressive phenotype, which is supported by an overexpression of receptors and transporters in the synovium (i. e. higher FLS expression of choline like transporter 1 and CTL2 [257], as well as an increased uptake of choline in the joint seen on choline C-11 PET scanning [256]). Another potential cause of decreased circulating metabolites is local consumption in an attempt to limit the inflammatory process, as is the case of the specialized pro-resolving mediators or other anti-inflammatory oxylipins.

We observed a different metabolomic profile in patients with inflammatory arthritis compared to controls, suggesting a dis-regulation of several metabolic pathways may be marker of disease in these patients.

In one of the studies, we explored oxylipins in patients with EORA. Despite the evidence for the involvement of classical oxylipins (PGE2, LTB4) in arthritis pathogenesis, no studies have addressed the role of other oxylipins in RA and specifically in the older adult population. Therefore, we conducted an extensive oxylipin profiling to comprehensively establish the association of circulating inflammatory oxylipins with arthritis in this population. To this end, we collaborated with the Lipidomics Core at the University of California San Diego, which has set up an LC/MS method that is able to identify more than 150 oxylipins in different types of specimens.

The omega-3 PUFAs, precursors of oxylipins, and particularly EPA and DHA, as well as an adequate balance of omega-6/omega-3 PUFAs, play a determinant role in most physiological and biochemical processes occurring in cells and organisms, having great significance in decreasing the risk of many diseases or even resolving their inherent inflammation condition [264]. In our study, patients had a decreased n-3/n-6 PUFA-derived oxylipin ratio compared

to controls, with a stronger decrease in EORA compared to PMR patients. This profound decrease of n-3 PUFAs in EORA didn't appear to be related to the degree of inflammation, as there were no differences in inflammatory biomarkers including CRP, ESR, or platelets count between PMR and EORA patients. Among the DHA-derived downregulated oxylipins are: 17-HDoHE, the precursor of resolvins, and 4-HDoHE, the precursor of maresins, while the EPA derived ones include hydroxy-eicosapentanoic acids (HEPE, also anti-inflammatory), 18-HEPE being a precursor of the resolvins. Patients also presented a decrease in the total free DHA and EPA compared to controls, which was more prominent in EORA compared to PMR patients, although without reaching a statistically significant difference. Interestingly, this was already observed in a previous study published by Rodríguez-Carrio and coworkers [124] who found decreased levels of palmitic, palmitoleic, oleic, arachidonic, total free EPA, and DHA in a Spanish cohort of YORA patients compared to healthy controls. Other AA derived anti-inflammatory oxylipins, including lipoxins A4 and B4, were upregulated in EORA, what would be expected to try to resolve a systemic inflammatory response.

We also explored potential alterations of the desaturase enzymes in patients with EORA. Δ5- and Δ6 desaturases exhibit affinity to metabolize n-3 over n-6 PUFA, provided that they exist in a ratio of 1:1–4. But, dietary intake of ALA is usually low compared to up to 30-fold higher intake of LA, which increases these enzymes' preference to metabolize n-6 PUFA, thus the conversion of ALA is poor in humans and only a small proportion is converted to EPA and DHA [265, 266]. Previous studies performed in RA patients observed that treatment with TNF inhibitors decreased the activity of Δ6 desaturase [267] and higher levels of EPA were associated with a greater decrease of DAS28 in response to treatment with TNF inhibitors, suggesting an increased activity of Δ6 desaturase. In our study, the EPA/ALA ratio, but not the DGLA/LA and AA/DGLA ratios, surrogates for the activity of Δ5 and Δ6 desaturase, was lower in arthritis patients compared to controls (although it did not reach a statistical significance, likely due to a small sample size). These data suggest a decreased activity of the desaturases only in the n-3 PUFA ALA pathway during inflammation.

We next compared the oxylipin profile in a cohort of patients with early RA, patients with clinically suspect arthralgia (CSA) and controls. We found an alteration of the oxylipin profiles across all the groups, which delineated distinct global patterns among the healthy controls, individuals with CSA, and patients with RA. Oxylipin levels were not related to demographic features or traditional cardiovascular risk factors in any of the study groups.

These findings, in a cohort of treatment-naive patients with early RA, ruled out the possibility of the influence of disease duration and treatment exposure, thus pointing to oxylipin networks as playing an active role in the disease, as opposed to being innocent bystanders. One of the the most interesting results from our study was the identification of alterations in oxylipins during the earliest stages of RA, including early RA as well as CSA, supporting the role of oxylipins as potential factors in this setting. These findings are in line with studies showing lower ω-3–derived PUFA levels in individuals at high risk of developing RA [268], and demonstrating changes in gene expression related to lipid metabolism at this stage [269]. The same findings, of decrease of DHA and EPA, were present in the EORA patients and reduced circulating DHA, EPA, and AA levels can be found in patients with RA at the time of disease onset [124], suggesting that lipid metabolism is potentially disturbed in RA. The results herein reinforce this hypothesis, and go further by identifying the actual species altered in the arthralgia stage downstream of the main PUFA precursors.

CSA itself is a very heterogeneous stage of arthritis, with a number of possible clinical outcomes reported [270], from resolution to progression to RA. The presence of individuals with CSA in both oxylipin clusters identified and the overlap in the PLS-DA findings between the healthy control and RA groups support this idea. Moreover, absolute levels of oxylipins also revealed specific alterations in the CSA group that were not present in patients with RA or healthy controls. Furthermore, an important number of oxylipins exhibited decreased levels in individuals with CSA, with a certain degree of recovery observed in patients with RA, although other trajectories were also noted and therefore, further research is warranted.

The correlation analyses comparing healthy controls to individuals with CSA or individuals with CSA to patients with RA led insights into the potential changes occurring in the multistep development of RA. The analysis comparing healthy controls to individuals with CSA identified 8 differentially expressed species originating from 4 different precursors and major pathways, whereas the comparison of individuals with CSA to patients with RA yielded fewer differentially expressed species, mostly derived from LAs and the LOX pathway. Overall, these findings suggest that distinct oxylipin alterations are associated with the different stages along the course of RA. A more diverse picture is evident in the earlier preclinical arthralgia stage, which is consistent with the different risk factors and mechanisms associated with the first events in the triggering of RA [271], while a more convergent effect is evident when comparing individuals with CSA to patients with RA, in which mechanisms

are thought to be shared among disease subsets [272, 273]. Importantly, these discrepancies may be the result of the natural regulation of eicosanoid pathways, hallmarked by an initial production of pro-inflammatory species (mostly AA-derived) that prompt a class-switch to an anti-inflammatory, homeostatic response [274, 275]. However, anti-inflammatory and pro-resolving functions are not equivalent [276] and it is plausible that a stronger shift toward pro-resolution may be needed to control the phase of CSA transitioning to RA.

Importantly, differences were noted between the 2-step analyses (healthy controls versus individuals with CSA and individuals with CSA versus patients with RA) and the global analyses (healthy controls versus individuals with CSA versus patients with RA). Although the latter global approach mostly supported the role of AA and LOX as a whole, a compartmentalization was noted in the 2-step process, which aligns with the different oxylipin trajectories and allows for the identification of potential targets for tailored strategies, the main goal of personalized medicine [277], thus supporting the rationale of our analyses.

Oxylipins showing a significant difference between individuals with CSA and patients with RA may be conceived as potential therapeutic targets to prevent disease progression. Actually, the LOX pathway has been recently described to be up-regulated at the synovial level in patients with RA in comparison to patients with osteoarthritis, while other enzymatic pathways remain unchanged [278]. Due to the relevance of LOX-derived species in this setting, LOX inhibition may be an attractive therapeutic candidate. In fact, zileuton-mediated LOX inhibition has already been studied in patients with RA, although no clinical efficacy was demonstrated in those with established disease [279]. However, in light of our results, further research on the effect of LOX inhibition on preventing or delaying the onset of inflammatory arthritis, rather than on management of the disease, must be considered. This is supported by studies in animal models in which treatment was administered very early [280]. The fact that LOX expression is persistent along the disease course, remaining unchanged by conventional treatments [129], emphasizes the need to initiate earlier intervention. Alternatively, and due to the synergistic effects among the oxylipins, dual COX/LOX inhibitors may also be considered [281].

Our results shed new light on the potential role of oxylipins in early RA. Decreased EPA and DHA levels, which were linked to the altered levels of their derived species, were observed at the time of disease onset, thereby strengthening our previous findings [124]. Cluster analyses revealed that 2 oxylipin profiles could be distinguished among RA patients. One of

the clusters was predominantly present in healthy controls, which may be a more homeostatic profile, whereas the other cluster identified a group of RA patients with more severe clinical features, including higher VAS pain scores. This is aligned with previous evidence from clinical trials assessing fish oils and omega-3 supplements, in which a protective effect on pain was demonstrated in patients with RA [122, 282]. Importantly, eicosanoid metabolites are known to activate nociceptive pathways [283], but the actual mediators are unknown.

Finally, oxylipin profiling led us to identify differences between seronegative and seropositive RA. Although previous metabolomics studies have shown distinct metabolomic signatures in seronegative RA patients, the exact compounds have not been elucidated [284]. Our results confirm that whereas both subsets could be distinguished from controls based on oxylipin signatures, the precursors and pathways greatly differed between them. These results underscore the differences between these 2 RA subsets and add another layer of complexity by identifying oxylipin networks as potential contributors. Whereas AA metabolism clearly dominated the oxylipin signature in seropositive RA patients, less impact was observed in seronegative RA patients, as demonstrated in the correlation and pathway analyses. Importantly, OA-derived nitrooleates, which are strong anti-inflammatory lipids [285], and DHA- and EPA-derived species, in addition to COX products, were associated with seronegative RA. Due to the complexity of the seronegative subset of the disease, these findings warrant further research into these pathways.

6.3 The role of metabolomics in identifying biomarkers of disease activity

Assessment of disease activity in inflammatory arthritis is critical for the management of the disease, allowing adequate treatment choice to avoid progression and disability. Disease activity in inflammatory arthritis is being evaluated at the moment using composite scores which include clinical examination of tender and swollen joints or enthesitis (for PsA), the patient's self-evaluation and a laboratory parameter, either ESR or CRP[286, 287]. However, these scores have disadvantages and ongoing research is trying to identify biomarkers of disease activity and of specific clinical manifestations, such as enthesitis, which are more challenging to assess in clinics. Metabolomics is a promising tool for biomarker activity, and it can be performed either in the inflamed tissue, or in blood samples, for a non-invasive

strategy. Our studies in RA patients included patients with high disease activity, which did not allow the study of metabolites in relation with degree of inflammation in these patients. However, our studies in PsA patients allowed the identification of biomarkers of disease activity.

In our first study, we studied the relation of choline related metabolites with disease activity in PsA patients. We found that circulating levels of TMAO, a metabolite of choline via the microbiome, correlated with skin and joint severity. PsA patients have a higher prevalence of metabolic syndrome and are at increased risk of cardiovascular disease. At the same time, previous studies showed that choline, betaine and TMAO were all associated with atherosclerosis and higher risk of CVD risk [288]. Moreover, TMAO concentration was dependent on the dietary intake of choline [133], hence it was suggested that changes in diet could be a therapeutic approach for decreasing CVD risk. Nonetheless, in our study, there was no correlation between the concentration of choline and TMAO, and, furthermore, only TMAO and not choline and betaine correlated with skin and joint disease activity. These data suggest that TMAO concentration does not depend on choline concentration in PsA patients, but more likely on the activity of FMO3 (TMAO producing enzyme), which might be elevated in patients with inflammation and obesity.

FMO3 has been studied in patients with obesity. The expression of FMO3 in adipose tissue in overweight patients positively correlated with BMI and waist-to-hip ratio, and negatively correlated with insulin sensitivity [289, 290], suggesting a link between TMAO producing enzyme FMO3 and obesity. In our cohort, the ratio TMAO/TMA was higher in the obese group (0.3 ± 0.2 in BMI 30 vs 0.7 ± 0.5 in BMI > 30, p=0.08) suggesting a higher activity of FMO3, similar to the cited study.

We also studied the relation of oxylipins with disease activity in patients with psoriatic disease. The involvement of oxylipins in the pathogenesis of psoriatic disease has been studied mainly in PsO. Skin lipidomic studies found higher concentrations of AA- derived 8- and 12-HETE and LTB4 in lesional skin compared to non-lesional skin and skin from healthy controls [291, 292, 156]. The existence of a natural skin COX inhibitor and a shift towards a LOX-mediated metabolism of AA was suggested to play a role in PsO pathogenesis [156, 154]. Using NMR spectroscopy, a different lipid profile was described in PsA compared to RA patients [284]. Yet, a lipidomic approach in psoriatic disease to investigate the components of eicosanoid biology, beyond AA metabolites and leukotrienes, has not been

used before.

We studied the oxylipins in 2 separate studies. The first study (Appendix 1) included 41 patients with PsA and different degrees of PsO. We found that several eicosanoids were positively correlated with parameters of joint disease activity (tender joint count -TJC-, swollen joint count -SJC-, CDAI, SDAI and DAS28CRP) including several AA-derived pro-inflammatory eicosanoids such as PGE2, HXB3 or 6,15- dk,dh,PGF1a, being HXB3 and tetranor 12-HETE, 12LOX derived eicosanoids. An even more interesting finding in these patients is that several EPA-derived anti-inflammatory eicosanoids, such as 11-HEPE, 12-HEPE and 15-HEPE, were upregulated in patients with higher joint disease activity, suggesting that EPA-derived anti-inflammatory eicosanoids amount might be increased in an effort to balance the inflammation induced by AA-derived pro-inflammatory eicosanoids. However, some DHA-derived anti-inflammatory eicosanoids negatively correlated with joint disease activity, including anti-inflammatory eicosanoids such as resolvin (Rv)D1 and 17-HDoHE. Resolvins (RvD1 and RvD3) have been shown to contribute to the resolution of inflammation, decreasing the joint inflammation (clinical score) and joint paw eicosanoids in the K/BxN serum transfer inflammatory arthritis model [293, 294]. 17-HDoHE, RVD1 precursor, also decreased pain in a different animal model of arthritis [295]. The negative correlation between these pro-resolving lipids mediators and joint activity could suggest that a disbalance between pro- and anti- inflammatory eicosanoid species might play a role in the pathogenesis of joint inflammation in PsA and that these could be useful to guide treatment.

The second study (under review) included the comparison of the oxylipin profile in 20 PsO without arthritis and 19 PsA patients with different degrees of skin involvement. In PsO patients, the oxylipins profile varied according to the severity of skin disease evaluated by PASI: patients with more severe disease (PASI > 2.5) had a decrease in several serum oxylipins. PGE2, derived from AA via COX, was the oxylipin that best discriminated between the 2 groups. It is the most abundant prostaglandin in the skin and has a role in the pathogenesis of PsO [151, 152], but also the resolution of inflammation, by stimulating the secretion of specialized pro-resolving mediators [153]. Previous studies have described similar oxylipin alterations: PGE2 and 12- and 15-HETE were described to be higher in psoriatic skin compared to skin from healthy controls, in contrast to serum concentrations that were found to be lower in PsO patients compared to healthy controls [154, 155]; HXB3 was also described to be higher in lesional skin compared to skin from healthy controls [156]. The

same studies also found that the concentrations of EPA (5-, 12-, 15- and 18-HEPE), LA (13-HODE, 9-oxoODE, 13-oxoODE), and DHA (4-, 7-, 14- and 17-HDoHE) derived oxylipins were higher in lesional skin compared to normal skin, but lower in serum of PsO patients versus healthy controls. Of interest, we reanalyzed the results from our first study, [296], and patients with a BSA > 3 (equivalent to higher skin inflammation) also had lower levels of several pro-inflammatory oxylipins in serum. Overall, several serum oxylipins derived from all the precursors via COX, 5-,12- and 15 LOX seem to be lower in patients with higher skin activity.

The reason for the decrease of serum oxylipins in patients with higher degrees of skin inflammation is unclear. Among the pro-inflammatory oxylipins, local consumption to amplify the inflammatory response could explain the decrease in the release of these oxylipin to the serum. The skin contains receptors for oxylipins which are involved in the pathogenesis of psoriasis. Ueharaguchi et al observed that TXA2 (AA derived via COX2 and precursor of TXB2) signaling through its receptor, TBXR2, may facilitate psoriatic dermatitis by promoting IL-17 production in psoriatic lesions in animal model [157]. Furthermore, mRNA expression of TBXAR2 was significantly increased in skin biopsies from psoriatic lesions compared to skin from healthy controls [157]. While IL23 induces the production of PGE2 by Th17 cells, PGE2 acts back on its receptors, EP2 and EP4 on these cells, to increase Il23r expression in a positive feedback manner. Interestingly, EP4 receptor (PTGER4) was overexpressed in human psoriatic lesional skins [151]. These data suggest that, in spite of increased levels of oxylipins in the psoriatic skin, they are probably consumed locally, due to the higher expression of their receptors.

In contrast to the results of the previous study, only a few oxylipins negatively correlated with disease activity: bicyclo PGE2, 12-oxoETE, 13-HOTrE, 8 iso PGF2a III and PGD3. Yet, oxylipins are also involved in joint inflammation. Data from RA shows that 5LOX and COX2 enzymes are upregulated in the synovium and they remain elevated despite treatment with disease modifying drugs [297, 298, 299]. In addition, 5LOX deficient mice developed less arthritis in a mouse model of RA [300]. PGE2's involvement in RA has been also studied. PGE2 receptors (EP1 to 4) are also expressed in the synovium, however only EP4 was found to be involved in arthritis development in a mouse model of collagen antibody–induced arthritis. [118]. Interestingly, polymorphisms of the human EP4 receptor gene PTGER4 have been identified as risk alleles for ankylosing spondylitis (AS) and its expression by Th17 cells

is associated with higher disease activity in AS, but not RA and PsA [301]. PGE2 induces IL17 secretion upon Th17-fibroblast interaction [302], and this is responsible for worsening arthritis severity in the collagen-induced arthritis model [303]. The lack of a significant association of the detected oxylipins with joint activity in our study could be due to the overlap with skin and entheseal inflammation, yet specific information on eicosanoids and their receptors in PsA synovial tissue is also lacking.

This study also included patients with entheseal involvement. Patients with PsA with higher enthesitis score had an increase of several oxylipins in serum, some of which were decreased in patients with PsO (PGE2 and 11bPGE2) or PsA (LTB4 and 5-oxoETE) and high PASI. Despite a better understanding of the anatomy and biology of the enthesis, data on the complex mechanisms of enthesis inflammation are lacking. There is limited data on eicosanoids in the enthesis. IL17 is produced in the enthesis by $\gamma\,\delta$ T cells and group 3 innate lymphoid cells, and by stimulating PGE2 and IL8 production facilitates neutrophil recruitment and enthesis inflammation. Moreover, expression of COX2 by resident mesenchymal cells reinforces the involvement of PGE2 in the entheseal inflammation [304]. Although more information on the role of eicosanoids in enthesis inflammation is required, our results suggest that specific oxylipins might not only play a role in enthesitis pathogenesis, but also function as a potential biomarker of enthesitis in psoriatic disease. These results should be taken with caution given previously described associations between PASI, BMI, and enthesitis [305]. However, in our study, there was a trend towards an inverse relation between PASI and enthesitis in PsA patients, although it wasn't statistically significant (r2 = -0.427, p = 0.674), and we did not observe any association between BMI and oxylipins in the PsO group.

Since oxylipins associated with enthesitis belong to COX2 and LOX pathways, dual inhibition could be beneficial in patients with enthesitis. Of note, even though TNF alpha inhibition decreased COX2 upregulation and PGE2 production in vitro, downregulation of COX2 expression after TNF treatment was not observed in ex vivo studies on synovial tissue from RA patients [299, 306]. Other studies in RA suggested that COX and LOX pathways remain overexpressed and can contribute to subclinical inflammation and relapse of rheumatic diseases [297]. Dual inhibition was explored in drug development. Yet, although these drugs were effective and had a better risk profile regarding cardiovascular and gastrointestinal damage compared to NSAIDs, they never reached the clinical stage mostly due their liver toxicity

[281]. In addition, evidence from animal models shows that 12/15 LOX, the murine enzyme equivalent to the human 15-LOX, has a protective role in inflammatory arthritis [128], and some LOX derived oxylipins were shown to have anti-inflammatory properties [129, 307]. A better understanding of oxylipin pathways is needed to help discover new therapeutic strategies.

Although our analysis revealed an overlap of the oxylipin profiles between PsO and PsA, this finding is not surprising, since PsA patients also had skin involvement. A previous study that compared the lipidomic profile in lymphocytes from patients with PsO, with PsA, and healthy controls also found that the profiles of PsA and PsO overlapped, although they were clearly different from healthy controls[308]. This overlap explains the difficulty of validating prior results when using such a heterogenous population, despite the statistical methods used to adjust for confounding factors.

Both studies on RA patients included patients with high disease activity, which did not allow the study of metabolites in relation with degree of inflammation in these patients.

6.4 The role of metabolomics in identifying biomarkers of response to treatment

In our first work on the relation between circulating metabolites and synovial inflammatory markers we found that the combination of only two or three metabolites identified in serum by NMR could discriminate between high or low levels of synovial TNF-α (decreased dimethysulfone, glutamine and trimethylamine in high TNF levels) and CD3E (carnitine and methionine) gene expression. This is a finding with potential of prediction of treatment response, since in previous studies higher TNF-α synovial expression was associated with disease activity (evaluated by DAS28-CRP)[309, 310] and it correlated with response to DMARDs: a good response to TNF-inhibitors [31], but a poor response to Methotrexate and Tocilizumab [310]. The patients included in the ARISE study received treatment with rituximab after a prior failure of at least one TNF inhibitor, and no correlations were found between synovial TNF-α expression and response [252]. However, our group previously identified serum metabolites related to response in these patients: 7 polar metabolites (phenylalanine, 2-hydroxyvalerate, succinate, choline, glycine, acetoacetate and tyrosine) [253].

In another study from our group (by J Murillo et al, currently under review), we investigated NMR identified metabolites in relation to response to treatment with Tocilizumab (TCZ). Sphingolipid and arginine/proline metabolism were the most enriched metabolic pathways in responders, while glutathione metabolism and glycolysis/gluconeogenesis pathways were the most important enrichment pathways in non-responders. Specifically, 3-hydroxybutyrate, isobutyrate, lysine, sn-glycero-3- phosphocholine, phenylalanine, tryptophan and tyrosine were up-regulated in responders. Interestingly, the levels of phenylalanine and tyrosine were upregulated in responders to TCZ, whereas they were downregulated in responders to rituximab. This is probably explained by the IL-6 dependency of these 2 metabolites, whose concentration was shown to vary after recombinant IL-6 infusion.

Studies in other cohorts of patients with active RA are needed to validate these results, yet the relationship between serum metabolic profiles and synovial biomarker profiling suggests that NMR, as well as LC/MS, may be promising tools for predicting specific pathogenic pathways in the inflamed synovium in RA. This might pave the way for personalized medicine in rheumatology, as recent studies started to include synovial biopsies as a guide in therapy adjustment [52]. Pitzalis and colleagues conducted a randomized clinical trial to determine whether the relative abundance of B cells in the synovium before treatment is related to the response to rituximab versus the response to TCZ. The authors found no significant difference between the treatment groups' response rates when they measured synovial B cells via histology. However, patients with limited B cell gene expression, as quantified by RNA sequencing, had significantly greater treatment responses to TCZ. It would also be of intereste to explore potential differences in metabolomics profile and their relation to treatment response. Another recent study examined the relation between synovial Krenn score at baseline and response to treatment at 6 months, and found that a lower Krenn score, a lower disease activity and a lower duration of disease at baseline were associated with a higher probability of disease remission [311]. However, this study did not explore which histological pathotype was associated with response.

The rest of our studies didn't have data from the synovial tissue available, but follow up information after treatment initiation was available, which allowed us to study the potential of oxylipin alterations in response to treatment. Nevertheless, no data was available for the cohorts of psoriasis and psoriatic arthritis patients, so no analysis concerning response to treatment were performed.

The majority of the patients in the EORA cohort received GC. Due to a good response to treatment in most of the patients (41), we couldn't evaluate whether or not changes in the oxylipin profile were related to therapeutic response or could potentially identify patients who will respond. However, we did observe that the ratio n-3/n-6 PUFA increased post-treatment in EORA patients. In addition, most of these patients were receiving a low-dose prednisone. Although GCs have very well-known anti-inflammatory properties, there is no data concerning the effect of GC on systemic oxylipins, which could partially be responsible for the upregulation of most of the previously downregulated n-3 PUFA-derived oxylipins. Moreover, while the effect of GC on COX enzymes is well known [312], little is published on its effect on LOX enzymes. Of note, a study showed that oral GC increased jejunal uptake of cholesterol and ileal uptake of lauric, palmitic, linoleic, and linolenic acid [313]. Interestingly, the ALA-derived oxylipins 9-HOTrE, 13-HOTrE, and 13-HOTRE(y) were still downregulated in EORA at 3 months.

Steroids (dexamethasone, hydrocortisone, and triamcinolone) are also known to reduce the activity of the Δ5 and Δ6 desaturases [314] and are likely responsible of some of the changes observed at 3 months. However, these enzymes are active in the tissue and no studies have been performed to evaluate the relationship between tissue and circulating metabolites, hence, it makes the interpretation of our results difficult. The baseline EPA/ ALA ratio, but not the DGLA/LA and AA/DGLA ratios, surrogates for the activity of Δ5 and Δ6 desaturases, was lower in arthritis patients compared to controls (although it did not reach a statistical significance, likely due to a small sample size), and returned to control values after treatment. These data suggest a decreased activity of the desaturases only in the n-3 PUFA ALA pathway during inflammation and these alterations might be affected by GC treatment.

In the YORA cohort we found correlations of certain oxylipins with response to DMARDs in patients with early RA. The oxylipins profile separated the RA patients in 2 clusters, which was associated not only to disease severity (higher VAS and pain), but also a lower probability of achieving a EULAR good response 6 months after DMARD initiation.Since early remission is an important aim in treat-to-target strategies [315, 316], oxylipin networks should be further studied either for their role as biomarkers or for their actionable mechanisms, to facilitate clinical management.

Limitations of our studies

As mentioned in the Introduction, several factors are involved in determining the circulating metabolites concentrations. We did take into account the comorbidities of the patients, such as diabetes, high blood pressure and cholesterol levels, as well as concomitant treatment, and these were equally distributed among the compared groups. However, we couldn't take into account other important factors such physical activity, number of hours of sleep, and perhaps the most important of all, diet. The objective of our new ongoing projects are to investigate the relation between environmental factors, specifically diet, circulating metabolites, microbiome and inflammmation in inflammmatory diseases.

Another very important aspect is the processing of the samples. The undertaken steps should be similar, all the way from blood draw until the sample is being stored and then analyzed in order to allow for reproducible results.

Yet, overall, our findings notably improve our understanding of the synovial pathology and its relation to circulating metabolites. Moreover, they provide information on the complex eicosanoid networks in rheumatoid arthritis and psoriatic disease, and should pave the ground for future, larger, multicentric and prospective studies to address this topic and validate our results, and ideally, to focus on the metabolomic profile of synovial tissue.

7 Conclusions

PUFA derived oxylipin metabolomic profile (identified by LC/MS) and energy-related metabolomic profile (identified by NMR) are different in patients with inflammatory arthritis compared to controls and change with treatment.

TMAO, a choline-derived metabolism, correlates with joint and skin disease activity in patients with psoriatic arthritis.

Decreased levels of the anti-inflammatory omega 3 PUFAs docosahexaenoic (DHA) and eicosapentanoic acid (EPA) and their derived oxylipins characterize both young and elderly onset RA patients, suggesting a pathogenic role.

Oxylipin profile is also altered in patients with clinically suspect arthralgia, hence the study of oxylipin profiles in these patients may reveal the underlying mechanisms in the early phases of disease and might represent a potential target to stop progression to RA in these patients.

8 Future directions

One of the future projects to undergo is to validate the results of our studies in new cohorts. We recently gained access to a large number of samples from the CORRONA registry, that includes biospecimens collected before and after treatment with different biological therapies. This will allow us to validate metabolic signatures associated with activity and with response, and to explore if the different DMARDs mechanisms of actions are characterized by different metabolic signatures.

One important field of study is the relation between synovial and circulating metabolites, to explore our hypothesis that circulating metabolites reflect the local pathogenic processes. The increase use of ultrasound synovial biopsies performed by rheumatologists will allow us to collect tissue from patients with inflammatory arthritis and perform metabolomic analysis. Together with Dr. Guma, Dr. Kavanaugh and Dr. Singh we have set up a synovial biopsy program at the University of California San Diego. We have already obtained tissue and plasma samples from patients with rheumatoid arthritis and psoriatic arthritis and we are performing metabolomic analysis at the moment. We will also compare the data with metabolomics analysis of synovial tissue from patients with end stage osteoarthritis who undergo total joint replacement.

Finally, the objective of another ongoing project is to further investigate the relation between diet, circulating metabolites, microbiome, and inflammation in inflammatory diseases. In our pilot study we observed improvement of RA clinical outcomes as well as changes in microbiome and metabolic fecal and plasma features after 2 weeks of "ITIS" (anti-inflammatory) diet intervention. A very interesting finding was that patients who responded to diet already had a healthier diet and a higher diversity of the microbiome before the intervention. A large number of patients is required to be able to study the interaction of diet-microbiome-metabolites with clinical outcomes, since existing evidence showed personalized responses of metabolites to the same food ingredients. With that in mind, we have

recently started a randomized clinical trial to evaluate long-term effects of dietary interventions in patients with rheumatoid arthritis. Moreover, we are performing a pilot trial of the ITIS diet in patients with osteoarthritis, a disease that also has an inflammatory component.

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10 Appendix

10.1 Pro-and anti-inflammatory eicosanoids in psoriatic arthritis

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ORIGINAL ARTICLE

Pro- and anti-inflammatory eicosanoids in psoriatic arthritis

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Abstract

Introduction Eicosanoids are biological lipids that serve as both activators and suppressors of inflammation. Eicosanoid pathways are implicated in synovitis and joint destruction in inflammatory arthritis, yet they might also have a protective function, underscoring the need for a comprehensive understanding of how eicosanoid pathways might be imbalanced. Until recently, sensitive and scalable methods for detecting and quantifying a high number of eicosanoids have not been available.
Objective Here, we intend to describe a detailed eicosanoid profiling in patients with psoriatic arthritis (PsA) and evaluate correlations with parameters of disease activity.
Methods Forty-one patients with PsA, all of whom satisfied the CASPAR classification criteria for PsA, were studied. Outcomes reflecting the activity of peripheral arthritis as well as skin psoriasis, Disease Activity Score (DAS)28, Clinical Disease Index (CDAI) and Body Surface Area (BSA) were assessed. Serum eicosanoids were determined by LC–MS, and the correlation between metabolite levels and disease scores was evaluated.
Results Sixty-six eicosanoids were identified by reverse-phase LC/MS. Certain eicosanoids species including several pro-inflammatory eicosanoids such as PGE2, HXB3 or 6,15-dk,dh,PGF1a correlated with joint disease score. Several eicosapentaenoic acid (EPA)-derived eicosanoids, which associate with anti-inflammatory properties, such as 11-HEPE, 12-HEPE and 15-HEPE, correlated with DAS28 (Disease Activity Score) and CDAI (Clinical Disease Activity Index) as well. Of interest, resolvin D1, a DHA-derived anti-inflammatory eicosanoid, was down-regulated in patients with high disease activity.
Conclusion Both pro- and anti-inflammatory eicosanoids were associated with joint disease score, potentially representing pathways of harm as well as benefit. Further studies are needed to determine whether these eicosanoid species might also play a role in the pathogenesis of joint inflammation in PsA.

Keywords Eicosanoids · Lipidomics · Biomarkers · Psoriatic arthritis

Abbreviations

PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
DAS28	Disease activity score 28
CDAI	Clinical disease index
BSA	Body surface area
EPA	Eicosapentaenoic acid
PUFA	Polyunsaturated fatty acids
AA	Arachidonic acid
LA	Linolenic acid
α-LA	α-Linolenic acid
DHA	Docosahexaenoic acid
COX	Cyclooxygenases
LOX	Lipoxygenases
SD	Standard deviation
sDMARDs	Synthetic disease modifying antirheumatic drug
NSAIDs	Non-steroidal anti-inflammatory drugs
TJC	Tender joints
SJC	Swollen joints
HAQ	Health Assessment Questionnaire
FLS	Fibroblast-like synovioctyes
CRP	C-reactive protein

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1 Background

Psoriatic arthritis (PsA) is a heterogeneous disorder with activity and severity ranging from mild synovitis to progressive debilitating erosive disease (Gravallese and Schett 2018; Ritchlin et al. 2017). The pathophysiology of PsA involves chronic inflammation mediated by pro-inflammatory cytokines, including TNF, IL-17 and IL-23 (Gravallese and Schett 2018; Ritchlin et al. 2017). It has become clear that many of the signaling pathways triggered by inflammatory cytokines that are activated during inflammation have a profound effect on core lipid metabolism of cells, and the study of the lipidome/metabolome has been successful in identifying new pathogenic targets (Johnson et al. 2016).

Although they represent only a small fraction of the total fatty acids in plasma, free fatty acids are highly metabolically active lipids and they also include the polyunsaturated fatty acids (PUFA): arachidonic acid (AA), linoleic acid (LA) and the nutritionally essential α-linolenic acid (α-LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Quehenberger et al. 2010; Quehenberger and Dennis 2011). Eicosanoids are a class of bioactive lipid mediators derived from the metabolism of AA and related PUFAs by cyclooxygenases (COX), lipoxygenases (LOX), cytochrome P450s, or non-enzymatic pathways (Buczynski et al. 2009). In general, AA gives rise to pro-inflammatory eicosanoids whereas α-LA, EPA and DHA give rise to anti-inflammatory eicosanoids (Buczynski et al. 2009). This class of lipids has been extensively studied over the years due to its implication in the inflammatory response. Some studies suggest that an alteration in AA metabolism is seen in the form of increased formation of pro-inflammatory eicosanoids and decreased production of PUFA-derived anti-inflammatory lipoxins, resolvins, protectins and maresins in several inflammatory diseases (Dennis and Norris 2015; Serhan 2014).

Until recently, sensitive and scalable methods for detecting and quantifying a large number of eicosanoids have not been available. Advances in mass spectrometry (MS) analytics now allow for high-throughput rapid and reliable quantification of more than 150 eicosanoids, both pro-and anti-inflammatory, in human plasma. This new technology addresses the question of how eicosanoid pathways may be active and involved in PsA pathogenesis. More important, it allows a more comprehensive, and detailed understanding of how eicosanoid pathways are active, imbalanced, and perturbed.

In this study, we describe the eicosanoid profiling in patients with PsA and its association with joint inflammation. We show that certain pro-inflammatory eicosanoids, such as PGE2, HXB3 or 6,15-dk,dh,PGF1a, but also some EPA-derived anti-inflammatory eicosanoids, such as 11-HEPE, 12-HEPE and 15 -HEPE, correlated with joint disease. Of interest, resolvin D1, a DHA-derived anti-inflammatory eicosanoid, was down-regulated in patients with high disease activity. These results suggest that an imbalance of pro- and anti-inflammatory eicosanoids might be associated with clinical inflammation in PsA patients and provide a foundation for more focused investigations into novel eicosanoid-based interventions to reduce PsA-related morbidity.

2 Patients and methods

2.1 Patient selection and assessment

41 adult patients with PsA fulfilling the classification for PsA (CASPAR) criteria were approached in University of California San Diego (UCSD) Arthritis Clinics for opportunistic sampling. The study was approved by the UCSD Institutional Review Board. Clinical assessment included the following: evaluation of the number of tender (TJC) and swollen joints (SJC) (out of 28); the number of tender entheseal sites; the percentage of the body surface affected by psoriasis; functional status as assessed by Health Assessment Questionnaire (HAQ); and assessments of pain; fatigue; global disease severity by patients; and a global assessment of disease by physicians, using a Visual Analogue Scale ranging from 0 to 10. Composite measures of peripheral arthritis were calculated using the above measures: Disease Assessment Score using a 28 joint count and C reactive protein (DAS28-CRP), Clinical Disease Activity Index (CDAI) and Simple Disease Activity Index (SDAI). Non-fasting blood samples were collected in the clinic by research personnel into 10 ml BD Vacutainer Blood Collection Tubes containing spray-coated silica and a polymer gel for serum separation. After 30 min incubation at room temperature, tubes were centrifuged for 10 min at 2000×*g* and sera were transferred into 1.7 ml tubes and immediately frozen and stored at − 80 °C until analysis.

2.2 Lipid extraction

All sera samples were stored at − 80 °C, thawed once, and immediately used for free fatty acid and eicosanoid isolation as described (Wang et al. 2014). Briefly, 50 µl sera was spiked with a cocktail of 26 deuterated internal standards that also included some selected PUFAs (individually purchased from Cayman Chemicals, Ann Arbor, MI) and brought to a volume of 1 ml with 10% methanol. The samples were then purified by solid phase extraction on Strata-X columns (Phenomenex, Torrance, CA), using an activation procedure consisting of consecutive washes with 3 ml of

100% methanol followed by 3 ml of water. The eicosanoids were then eluted with 1 ml of 100% methanol, and the eluent was dried under vacuum, dissolved in 50 µl of buffer A (consisting of water-acetonitril-acetic acid, 60:40:0.02 (v/v/v)), and immediately used for analysis).

2.3 LC–MS measure of eicosanoids

Eicosanoids in sera were analyzed and quantified by LC/MS/MS as previously described (Quehenberger et al. 2010; Wang et al. 2014). The methods used are in line with the proposed minimum reporting standards published in 2007 by Sumner et al. (2007). Briefly, eicosanoids were separated by reverse-phase chromatography using a 1.7 µm 2.1 × 100 mm BEH Shield Column (Waters, Milford, MA) and an Acquity UPLC system (Waters). The column was equilibrated with buffer A, and 10 µl of sample was injected via the autosampler. Samples were eluted with a step gradient starting with 100% buffer A for 1 min, then to 50% buffer B (consisting of 50% acetonitril, 50% isopropanol, and 0.02% acetic acid) over a period of 3 min, and then to 100% buffer B over a period of 1 min. The LC was interfaced with an IonDrive Turbo V ion source, and mass spectral analysis was performed on a triple quadrupole AB SCIEX 6500 QTrap mass spectrometer (AB SCIEX, Framingham, MA). Eicosanoids were measured using electrospray ionization in negative ion mode and multiple reaction monitoring (MRM), using the most abundant and specific precursor ion/product ion transitions to build an acquisition method capable of detecting 158 analytes and 26 internal standards. The ionspray voltage was set at − 4500 V at a temperature of 550 °C. Collisional activation of the eicosanoid precursor ions was achieved with nitrogen as the collision gas with the declustering potential, entrance potential, and collision energy optimized for each metabolite. Eicosanoids were identified by matching their MRM signal and chromatographic retention time with those of pure identical standards.

Eicosanoids and free fatty acids were quantitated by the stable isotope dilution method. Briefly, identical amounts of deuterated internal standards were added to each sample and to all the primary standards used to generate standard curves. To calculate the amount of eicosanoids and free fatty acids in a sample, ratios of peak areas between endogenous metabolite and matching deuterated internal standards were calculated. Ratios were converted to absolute amounts by linear regression analysis of standard curves generated under identical conditions (Loomba et al. 2015). Eicosanoid levels are shown as pmol/ml.

2.4 Data analysis

The data, consisting of 41 patient samples measured across clinical outcomes were processed using R, Version 3.4.1. (www.r-project.org). Continuous variables were expressed as mean ± standard deviation (SD) and the categorical variables as percentage. Pearson correlation was used to check correlation between disease activity scores. *T* test was used to analyze statistically significant difference between the means of two groups, and the comparisons were then adjusted for BMI, therapy, and disease activity, by including the latter as covariates in a logistic regression model. Hierarchically clustered heatmaps were generated for correlations between each eicosanoid and clinical outcomes. Dendrograms were divided into flat clusters using a cophenetic distance metric. Linear regression was performed between each eicosanoid metabolite- clinical outcome pair, controlling for patient BMI, therapy, and disease activity using the Ordinary Least Square method. Normally distributed independent variables were standardized to a mean of 0 and a standard deviation of 1. Benjamini–Hochberg method was used to adjust for multiple comparisons.

3 Results

3.1 Patient demographics and disease characteristics

Patient characteristics are summarized in Table 1. Of the 41 PsA patients included in this study, 61% were males (N = 25)

Table 1 Demographic and disease characteristics of patients included in our study

Characteristic	Percentage/mean (standard deviation)
Male	61%
Age	49.2 (11.1)
BMI	27.4 (5.5)
Duration of PsA in years	11.3 (10)
Duration of psoriasis in years	18.4 (14.1)
Number of tender joints	1.7 (range 0–10)
Number of swollen joints	2.09 (range 0–10)
Pain	2.8 (2.8)
Fatigue	3.3 (3.3)
HAQ	0.45 (0.55)
DAS28-CRP	2.72 (1.28)
CDAI	9.1 (9.6)
SDAI	10.84 (10.25)
Enthesitis	19.5%
Body surface affected by psoriasis (BSA)	7.3 (range 0–95)
Treatment with TNF inhibitors	56%
Diabetes mellitus type 2	17%
High blood pressure	44%
Dyslipidemia	29.2%

and the mean age was 49.2 ± 11.1 years. The mean number of tender joints and swollen joints were 1.7 ± 3 (range 0 to 10) and 2.1 ± 3.2 (range 0 to 10) respectively. The average DAS28-CRP score was 2.72 ± 1.3 (range 1–5.2). Twenty-seven patients (71%) had active skin disease, with an average BSA of 7.3 ± 15.7 (range 0–95%). Eight patients had enthesitis. The average body mass index (BMI) was 27.4 ± 5.5. Sixty-five percent were receiving biological therapy, 56.5% of them in association with a synthetic disease modifying antirheumatic drug (sDMARDs), mostly methotrexate. 14.6% received sDMARDs as monotherapy, and 29.2% were on daily non-steroidal anti-inflammatory drugs (NSAIDs). Comorbidities are also summarized in Table 1.

3.2 Eicosanoid profiling and clustering

Sixty-six eicosanoids, which are derived from AA, EPA, LA, DGLA, αLA and DHA, were identified by Reverse-phase LC/MS. Among these, 18 (22.38%) belong to the cyclooxygenase (COX) pathway, 12 (11.94%) belong to the 5 lipoxygenase (LOX) pathway, 13 (23.88%) belong to the 15LOX pathway, 7 (13.43%) belong to the 12LOX pathway, 14 (20.89%) belong to the CYP pathway and the rest (2) are synthetized by non-enzymatic pathways. Twenty-seven of the detected eicosanoids are considered pro-inflammatory and 39 are anti-inflammatory species. Figure 1a shows the pro- (in red) and anti-inflammatory (in blue) eicosanoids and the different enzymatic eicosanoid pathways of the eicosanoids detected in our samples. We also analyzed eicosanoid clustering (Fig. 1b, c). Concentrations of the circulating eicosanoids detected in our samples are shown in Table 2.

3.3 Linear regression analysis between grouped serum eicosanoids and clinical parameters of PsO and PsA

Linear regression was performed between each eicosanoid-clinical parameter pair, controlling for BMI, NSAIDs and biological therapy, by including these factors as covariates in the model. We also adjusted joint disease scores (TJC, SJC, DAS28, CDAI and SDAI) for BSA, and BSA score was also adjusted for CDAI. The regression coefficients for each eicosanoid-clinical parameter pair were used to form a clustered heatmap to lend insight into which groups of eicosanoids were correlated with which clinical parameters (Fig. 2a). p-values and q-values of the regression analysis of metabolite levels are displayed in Figs. 2b and S1A, where the row and column order are preserved from Fig. 2a. Several eicosanoids positively correlated with parameters of joint disease activity (tender joint count-TJC-, swollen joint count-SJC-, CDAI, SDAI and DAS28-CRP) including several pro-inflammatory eicosanoids, such as PGE2, 12-oxo-HETE, HXB3 or 6,15-dk,dh,PGF1a, but interestingly, also several anti-inflammatory eicosanoids, such as 11-HEPE, 12-HEPE and 15-HEPE among others. Interestingly, we also detected some eicosanoids that negatively correlated with joint disease activity including some anti-inflammatory eicosanoids such as 8,9-diHETrE, 11,12-diHETrE,14,15-diHETrE, 19,20-diHDPA and 7,17 DHDPA. Other clinical scores that cluster together were patients' clinical scores (pain, HAQ, fatigue and global patient score), with a similar eicosanoid profiling as CDAI, TJC and TJC.

3.4 Logistic regression analysis between grouped serum eicosanoids and DAS28CRP

We further analyzed whether some eicosanoids could differentiate between high and low disease activity scores. Figures 3a, b, S1B and Supplemental Table S1 show logistic regression analysis adjusted by BMI, BSA, NSAIDs and biological therapy of each eicosanoid in patients in remission or with low disease activity (DAS28CRP ≤ 2.32), compared to patients with high disease activity (DAS28CRP > 2.32). Figure 3c shows the significant eicosanoids within the different PUFA and enzymatic pathways. Of interest, while most of the EPA-derived anti-inflammatory eicosanoids were elevated in the high disease activity, some of the DHA-anti-inflammatory eicosanoids, including resolvin D1 and 17-HDoHE, were down-regulated in these group of patients.

4 Discussion

Data regarding the role of eicosanoids in PsA is very scarce, as most of the research is associated with rheumatoid arthritis (RA) pathogenesis. Evidence that the COX pathway might be involved in the pathogenesis of RA dates back to the 1970s, when elevated prostaglandin levels were reported in synovial fluid from patients with RA (Korotkova and Jakobsson 2014). Since then, studies in animals and patients have established a pivotal and complex role of prostaglandins and other eicosanoids in RA. For instance, expression of 5LOX and 15LOX was also increased in rheumatoid synovium (Gheorghe et al. 2009). LTB4, a metabolite of AA via 5LOX, has been related to inflammation in arthritis for a long time. Two independent studies demonstrated that LTB4 and BLT1 receptors are indispensable for the development of arthritis in the K/BxN model of RA (Chen et al. 2006; Kim et al. 2006). Another study demonstrated that inhibition of 5LOX in fibroblast-like synovioctyes (FLS) and knocking out 5LOX gene in a mouse model of RA decreased inflammatory cytokines expression and paw inflammation (Lin et al. 2014). More importantly, in RA, despite the wide range of sDMARDs and biological therapies that potently suppress specific inflammatory mechanisms, the COX and LOX pathways remain overexpressed in inflamed tissue in some patients with RA, which might

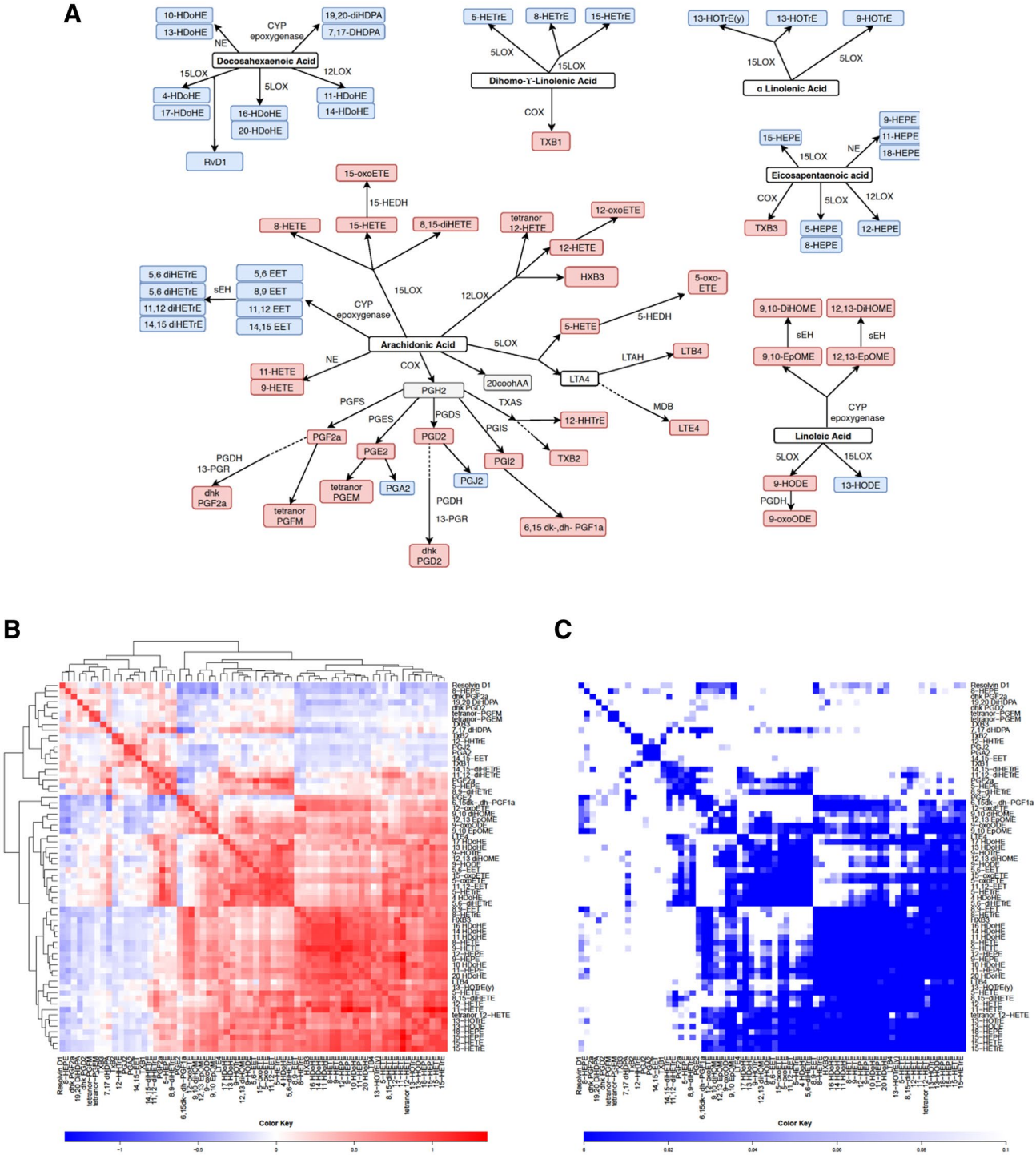

Fig. 1 Pro-inflammatory and anti-inflammatory eicosanoids. **a** Pro-inflammatory and anti-inflammatory eicosanoids detected in our patients, divided by pathways and origin PUFA. In grey, metabolites not identified by *MS COX* cyclooxygenase, *LOX* lypooxigenase, *CYP* cytochrome P450, *NE* non-enzymatic, *PGFS* prostaglandin F synthase, *PGES* prostaglandin E synthase, *PGDS* prostaglandin D synthase, *PGIS* prostaglandin I synthase, *TXAS* thromboxane A2 synthase, *LTAH* leukotriene A4 hydrolase, *MDB* membrane dipeptidase, *HEDH* hydroxyeicosanoid dehydrogenase, *PGDH* hydroxyprostaglandin dehydrogenase, *13-PGR* 15-ketoprostaglandinΔ_{13} reductase, *sEH* soluble epoxide hydrolase. **b** Heat map and hierarchical cluster analysis indicate positive relationships between circulating eicosanoids identified in blood from PsA patients (the heatmap was created using regression coefficients adjusted for BMI, BSA, NSAIDs and biological therapy). **c** Eicosanoid regression p-values are displayed in **c**, where the row and column order are preserved from **b**

Table 2 Mean (SD) of each circulating eicosanoid detected in 41 PsA patients

Eicosanoid	Mean (SD) pmol/ml	Eicosanoid	Mean (SD) pmol/ml
TxB2	27.332 (32.049)	13 HDoHE	0.43 (0.993)
PGF2a	1.269 (1.286)	5-HEPE	1.059 (2.126)
PGE2	1.029 (1.232)	8-HEPE	2.999 (2.862)
6,15dk-,dh-PGF1a	5.435 (6.886)	9-HOTrE	1.656 (1.477)
dhk PGF2a	0.813 (1.018)	5-HETrE	0.176 (0.463)
dhk PGD2	13.341 (5.663)	16 HDoHE	4.736 (6.834)
tetranor-PGFM	1.609 (3.016)	20 HDoHE	2.585 (4.27)
tetranor-PGEM	26.919 (63.712)	tetranor 12-HETE	14.622 (38.727)
9-HETE	9.747 (13.861)	12-HEPE	32.511 (45.631)
TXB1	0.278 (0.424)	11 HDoHE	6.285 (9.566)
TXB3	0.133 (0.244)	14 HDoHE	109.937 (162.784)
LTB4	0.31 (0.455)	15-HETE	14.395 (21.968)
LTE4	0.139 (0.182)	15-HEPE	0.877 (1.484)
5-HETE	5.242 (6.475)	13-HODE	53.761 (64.177)
5-oxoETE	0.203 (0.505)	13-HOTrE	3.588 (5.313)
9-HODE	21.767 (20.02)	13-HOTrE(y)	0.795 (1.427)
9-oxoODE	5.177 (4.807)	8-HETrE	0.841 (1.184)
12-HETE	581.357 (988.653)	15-HETrE	1.903 (2.789)
12-oxoETE	3.246 (7.153)	4 HDoHE	0.529 (1.323)
HXB3	7.054 (7.689)	17 HDoHE	4.201 (11.148)
8-HETE	7.764 (12.175)	Resolvin D1	0.273 (0.269)
8,15-diHETE	0.619 (1.171)	5,6-EET	0.113 (0.276)
15-oxoETE	0.349 (0.852)	8,9-EET	2.294 (5.015)
9,10 EpOME	4.258 (2.809)	11,12-EET	0.127 (0.335)
12,13 EpOME	5.052 (4.786)	14,15-EET	0.215 (0.397)
9,10 diHOME	8.081 (8.241)	5,6-diHETrE	1.894 (3.549)
12,13 diHOME	8.804 (7.916)	8,9-diHETrE	0.983 (1.393)
PGA2	0.263 (0.595)	11,12-diHETrE	0.822 (0.337)
PGJ2	0.127 (0.229)	14,15-diHETrE	1.181 (0.41)
12-HHTrE	71.954 (76.621)	19,20 DiHDPA	1.55 (0.735)
11-HETE	5.961 (8.577)	7,17 dHDPA	1.073 (0.782)
9-HEPE	3.316 (5.152)	18-HEPE	0.546 (0.768)
11-HEPE	0.67 (1.049)	10 HDoHE	2.03 (3.004)

contribute to subclinical inflammation and disease relapse (Korotkova and Jakobsson 2014).

In PsA, pharmacological therapies begin with NSAIDs, suggesting that at least COX pathway is critical in PsA pathogenesis. However, the role of LOX and cytochrome P450 (CYP) enzymes, which catalyze several AA-derived pro-inflammatory eicosanoids and most of the PUFA-derived anti-inflammatory eicosanoids, is unknown in this disease. In our study, several eicosanoids were positively correlated with parameters of joint disease activity (tender joint count -TJC-, swollen joint count -SJC-, CDAI, SDAI and DAS28CRP) including several AA-derived pro-inflammatory eicosanoids such as PGE2, HXB3 or 6,15-dk,dh,PGF1a, being HXB3 and tetranor 12-HETE, 12LOX derived eicosanoids. More interestingly several EPA-derived anti-inflammatory eicosanoids, such as 11-HEPE, 12-HEPE and 15-HEPE, were also upregulated in active patients, suggesting that EPA-derived anti-inflammatory eicosanoids amount might be increased to try to balance the inflammation induced by AA-derived pro-inflammatory eicosanoids. Yet, we also detected some DHA-derived anti-inflammatory eicosanoids that did negatively correlate with joint disease activity, including anti-inflammatory eicosanoids such as resolvin (Rv)D1 and 17-HDoHE. RvD1 is one of the pro-resolving lipid mediators shown to actively contribute to the resolution of several inflammatory processes (Serhan 2014). The injection of RvD3 reduced joint leukocytes as well as paw joint eicosanoids, clinical scores and edema in the K/BxN serum transfer inflammatory arthritis model (Arnardottir et al. 2016). Resolvins are also potential analgesics: systemic treatment with 17-HDoHE, the precursor of RvD, reduced inflammatory pain in an

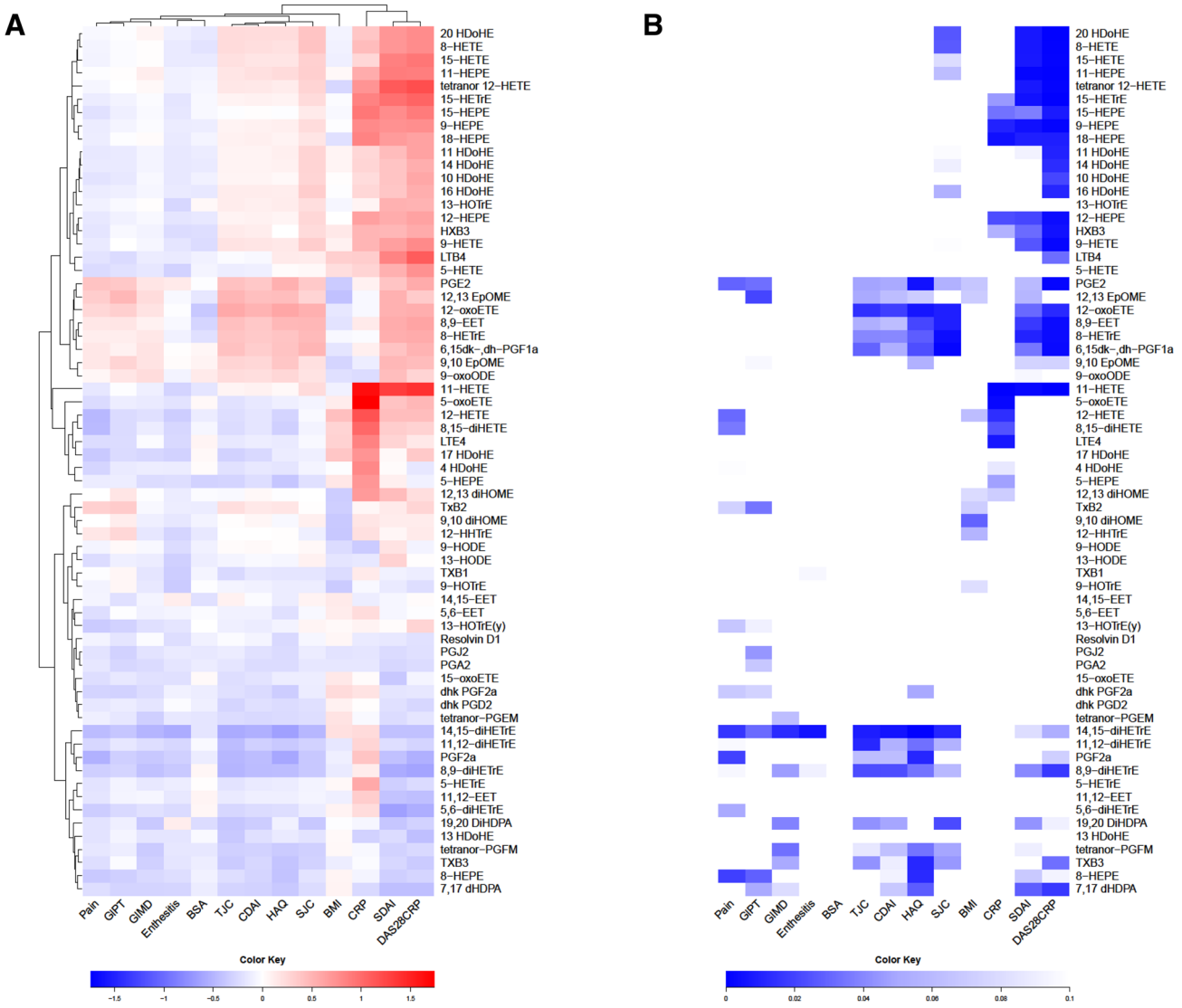

Fig. 2 Clinical score correlations with eicosanoids. **a** Linear regression was performed between each clinical score—eicosanoid pair, controlling for BMI, NSAIDs and biological therapy. We also adjusted joint disease scores (TJC, SJC, DAS28, CDAI and SDAI) for BSA, and BSA score was also adjusted for CDAI. Other factors, including comorbidities, sex and age were not found to influence eicosanoid levels and were not included in the model. The regression coefficients for each pair were used to form a clustered heatmap, to lend insight into which clinical scores were correlated with which groups of eicosanoids. Row clusters have been identified by cophenetic cutting of the row dendrogram. **b** Metabolite regression p-values are displayed in **b**, where the row and column order are preserved from **a**. *TJC* tender joint count, *SJC* swollen joint count, *CDAI* clinical Disease Activity Index, *SDAI* simple disease Activity Index, *HAQ* Health Assessment Questionnaire, *GIMD* physician global assessment, *GIPT* patient global assessment, *BMI* body mass index, *BSA* body surface area, *DAS28CRP* disease assessment score 28, *NSAIDS* non-steroidal anti-inflammatory drugs

adjuvant-induced arthritis model (Xu and Ji 2011). The fact that we detected a negative correlation between these pro-resolving lipids mediators and joint activity could suggest that a disbalance between pro- and anti- inflammatory eicosanoid species might play a role in the pathogenesis of joint inflammation in PsA and that these could be used for the treatment of inflammatory arthritis.

Although these findings are certainly promising, this study is not without limitations, the heterogeneity of our PsA patients being the main limitation. Patients had long-standing disease and were exposed to various therapies prior to the study which could represent activated pathways resistant to current DMARDs and biological therapies. Confirmation of our results with a larger sample size from prospective cohorts of patients with new onset inflammatory arthritis before treatment initiation is necessary to strengthen our conclusions. Comparison with other arthritides would help to determine if the described eicosanoid changes are specific

A

Pathway	PUFA	Eicosanoid	Mean (SD) pmol/ml DAS28CRP ≤ 2.32	Mean (SD) pmol/ml DAS28CRP > 2.32	p	p adjusted
COX	AA	PGF2a	1.6 (1.3)	0.7 (0.7)	0.01	0.03
		PGE2	0.7 (0.6)	1.4 (1.5)	0.08	0.04
		6,15dk-,dh-PGF1a	3.1 (3.6)	7.8 (8.8)	0.03	0.05
		dhk PGD2	15.1 (6.9)	11.5 (3.6)	0.05	0.06
		12-HHTrE	83.7 (81.9)	62.2 (74.4)	0.39	0.04
	EPA	TXB3	0.2 (0.3)	0	0.02	0.05
12LOX	AA	tetranor 12-HETE	9.4 (36.2)	12.4 (22.5)	0.76	0.03
		HXB3	4.1 (5.4)	8.6 (7.6)	0.04	0.06
Non-enzymatic	AA	11-HETE	4.6 (9.8)	5.4 (4.9)	0.74	0.02

B

Pathway	PUFA	Eicosanoid	Mean (SD) pmol/ml DAS28CRP ≤ 2.32	Mean (SD) pmol/ml DAS28CRP > 2.32	p	p adjusted
COX	AA	PGA2	0.4 (0.7)	0.1 (0.2)	0.11	0.02
		PGJ2	0.1 (0.2)	0 (0.1)	0.07	0.02
5LOX	DHA	20-HDoHE	1.3 (3.8)	2.9 (3.8)	0.21	0.08
12LOX	AA	12-HEPE	15.7 (26.2)	38.7 (46.6)	0.06	0.05
	DHA	14-HDoHE	59.9 (114.3)	129.1 (165)	0.13	0.03
15LOX	EPA	15-HEPE	0.5 (0.8)	0.7 (0.8)	0.34	0.09
	LA	13-HODE	48.1 (50.3)	43.7 (58.4)	0.8	0.07
	ALA	13-HOTrE	1.9 (3.6)	4.1 (5.6)	0.16	0.05
		13-HOTrE(γ)	0.5 (1.1)	0.7 (1.3)	0.6	0.04
	DHA	17-DHoHE	3 (12.6)	2.9 (6.3)	0.97	0.08
		Resolvin D1	0.3 (0.2)	0.2 (0.3)	0.52	0.04
CYP	AA	11,12 EET	0.1 (0.1)	0 (0.1)	0.29	0.04
		5,6 diHETrE	1.7 (2.1)	0.7 (0.8)	0.07	0.04
	DHA	19,20-diHDPA	1.7 (0.5)	1.3 (0.8)	0.12	0.02
Non-enzymatic	EPA	18-HEPE	0.3 (0.5)	0.5 (0.6)	0.19	0.05
		11-HEPE	0.3 (0.7)	0.7 (0.7)	0.09	0.02
	DHA	10-HDoHE	1.2 (2.5)	2.2 (2.7)	0.24	0.03
		13-HDoHE	0.4 (0.8)	0.2 (1)	0.49	0.06

C

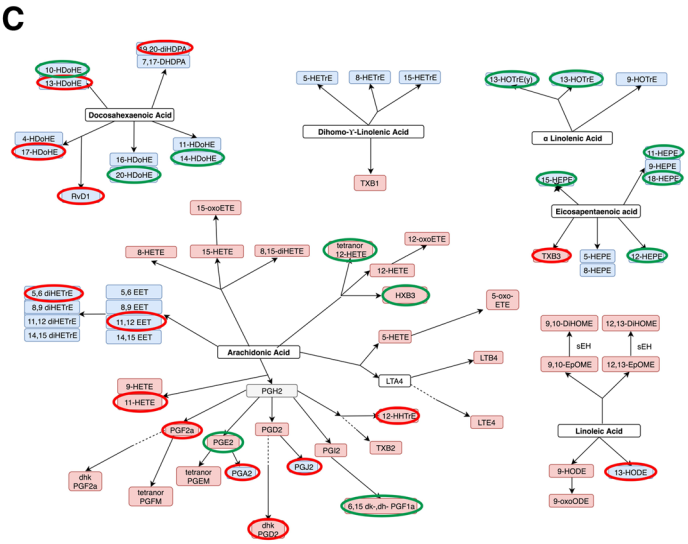

Fig. 3 Pro- and anti-inflammatory eicosanoids associated with DAS28-CRP. Logistic regression was performed between each eicosanoid (pmol/ml) in patients with DAS ≤ 2.32 compared with patients with DAS > 2.32. **a** Pro-inflammatory eicosanoids with p value < 0.1 after adjusting for BMI, BSA, NSAIDs and biological therapy. **b**) Anti-inflammatory eicosanoids with p value < 0.1 after adjusting for BMI, BSA, NSAIDs and biological therapy. Other factors, including comorbidities, sex and age were not found to influence eicosanoid levels and were not included in the model. **c** Significant eicosanoids (p < 0.1) are circled in green if upregulated in patients with DAS > 2.32, and in red if downregulated in these patients. *DAS28* disease activity score, *NSAIDs* non-steroidal anti-inflammatory drugs

to PsA or secondary to systemic inflammation. Yet, we believe that this work can improve our limited understanding of role of eicosanoids beyond arachidonic acid metabolites and leukotrienes in PsA and may lay the groundwork for a more targeted investigation of novel eicosanoid- and lipidomics-based studies in PsA.

5 Conclusions

Sensitive and scalable methods for detecting and quantifying a high number of eicosanoids are now available. In this study 66 eicosanoids were identified, and both pro- and anti-inflammatory eicosanoids were associated with joint disease scores, potentially representing pathways of harm as well as benefit. Further studies are needed to determine whether these eicosanoid species might also play a role in the pathogenesis of joint inflammation in PsA.

Author contributions MG designed and supervised the overall project. Study conception and design: MG, OQ and AK. Patients recruitment: AK, DH, TB. Acquisition of Mass Spectrometry data: AMA, SDW, OQ. Analysis and interpretation of data. RC, AK, BP, SM, MJ, TP, OQ, MG.

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Availability of data and materials All data generated or analyzed during this study are included in this published article .

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Patients were enrolled following written informed consent. Ethical approval was granted by the Institutional Review Board (IRB) at UCSD.

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10.2 Circulating Pro- and Anti-Inflammatory Metabolites and Its Potential Role in Rheumatoid Arthritis Pathogenesis

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Review

Circulating Pro- and Anti-Inflammatory Metabolites and Its Potential Role in Rheumatoid Arthritis Pathogenesis

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Abstract: Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that affects synovial joints, leading to inflammation, joint destruction, loss of function, and disability. Although recent pharmaceutical advances have improved the treatment of RA, patients often inquire about dietary interventions to improve RA symptoms, as they perceive pain and/or swelling after the consumption or avoidance of certain foods. There is evidence that some foods have pro- or anti-inflammatory effects mediated by diet-related metabolites. In addition, recent literature has shown a link between diet-related metabolites and microbiome changes, since the gut microbiome is involved in the metabolism of some dietary ingredients. But diet and the gut microbiome are not the only factors linked to circulating pro- and anti-inflammatory metabolites. Other factors including smoking, associated comorbidities, and therapeutic drugs might also modify the circulating metabolomic profile and play a role in RA pathogenesis. This article summarizes what is known about circulating pro- and anti-inflammatory metabolites in RA. It also emphasizes factors that might be involved in their circulating concentrations and diet-related metabolites with a beneficial effect in RA.

Keywords: metabolomics; microbiome; diet; lifestyle; circulating

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory arthritis that affects approximately 1% of the world's population. It is a potentially debilitating disease that affects women two to three times more frequently than men [1]. It is characterized by pain and swelling in joints and produces irreversible joint damage that negatively affects patients' quality of life in the absence of treatment. In our clinical practice, patients often mention changes in their symptoms after the consumption or avoidance of certain foods and inquire about the adequate type of diet for this disease. However, there is very little knowledge on how diet or specific ingredients affect pain and inflammation in RA. Recently, a lot of research on diet, gut microbiome, and gut-microbe-derived metabolites has focused on explaining how this diet–microbiome-metabolomic axis can explain different symptoms and overall health status.

Several studies have employed different analytical methods (mass spectrometry, MS, nuclear magnetic resonance, NMR) to characterize the metabolomic profile in the blood (serum or plasma), urine, or synovial fluid in patients with rheumatoid arthritis. Due to the heterogeneity of the methods that were used, the results of most of the studies are not comparable; however, there are metabolites with similar changes across multiple studies (Figure 1 and Table 1). The objective of this work is to review the existing evidence for the relationship between diet, metabolites, and inflammation in RA.

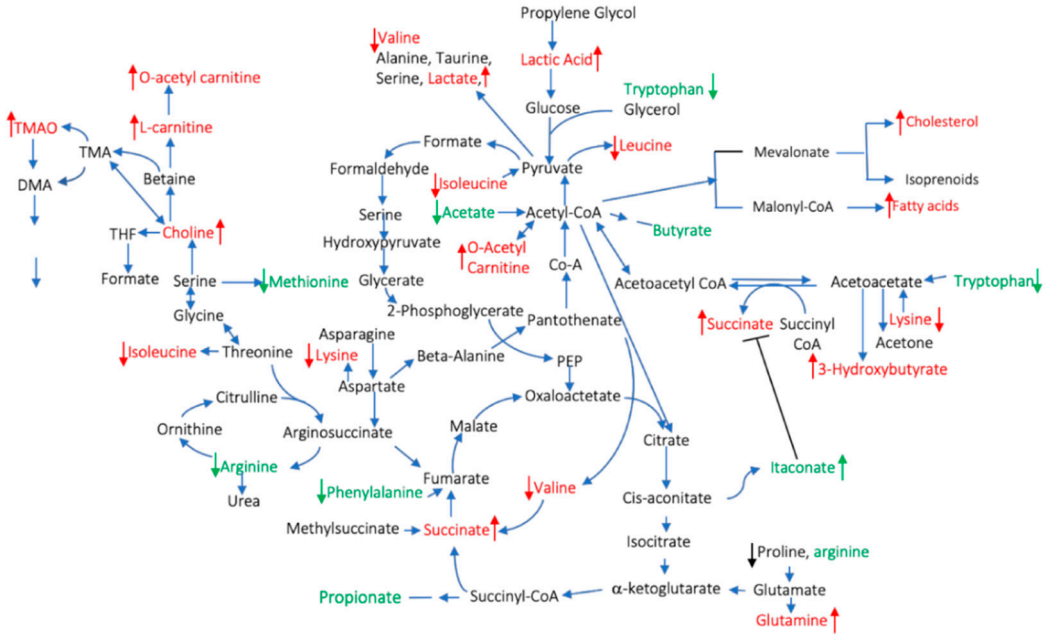

Figure 1. Pro- and anti-inflammatory circulating metabolites described in rheumatoid arthritis (RA) patients. The red color indicates pro- and the green indicates anti-inflammatory metabolites. The arrows indicate increases/decreased concentrations of the metabolites compared to healthy controls.

Table 1. Metabolic profile changes in plasma or serum of patients diagnosed with rheumatoid arthritis (RA). DMARD: disease-modifying antirheumatic drugs; GC: glucocorticoids; pSS primary Sjogren syndrome.

Type of Study	Number of Participants	Metabolite Changes
Plasma		
Prospective. RA patients vs. controls	47 RA patients on DMARDs (23 active and 24 in remission) and 51 controls. Sample collected at 0, 2, 4 weeks and 6, 12 months.	Elevated metabolites in RA patients compared to controls: choline, cholesterol, acetylated glycoprotein, lactate, and unsaturated lipid. Decreased HDL in RA patients compared to controls [2]
Cross-sectional	24 RA patients on methotrexate and less than 10 mg prednisolone daily	Positive correlation with fatigue in RA: Fructose, arachidonic acid (ARA), glycerol-3-phosphate, indole-3-acetic acid, and proline. Negative correlation with fatigue in RA: 2-oxoisocaproate, cystine, hydroxyproline, decosahexaenoic acid, tryptophan, pipecolic acid, valine, ornithine, arginine, urea, tyrosine, and linoleic acid [3]
Cross-sectional. RA patients vs. control	132 established RA patients and 104 controls	Metabolites increased in RA vs. control: prolyglycine. Metabolites decreased in RA vs. control: 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, and sarcosine. * Steroids in those with past corticosteroids treatment vs. those who never received them or are currently taking them [4]

Table 1. *Cont.*

Type of Study	Number of Participants	Metabolite Changes
Serum		
Cross-sectional. RA patients vs. controls	14 healthy controls 16 established RA patients, and two groups of early RA patients (89 and 127 RA patients)	High in RA patients compared to controls: 3-hydroxybutyrate, lactate, acetylglycine, taurine, glucose. Low in RA patients compared to healthy controls: LDL-CH3, LDL-CH2, alanine, methylguanidine, and lipid [5]
Cross-sectional. RA patients vs. controls	33 established RA patients and 32 controls	Metabolites increased in RA compared to controls: glycerol, citrate, pyruvate, cholesterol, fatty acids. Metabolites decreased in RA compared to controls: glucose, urate, alanine, serine, methionine, threonine, leucine, valine, isoleucine, aspartate, phenylalanine, tyrosine, proline, and urea [6]
Cross-sectional. RA on GC vs. RA that did not receive GC	281 RA patients 73 Males taking GC 42 Females taking GC	Higher in women on GC: lysophosphatidylcholines and lysophosphatidylethanolamines. In men, lysophospholipids levels were similar between GC users and nonusers [7]
Cross-sectional. RA and pSS patients vs. controls	30 active RA patients and 30 pSS as a disease control 32 controls	Metabolites increase in RA vs. pSS and control: L-Leucine, L-phenylalanine, glutamic acid, and L-proline, 4-methoxyphenylacetic acid. Metabolites decrease in RA vs. pSS and control: Tryptophan, argininosuccinic acid, and capric acid [8]

2. Factors That Influence Circulating Metabolites and Their Potential Role in Rheumatoid Arthritis

Metabolites reflect an organism's state, which results from the interaction of internal and external factors, such as genetic and environmental/lifestyle factors, respectively. In disease states, the circulating metabolites are also affected by the pathological processes, and there are already well-studied metabolites that are considered to be disease reporters, like the increase of blood glucose levels in diabetes mellitus. In systemic diseases such as RA, the abnormal circulating metabolomic profile might reflect genetic predisposition, local inflammation, comorbidities, and several environmental factors including diet, smoking, or microbiome (Figure 2).

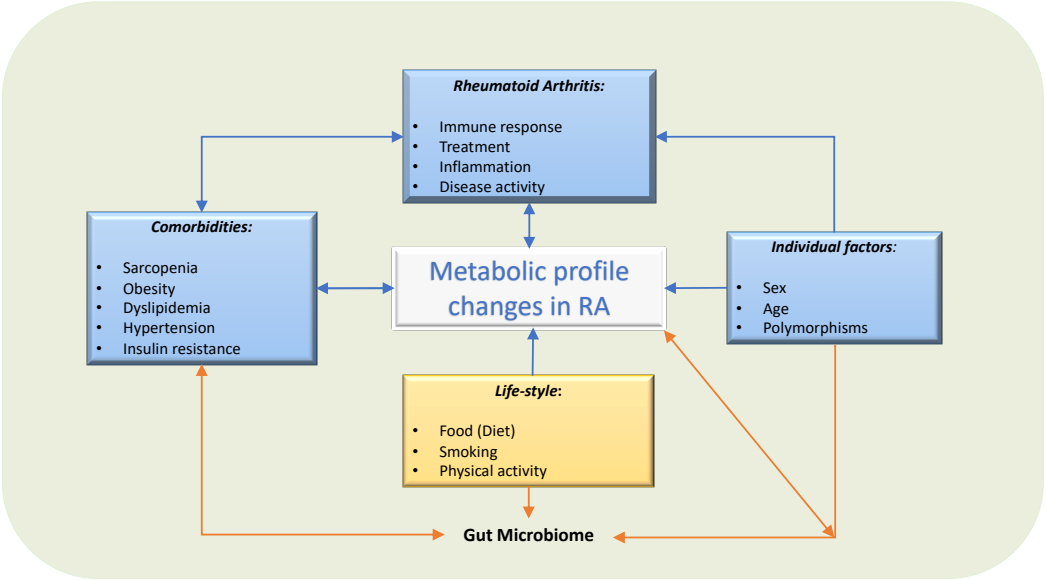

Figure 2. Factors involved in circulating metabolic profile in patients with RA. Several factors influence the circulating metabolites levels. Not only dietary factors or local synovial metabolites, but also comorbidities, treatment and individual factors, such as sex, age and genetics, will modify their metabolism, gut microbiome, and therefore, the circulating metabolic profile.

2.1. Diet

Amongst environmental factors, diet is one that directly affects circulating metabolites. For example, essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and essential fatty acids (alpha-linolenic acid and linoleic acid) come from the diet. Of interest, some of these essential nutrients were found to be low in RA patients, including linoleic acid, and several amino acids (Table 1), suggesting a link between diet and inflammation in RA.

Epidemiological studies have shown a relationship between diet and RA; thus, some of the metabolomic changes observed in several fluids (serum/plasma or urine) in early arthritis could be related to differences in dietary patterns between RA patients and the healthy population. A study that included a large number of patients (15770 adult males and females) found that patients with arthritis (including RA and osteoarthritis) had lower quality diets compared to people without arthritis, based on HEI-2015, a healthy eating index created by the USDA and based on the Dietary Guidelines for Americans [9]. Patients with arthritis consumed less fruit, vegetables, greens and beans, whole grains, seafood, and plant protein, but more added sugars, saturated fats, and empty calories compared to those without arthritis [10]. The association of poor dietary quality with RA was also observed in other studies, in which RA patients had an inadequate intake of fruit, vegetables, dairy, fatty acids, and whole grains [11–13]. A study in a Chinese population found that RA patients were consuming different amounts of chicken, fish, mushrooms, beans, citrus, dairy products, and organ meats than healthy controls [14]. Another study that included a white population found that both women and men on a nonvegetarian diet were at higher risk of developing RA [15]. Hu et al. analyzed the cohort of women included in the Nurses' Health Study and Nurses's Health Study II that were followed from 1984 to the present-day, and found that good dietary quality, moderate alcohol consumption, and low intake of red meat were associated with a lower rate of RA incidence [13].

In the past, due to advances in the field of metabolomics, efforts have been made to predict food intake by measuring blood/urine/fecal metabolites. Two main techniques are being used: MS coupled with liquid- or gas-phase chromatography and proton (^{1}H) NMR [16]. There are numerous metabolomics studies that have identified candidate biomarkers for different dietary patterns, as well as for different types of foods, ranging from meat to fruits and vegetables. Table 2 shows a summary

of foods and the metabolites that have been found to be markers of their intake using metabolomics. Several studies have also found metabolites related to dietary patterns, like Mediterranean, high fat, or Western diets [17–21].

Table 2. Food intake and candidate biomarkers identified by mass spectrometry (MS) and/or nuclear magnetic resonance (NMR).

Type of Food	Sample Type	Candidate Biomarker Metabolite
Meat (red meat, low-fat meat, chicken)	Urine Plasma	1-Methylhistidine; 3-methylhistidine; acetyl carnitine; creatinine; taurine; carnitine; trimethylamine N-oxide; creatine; histidine; urea; anserine; carnosine; guanidoacetate [19,22–25]
Beef	Plasma	β-Alanine; 4-hydroxyproline; 2-aminoadipic acid; leucine [26]
Fish	Urine Plasma	Trimethylamine N-oxide; anserine; 1-methylhistidine; 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; docosahexaenoic acid (DHA); eicosapentaenoic acid (EPA); 1-docosahexaenoylglycero- phosphocholine; cetoleic acid [25–31]
Vegetables		
Vegetarian and lactovegetarian diet)	Urine	p-Hydroxyphenylacetate Hippurate; phenylacetylglutamine; lysine; hippurate; N-acetyl glycoprotein; succinate [19,32,33]
Broccoli	Urine	Ascorbate; tetronic acids; l-xylonate/l-lyxonate; naringenin glucuronide [28]
Onion	Urine	*N*-acetyl-S-(1Z)-propenyl-cysteine-sulfoxide 4-Ethyl-5-amino-pyrocatechol [34]
Lettuce, spinach, green peppers	Serum	3-Carboxy-4-methyl-5 -propyl-2-furanpropanoic [30]
Cabbage, brussels sprouts, pointed cabbage	Urine	*N*-acetyl-*S*-(*N*-3-methylthiopropyl)cysteine; *N*-acetyl-*S*-(*N*-allylthiocarbamoyl)cysteine; iberin *N*-acetyl-cysteine; erucin *N*-acetyl-cysteine; *N*-acetyl-(*N*′ -benzylthiocarbamoyl)-cysteine; sulforaphane *N*-acetyl-cysteine; sulforaphane *N*-cysteine,3-Hydroxy-hippuric acid sulfate; 3-hydroxy-hippuric acid; iberin N-acetyl-cysteine [29]
Fruit		
Apples and pears	Urine	Phloretin [35,36]; rhamnitol [34]
Citrus	Urine	Proline betaine; limonene 8,9-diol glucuronide; nootkatone 13,14-diol glucuronide; hesperetin 3′-O-glucuronide; hydroxyproline betaine; *N*-methyltyramine sulfate; naringenin 7-O-glucuronide; stachydrine; scyllo- and chiro-inositol [28,30,35–40]
Orange juice	Urine	*N*-methyl proline; methyl glucopyranoside (α+β); stachydrine; betonicine; *N*-acetyl putrescine; dihydroferulic acid [41]

Table 2. *Cont.*

Type of Food	Sample Type	Candidate Biomarker Metabolite
Raspberries	Urine	Sulfonated caffeic acid; methyl-epicatechin sulfate; 3-hydroxyhippuric acid; naringenin glucuronide; ascorbate [28]
Strawberries	Urine	4-Hydroxyhippuric acid; 4-hydroxy-2,5-dimethyl- 3(2H)-furanone (furaneol) glucuronide; pelargonin glucuronide; p-coumaric acid sulfate; dihydrokaempferol glucuronide; furaneol sulfate; 2,5-dimethyl-4-methoxy -2,3-dihydro-3-furanone (mesifurane); mesifurane sulfate; leucopelargonidin; catechin sulfate [28]
Cereals		
Whole-grain rye	Urine	Alkylresorcinol metabolites; caffeic acid sulfate; hydroxyhydroxyphenyl acetamide sulfate; 3,5-dihydroxyphenylpropionic acid sulfate; hydroxyphenyl acetamide sulfate [31]
Whole-grain sourdough rye bread	Urine Plasma	Benzoxazinoid derivatives; hydroxylated phenyl acetamide derivatives; sulfonated hydroxyl-*N*-(2-hydroxyphenyl) acetamide; *N*-(2-hydroxyphenyl)acetamide; 2,4-dihydroxy- 1,4-benzoxazin-3-one; 1,3-benzoxaxazol-2-one [42,43]
Whole-grain bread	Urine	Glucuronidated alk(en)ylresorcinols; 2-hydroxy-*N*-(2-hydroxyphenyl) acetamide; 2-hydroxy-1,4-benzoxazin-3-one glycoside; 3-(3,5-dihydroxyphenyl) propanoic acid glucuronide; 5-(3,5-dihydroxyphenyl) pentanoic acid sulfate; dihydroferulic acid sulfate; enterolactone glucuronide; pyrraline; 3-indolecarboxylic acid glucuronide; 2,8-dihydroxyquinoline glucuronide [43]
Dairy products		
Cheese	Urine	Indoxyl sulfate; xanthurenic acid; tyramine sulfate; 4-hydroxyphenylacetic acid; isovalerylglutamic acid; acylglycines; 3-phenyllactic acid [44]
Butter	Urine	3-Phenyllactic; alanine, proline; pyroglutamic acid; methyl palmitate (15 or 2); pentadecanoate (15:0); 10-undecenoate (11:1n–1) [30]
Milk	Urine Serum Plasma	Trimethyl-*N*-aminovalerate; uridine; hydroxysphingomyelin C14:1; diacylphosphatidylcholine C28:1; lactose; galactose; galactonate; allantoin; hippurate; galactitol; galactono-1,5-lactone [44–46]

Table 2. *Cont.*

Type of Food	Sample Type	Candidate Biomarker Metabolite
Beverages		
Coffee	Urine	Caffeic; chlorogenic acid; Dihydrocaffeic acid-3-O-sulfate; feruloylglycine [35,47] Atractyligenin glucuronide; diketopiperazine cyclo(isoleucyl-prolyl); trigonelline; paraxanthine; 1-methylxanthine, 1-methyluric acid, 1,7-dimethyluric acid, 1,3- or 3,7-dimethyluric acid; 1,3,7-trimethyluric acid; 5-acetylamino-6 -formylamino-3-methyluracil [48]
	Serum/Plasma	Trigonelline (*N*′-methylnicotinate); quinate; 1-methylxanthine; paraxanthine; *N*-2-furoyl-glycine; catechol sulfate [30] Pathways: xanthine metabolism; benzoate metabolism; steroid; fatty acid metabolism (acylcholine); endocannabinoid [49]
Black tea	Urine	Hippuric acid; 1,3-dihydroxyphenyl-2-O-sulfate gallic; 4-O-methylgallic acids [35,50]
Black/Green tea	Urine	Hippuric acid; 1,3-dihydroxyphenyl-2-O-sulfate; hydroxybenzoic glycine conjugate; vanilloylglycine; pyrogallol-2-O-sulfate [51–53]
Wine	Urine	Tartaric acid, microbial-derived phenolic metabolites (5-(dihydroxyphenyl)-γ-valerolactones and 4-hydroxyl-5-(phenyl)-valeric acids) [54]
	Plasma	Gallic acid and ethylgallate metabolites; resveratrol and resveratrol microbial metabolites; 2,4-dihydroxybenzoic acid; (epi)catechin; valerolactone metabolites [55]
Other		
Walnuts	Urine	10-Hydroxy-decene-4,6-diynoic acid sulfate; tridecadienoic/tridecynoic acid glucuronide; sulfate conjugates of urolithin A; 3-indolecarboxylic acid glucuronide; 5-Hydroxyindole-3-acetic acid [29,56]
Peanuts	Urine	4-Vinylphenol sulfate; tryptophan betaine [30]
Cocoa	Urine	Theobromine metabolism (AMMU; 3-methyluric acid; 7-methylxanthine; 3-methylxanthine; 3,7-dimethyluric acid; theobromine). Polyphenol microbial metabolites [methoxyhydroxyphenylvalerolactone; glucuronide and sulfate conjugates of 5-(3′,4′ -dihydroxyphenyl)-valerolactone] [57,58]
Chocolate	Urine	6-Amino-5-[*N*-methylformylamino] -1-methyluracil; theobromine; 7-methyluric acid [29]

The identification of food biomarkers is an ongoing process; a consensus has not been reached as to which metabolites would be the most adequate biomarkers for different types of foods. Moreover, some metabolites are markers of categories of food, not being able to discriminate between the exact types of foods being analyzed (1-methylhistidine and 3-methylhistidine are found in meat and are not useful in discriminating between types of meat). It is possible that for some foods, a combination of metabolites would be more suited as a marker than a single metabolite. Unfortunately, as of now, the metabolomic studies in RA (Table 1) have not collected food intake data nor used the same metabolomic platforms, making it difficult to associate specific food intake with metabolic changes in RA patients. However, some metabolites from Tables 1 and 2 suggest an interaction between circulating metabolites and diet. For instance, some of these studies [5,59] showed higher levels of carnitine and taurine in RA patients, which are potential biomarkers of meat intake.

2.2. Drugs

Researchers have used a metabolomics approach to evaluate the changes in circulating metabolites from drugs used in RA treatment (Table 3). The study of these changes might help to understand RA pathogenesis, since the therapeutic effects of these drugs could potentially be driven by metabolic changes either by normalizing their abnormal values or by increasing anti-inflammatory metabolites. For instance, using a targeted metabolomic approach, Fu et al. compared the effect of oral glucocorticoids (GC) on serum polar lipids and observed an increase in lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE) in females but not in male patients with RA [7]. GC inhibits phospholipase A, a key enzyme that hydrolyzes membrane phospholipids which is increased in inflammatory tissues. The effect of GC on phospholipase A will likely modify the phospholipid profile. Of interest, polyunsaturated acyl LPC and LPE presented an anti-inflammatory effect on animal models [60]. The effect of low dose GC (<10 mg/day) on arginine metabolism and cardiovascular risk in RA patients was also studied [61]. This study from Australia that included 36 RA patients, 18 of which were on GC (GC users) and 18 that were not receiving GC (non-GC-users), found that asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA) levels were lower in patients on chronic GC compared to non-GC users, suggesting that long-term treatment with GC had an improved endothelial function and a cardiovascular protective effect. by modulating arginine metabolism [61].

Wang et al. [59] studied the change of the plasma metabolic profile in 29 RA patients after the initiation of treatment with methotrexate (14 patients) or a combination of methotrexate with a Chinese medicinal herb (15 patients). They found decreased levels of several amino acids (tryptophan, threonine, histidine, methionine, and glycine) as well as other metabolites (carnitine, hypoxanthine, cytosine, uracil, and uric acid), while taurine, aspartate, alanine, lactic acid, adenosine, and guanine were significantly increased in RA patients compared to controls. Interestingly, the treatment with methotrexate brought the levels of all these metabolites back to normal levels, suggesting a causative role of these amino acids in RA pathogenesis. The combination of MTX with tripterygium glycosides tablets was more effective in obtaining these results compared to monotherapy with MTX. Although more data is needed to link amino acid changes to abnormal immune response in RA, data in immune cells suggest a direct link between amino acid metabolism and T cell and macrophage responses by promoting and modulating inflammation, which could potentially be involved in RA pathogenesis [62–65]. In RA, tryptophan is the substrate of indoleamine-2,3-dioxygenase IDO2, which was demonstrated to be required for the activation of CD4+ Th cells, the production of pathogenic autoantibodies, and the subsequent development of arthritis in a KRN mouse model of arthritis [66–68]. This offers a possible explanation for the decrease in tryptophan levels that is then reversed by the addition of methotrexate. On the other hand, levels of S-adenosy-L-homocysteine, 5-formyltetrahydrofolate, and 5-methyltetrahydrofolate were similar between controls and RA patients before treatment, and decreased after 3 months of methotrexate, pointing to these methotrexate-associated metabolites as adherence biomarkers [59].

Table 3. Metabolic profile modifications by drugs used in the treatment of RA.

Samples	Decreased	Increased
Methotrexate [59]		
Plasma	Taurine, aspartate, alanine, hypoxanthine, cytosine, uric acid, uracil, lactic acid, S-adenosyl-L-homocysteine, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate.	Tryptophan, threonine, histidine, methionine, glycine, carnitine, guanine, and adenosine.
Glucocorticoids		
Serum [7]	None reported	Lysophosphatidylethanolamines and lysophosphatidylcholines (Females).
Plasma [61]	Asymmetric dimethyl arginine, symmetric dimethyl arginine	None reported.
Anti-tumor necrosis factor (TNF)		
Serum [69]	3-hydroxyisobutyrate, lysine, acetoacetate, acetylphosphocholine, creatine sn-glycero-3-phosphocholine, histidine, and phenylalanine.	Leucine, acetate, betaine, and formate.
Serum [70]	3-hydroxybutyrate.	Isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose.
Urine [71]	Eanolamine, p-hydroxyphenylpyruvic acid, and phosphocreatine.	Hippuric acid, citrate, and lactic acid (Infliximab). Choline, phenylacetic acid, urea, creatine, and methylamine (Etanercept). Histamine, glutamine, phenylacetic acid, xanthine, xanthurenic acid, and creatinine.

TNF is a potent pro-inflammatory cytokine that plays key role in cell metabolism, including glucose and lipid metabolism [72]; thus, changes in metabolic profile are expected after the administration of a TNF inhibitor. The first study that evaluated the changes in the metabolic profile of 16 RA and psoriatic arthritis (PsA) patients after TNFi treatment (etanercept and infliximab) used urine samples. The study described increases in hippuric acid, citrate, and lactic acid after infliximab treatment, while increases in choline, phenylacetic acid, urea, creatine, and methylamine were seen after etanercept treatment [71]. Another group evaluated the serum metabolomic profile in 20 patients with RA before and after treatment with TNFi (etanercept or adalimumab). Of the 20 patients, 55% of patients had a moderate EULAR response, while only 20% reached a good response. At 3 months posttreatment, 3-hydroxyisobutyrate, lysine, acetoacetate, acetylphosphocholine, creatine sn-glycero-3-phosphocholine, histidine, and phenylalanine levels decreased, while leucine, acetate, betaine, and formate levels increased, but they did not reach those of the healthy control [69]. The changes of the serum metabolic profile in response to treatment with a TNFi, etanercept, in 27 patients with active RA were also evaluated by Priori et al. These patients were receiving concomitant therapy with GC and disease-modifying antirheumatic drugs. After 3 months of treatment, isoleucine, leucine, valine, alanine, glutamine, tyrosine, and glucose levels were found to be increased in good responders as defined by EULAR-ESR criteria, whereas 3-hydroxybutyrate levels were reduced [70]. The decrease of 3-hydroxybutirate, acetoacetate, and acetylphosphocholine levels suggests a modulation of lipid metabolism after TNF inhibition, especially in responders. In addition, the increase of glucose and other amino acids suggests a decrease of glucose and amino acid metabolism by the inflamed tissues.

2.3. Comorbidities

RA patients present several comorbidities including obesity, metabolic syndrome, and sarcopenia, probably triggered by a disbalance of proinflammatory cytokines including TNF and IL-6 among other causes [73–77], that will modify the circulating metabolites [78]. Several studies have investigated circulating metabolic changes related to the metabolic syndrome and obesity [79,80]. Of interest, a lot of circulating metabolites that are different in RA patients compared to controls could be related to associated metabolic syndrome, since choline metabolism (especially TMAO and carnitine), aminoacids (alanine, glutamine, glutamate, arginine, aspartate, asparagine, histidine, methionine, cysteine, lysine,

branched-chain amino acids (BCAA), phenylaniline, tyrosine, and tryptophan) and phospholipids (phosphatydilcholines) also change in those with metabolic syndrome [80]. Several works on muscle mass have also suggested that some circulating metabolites can be biomarkers of muscle mass and sarcopenia [81]. Even though both fat tissue and muscle, as well as associated immune cells in these inflamed tissues, can be sources of metabolites, it is unknown how much they can contribute to the pool of circulating metabolites. For example, studies measuring the metabolomics profile in visceral adipose tissue and serum from obese patients found low correlations between serum and adipose tissue metabolites [82]. On the other hand, we can speculate that there might be a competition between inflamed tissues (adipose tissue vs. synovial tissue) for the uptake of circulating anti-inflammatory metabolites.

2.4. Sex and Age

Several epidemiological studies have shown differences in metabolite concentrations according to sex and gender. A cross-sectional study in urine samples showed that some metabolites from the tricarboxylic acid cycle (TCA) cycle such as citrate and fumarate were elevated in women, while carnitine, acetylcarnitine, acetone, and creatinine were higher in men [83]. In addition, Fan et al. found that 2-hydroxyglutaric acid, α-ketoglutarate, and 2-oxyglutaric acid were higher in women. However, UDP-glucoronic acid was higher in men, suggesting that this could be linked to sex hormones [84]. Another study showed differences between sex and metabolic profile in serum, suggesting that glycine, serine, and sphingomyelines are upregulated in women, and ornithine, arginine, acyl carnitines, and amino acids derived from glutamine pathway are elevated in males [85]. Finally, a longitudinal cohort of adults showed a positive correlation of levels of glutamine, tyrosine, long chain fatty acids, acyl-carnitines, and sphingolipids, and a negative correlation of histidine, tryptophan, threonine, serine, and leucine levels with age [86].

2.5. Smoking and Exercise

Smoking is a known risk factor for RA and is associated with an increased risk of more severe arthritis, and less likelihood of achieving remission. Smoking also decreases the effectiveness of some disease-modifying antirheumatic drugs (DMARDs) [87,88]. The exact reason of these associations is not well understood, although the effect of smoking on immune cells, and cytokine production, and the increase of oxidative stress that it causes, have been described [89–91], and these likely affect the immune response in RA. Metabolomics has also identified blood biomarkers associated with chronic tobacco smoking. One study performed on a large number of healthy participants (892) from around the world found an association between smoking and three well-established nicotine metabolites (cotinine, hydroxycotinine, and cotinine N-oxide), and an additional 12 xenobiotic metabolites involved in benzoatic (e.g., 3-ethylphenylsulphate) or xanthine metabolism (e.g., 1-methylurate), three amino acids (o-cresol sulphate, serotonin, indolepropionate), two lipids (scyllo-inositol, pregnenolone sulphate), four vitamins or cofactors, and one carbohydrate (oxalate) [92]. Several of these metabolites, especially nicotine-derived metabolites, have been described to modulate the immune response [90], and other metabolic changes could be involved in smoking-induced methylation changes in immune cells [93]. Another study looked at the immediate effects of smoking on the metabolic profile. Thirty-one metabolites were shown to be acutely affected by cigarette smoking, including menthol-glucuronide, the reduction of glutamate, oleamide, and 13 glycerophospholipids. Moreover, detailed analysis revealed changes in 12 cancer-related metabolites, notably related with cAMP inhibition [94]. Since a known mechanism of methotrexate in treatment of RA is to induce an increase of cellular cAMP [95], the inhibition of this metabolite by smoking could explain the decrease in the effectiveness of this drug in RA.

Exercise is another factor that might change the metabolomic profile. However, these changes depend of the quantity and type of exercise. For example, in people who exercise more than 2 h per day, some metabolites, including medium and long fatty acids, ketones, sulfated bile acids, palmitate,

linoleate, stearate, and palmitoleate, increased two-fold in their plasma concentration. Decreases of pyruvate and lactate, among others intermediates of TCA, have been reported after a short running period [96]. The reader can find an extensive review of these changes in a recently published review [96] about metabolic changes after exercising.

2.6. Genetics: Polymorphisms and Metabolism

Genome-wide association studies (GWAS) uncovered multiple loci that are associated with the level of metabolites, which involve a large number of metabolic pathways, indicating widespread genetic influences on the human metabolome (Figure 3). The loci that have been described involve amino acids, intermediates of lipid metabolism, including sterols, carnitines, and intermediates of inositol and fatty acid metabolism, intermediates of purine and pyrimidine metabolism, glucose homeostasis, and vitamin and cofactor levels [97–100]. Polymorphisms in these metabolite-associated genes were also described in RA GWAS. In Figure 3, we put together a summary of metabolism-related genes described in genome-wide association studies (GWAS; https://www.ebi.ac.uk/gwas/). Polymorphisms in the genes underlined in red were found to be associated with RA. These genes are mostly related to lipid metabolism. Interestingly, lipid metabolites are considered pro-inflammatory metabolites (see Section 3), and higher levels of lipids were described in serum of RA patients compared to control subjects (Table 1). *DLG2* (Discs Large MAGUK Scaffold Protein 2), which was found to be associated with glycerophospholipid metabolism [101], was also found to be related to response to TNF inhibitors in RA patients [102]. *FADS1 and 2* (Fatty Acid Desaturase) and *BLK* (BLK Proto-Oncogene, Src Family Tyrosine Kinase), involved in fatty acid metabolism, and *STAG1* (Stromal Antigen 1) and *FCGR2B* (Fc Fragment Of IgG Receptor IIb), involved in lipoprotein metabolism, were found to be associated with susceptibility to developing RA in several studies [103–107]. SLC22A4, a transporter related to isovaleryl/carnitine, was found to be associated with RA in a Japanese population [108], but not in a Canadian one [109]. Finally, Geiger et al. described 2 SNPs (single nucleotide polymorphism), rs9309413 and rs4775041, found on PLEK (Pleckstrin) and LIPC (Hepatic Triacylglycerol Lipase) genes, associated with sphingomyelin associated and phosphatidylethanolamine (PE) [110], that were associated with RA in a previous study [111]. Little is known about the role of these genes in inflammation and autoimmunity, so more studies are needed to determine whether some of these pathways are critical for the pathogenesis of RA.

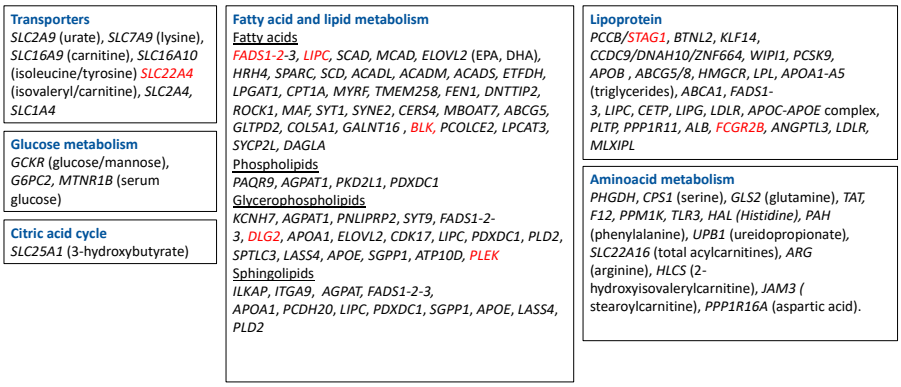

Figure 3. Metabolism-related genes described in GWAS. Highlighted in red are the genes that were found to be associated with RA. SLC2A9—Solute Carrier Family 2 Member 9; SLC7A9—Solute Carrier Family 7 Member 9; SLC16A9—Solute Carrier Family 16 Member 9; SLC16A10—Solute Carrier Family 16 Member 10; SLC22A4—Solute Carrier Family 22 Member 4; SLC22A4—Solute Carrier Family 2 Member 4; SCL1A4—Solute Carrier Family 1 Member 4; SLC25A1—Solute Carrier Family 25 Member 1; FADS—fatty acid desaturase; LIPC—Hepatic Triacylglycerol Lipase; SCAD—Short-chain acyl-CoA dehydrogenase; MCAD—Medium-chain acyl-CoA dehydrogenase; ELOVL2—Fatty Acid Elongase 2; HRH 4—Histamine Receptor 4; SPARC—Secreted Protein Acidic And Cysteine Rich; SPTLC3—Serine

Palmitoyltransferase Long Chain Base Subunit 3; LASS4—Ceramide Synthase 4, SGPP1—Sphingosine-1-phosphate, ATP10D—ATPase Phospholipid Transporting 10D (Putative), SCD—Stearoyl-CoA Desaturase; ACADL—Acyl-CoA Dehydrogenase Long Chain; ACADM—Acyl-CoA Dehydrogenase Medium Chain; ACADS—Acyl-CoA Dehydrogenase Short Chain; ETFDH—Electron Transfer Flavoprotein Dehydrogenase; LPGAT1—Lysophosphatidylglycerol Acyltransferase 1; CPT1—Carnitine Palmitoyltransferase 1; PHGDH—Phosphoglycerate Dehydrogenase; CPS1—Carbamoyl-Phosphate Synthase 1; GLS2—glutaminase; EPA—eicosapentaenoic acid; DHA—docosahexaenoic acid; GCKR—Glucokinase Regulator; APOA—Apolipoprotein; MYRF—Myelin Regulatory Factor; TMEM258—transmembrane protein 258; FEN1—flap structure-specific endonuclease 1; DNTTIP2—deoxynucleotidyl transferase terminal interacting protein 2; ROCK1—Rho Associated Coiled-Coil Containing Protein Kinase 1; MAF—MAF BZIP Transcription Facto; SYT1—Synaptotagmin 1; SYNE2—Spectrin Repeat Containing Nuclear Envelope Protein 2; CERS4—Ceramide Synthase 4; MBOAT7—Membrane Bound O-Acyltransferase Domain Containing 7; ABCG5—ATP Binding Cassette Subfamily G Member 5; GLTPD2—Glycolipid Transfer Protein Domain Containing 2; COL5A1—Collagen Type V Alpha 1 Chain; GALNT16—Polypeptide N-Acetylgalactosaminyltransferase 16; BLK—BLK Proto-Oncogene, Src Family Tyrosine Kinase; PCOLCE2—Procollagen C-Endopeptidase Enhancer 2; LPCAT3—Lysophosphatidylcholine Acyltransferase 3; SYCP2L—Synaptonemal Complex Protein 2 Like; DAGLA—Diacylglycerol Lipase Alpha; PAQR9—Progestin And AdipoQ Receptor Family Member 9; AGPAT1—1-Acylglycerol-3-Phosphate O-Acyltransferase 1; PKD2L1—Polycystin 2 Like 1, Transient Receptor Potential Cation Channel, PDXDC1—yridoxal Dependent Decarboxylase Domain Containing 1; KCNH7—Potassium Voltage-Gated Channel Subfamily H Member 7; PNLIPRP2—Pancreatic Lipase Related Protein 2 (Gene/Pseudogene); SYT9—Synaptotagmin 9; DLG2—Discs Large MAGUK Scaffold Protein 2; CDK17—Cyclin Dependent Kinase 17; PDXDC1—Pyridoxal Dependent Decarboxylase Domain Containing 1; PLD2—Phospholipase D2; APOE—Apolipoprotein E; ILKAP—ILK Associated Serine/Threonine Phosphatase; ITGA9—Integrin Subunit Alpha 9; PCDH20—Protocadherin 20; GCKR—Glucokinase Regulator; G6PC2—Glucose-6-Phosphatase Catalytic Subunit 2; MTNR1B—Melatonin Receptor 1B; PCCB—Propionyl-CoA Carboxylase Subunit Beta; STAG1—Stromal Antigen 1; BTNL2—Butyrophilin Like 2, KLF14—Kruppel Like Factor 14, CCDC9—Coiled-Coil Domain Containing 9; DNAH10—Dynein Axonemal Heavy Chain 10; ZNF664—Zinc Finger Protein 664; WIPI1-WD Repeat Domain, Phosphoinositide Interacting 1; PCSK9 -, Proprotein Convertase Subtilisin/Kexin Type 9; APOB—Apolipoprotein B; HMGCR—3-Hydroxy-3-Methylglutaryl-CoA Reductase; LPL—Lipoprotein Lipase; ABCA1—ATP Binding Cassette Subfamily A Member 1; CETP—Cholesteryl Ester Transfer Protein; LIPG—Lipase G, Endothelial Type; LDLR—Low Density Lipoprotein Receptor; PLTP—Phospholipid Transfer Protein; PPP1R11—Protein Phosphatase 1 Regulatory Inhibitor Subunit 11; ALB—Albumin; FCGR2B—Fc Fragment Of IgG Receptor IIb; ANGPTL3—Angiopoietin Like 3; MLXIPL—MLX Interacting Protein Like; GLS2—Glutaminase 2, TAT—Tyrosine Aminotransferase; F12—; Coagulation Factor XII; PPM1K—Protein Phosphatase, Mg2+/Mn2+ Dependent 1K, TLR3—Toll Like Receptor 3; HAL—Histidine Ammonia-Lyase; PAH—Phenylalanine Hydroxylase; UPB1—Beta-Ureidopropionase 1; SLC22A16—Solute Carrier Family 22 Member 16; ARG—Arginase; HLCS—Holocarboxylase Synthetase; JAM3—Junctional Adhesion Molecule 3; PPP1R16A—Protein Phosphatase 1 Regulatory Subunit 16A.

2.7. Gut Microbiome/Absorption

The gut microbiome represents the collection of microbes that inhabit the intestines. Its composition is shaped by several factors, like genetics, age, delivery pattern, diet, antibiotic use, and other treatments [112–115]. It can also be modulated by prebiotics [116,117], probiotics [118], and fecal microbiota transplantation. Bacteria in the gut are important not only in the absorption of certain vitamins and in the synthesis of bile acids, but they also have the potential to modify circulating pro- or anti-inflammatory mediators, since they are involved in the metabolism of some dietary components [119]. For example, trimethylamine-*N*-oxide, a pro-inflammatory metabolite that derives

from choline and carnitine present in red meat, eggs, and dairy products, is produced by *Prevotella copri* among other bacteria [120,121]. An increased abundance of *Prevotella copri* was found in new-onset untreated RA patients, suggesting *P. copri* may be pathogenic in this disease [122]. In contrast, bacteria that have an almost exclusive saccharolytic metabolism, such as lactobacilli and bifidobacterial, are considered potentially beneficial [123], since they produce a variety of tryptophan catabolites (indole, tryptamine, indoleethanol (IE), indolepropionic acid (IPA), indolelactic acid (ILA), indoleacetic acid (IAA), skatole, indolealdehyde (IAld), and indoleacrylic acid (IA)) which are critical for intestinal homeostasis by decreasing intestinal permeability [124]. In addition, some of these catabolites enter the bloodstream and may have anti-inflammatory and anti-oxidative effects [124]. The microbial degradation of whole-grain complex carbohydrates increases short-chain fatty acids (SCFA; butyrate, acetate and propionate), which were also shown to be beneficial to the intestinal immune response [125]. Microbial bile acid metabolites have recently been linked to colonic homeostasis [126].

The modulation of the microbiome through diet interventions is a potential strategy in the treatment of diseases, since microbiome alterations are related to disease, i.e., inflammatory bowel disease, obesity, cardiovascular diseases, autoimmune diseases, and others. It seems that the microbiome response to diet is variable and is highly influenced by the subject's baseline microbiome. Several studies found differences in the baseline microbiome of responders versus nonresponders to different diet interventions. Additionally, individuals with differing bacterial gene richness appear to have differing baseline gut microbiota communities that respond distinctively to a given dietary intervention which will influence the diversity of the gut microbe-derived specialized metabolites and circulating metabolites [127–136].

2.8. Metabolite Released from or Uptaken by Inflamed Tissues

Another potential factor that determines the concentrations of the circulating metabolites is represented by the release of metabolites from the inflamed joint or their uptake by the synovium. Little is known about metabolic or lipidomic profiling of synovial tissue [137,138]. In addition, no study has, to date, evaluated the relation between circulating metabolites in serum or plasma and synovial metabolites, although there might be a correlation. For instance, the synovial tissue of RA patients presents an enhanced level of lactate compared to noninflamed synovial tissue [138], which suggests an increase in the anaerobic cellular metabolism of resident cells [139,140]. Lactate has also been one of the metabolites described to be upregulated in patients with RA [5]. Of note, inflammatory pathways increase the expression of nutrient transporters [141–144]; therefore, this highly metabolic tissue will consume high amounts of metabolites, either to feed the increased metabolism of activated cells (pro-inflammatory metabolites) or to resolve inflammation (anti-inflammatory metabolites); this could be reflected by a decrease of circulating metabolites described in RA (Table 1 and Figure 1): such as glucose and amino acids (alanine, serine, methionine, threonine, leucine, valine, isoleucine, aspartate, phenylalanine, tyrosine, and proline) [6,145].

Fibroblast-like synoviocites (FLS), key cells in the pathogenesis and progression of RA, have an activated metabolism and can potentially release metabolites into the bloodstream [146]. Ahn et al. [147] characterized the intracellular metabolic profile of RA and osteoarthritis (OA) by an untargeted metabolomic approach using GC/TOF-MS. The results revealed that a high number of metabolites were increased in RA compared to OA FLS; these metabolites were amines (inosine, urate, 5′-deoxy-5′-methylthioadenosine, guanine, benzamide), fatty acids (behenic acid, palmitoleic acid, arachidic acid, oleic acid, myristic acid, stearic acid, palmitic acid, octadecanol, linoleic acid, lauric acid), phosphates (glucose-6-phosphate, phosphogluconic acid, adenosine-5-monophosphate, phosphate, fructose-6-phosphate), organic acids (aspartate, adipate, 2-ketoisocaproate 3-phenyllactate, 2-hydroxyvaleric acid, phenylacetate, glycolate, oxalate, benzoate), amino acids (asparagine, glutamine), sugars and sugar alcohols (lactose fucose, mannose) and salicylaldehyde. Other metabolites, mostly amino acids (isoleucine, leucine, histidine, valine, ornithine, lysine, methionine sulfoxide, tryptophan, N-methylalanine, tyrosine, phenylalanine, citrulline, oxoproline, threonine, serine) were decreased in

RA compared to OA FLS. At the same time, the glycolysis and pentose phosphate pathways were more activated in RA than OA FLS. RA FLS are aggressive cells, similar to cancer cells, and require high amounts of energy to fulfill their pathogenetic functions in RA, which include proliferation, migration, and invasion [148,149].

Macrophages and T cells are the other dominant type of synovial cells in the inflamed joint, and are important in the progression of the disease, with their abundance being correlated with disease activity but also response to treatment [150]. Similar to the FLS, activated macrophages and T cells also rely on glycolysis and have alterations of the TCA cycle [150], which is consistent with the high levels of lactic acid, citrate, and succinate found in the synovial fluid of RA patients [151]. Although metabolic profiling of RA synovial macrophages and T cells hasn't yet been undertaken, they are probably a source of circulating metabolites, while metabolites also exert their effect on synovial cells [152–155].

3. Evidence for a Pro-/Anti-Inflammatory Role of Metabolites in RA

RA is a chronic autoimmune disease, with a systemic immune response to autoantigens that may exist years before the onset of clinical symptoms, and a local immune activation of the synovial tissue which becomes inflamed, hyperplastic, and invasive of local cartilage and bone [156]. Whether or not pro- or anti-inflammatory metabolites play a role in RA pathogenesis is still unknown. However, the explosive growth of the field of tissue immunometabolism and its description of multiple critical metabolic pathways in the activation and differentiation of immune cells such as T and B lymphocytes, macrophages, dendritic cells, and fibroblasts, among others (see reviews in [157–161]), suggests that most of the metabolites involved in the immune response can also be important in RA. Here, we describe pro- and anti-inflammatory metabolites associated with RA pathogenesis (Figure 4).

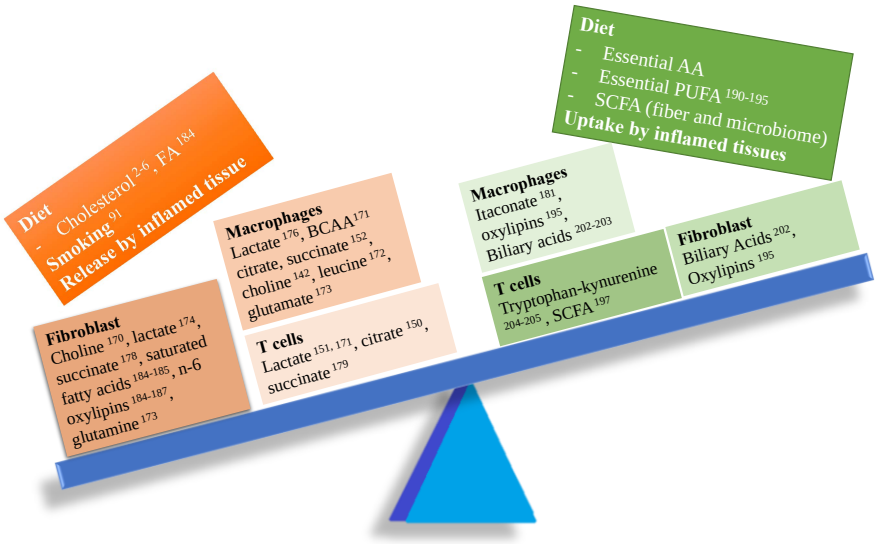

Figure 4. Imbalance between pro- and anti-inflammatory metabolites in RA. Several pro-inflammatory metabolites (left side of the balance) might play a key role in RA pathogenesis modulating the function of several cell types involved in synovial inflammation.

3.1. Pro-Inflammatory Metabolites

Choline and Trimethylamine-N-oxide (TMAO). Metabolites related to the choline pathway were identified in several studies in synovial tissue, synovial fluid, and blood (serum/plasma) samples in both animal models and human studies. Diet is the main source of choline [162], whose metabolites (trimethylamine-N-oxide, TMAO) have already been related with cardiovascular inflammation [121,163]. Choline and other dietary trimethylamine (TMA) containing species like carnitine are metabolized to TMA by the gut microbiota. TMA is subsequently oxidized by at least one

member of the flavin-containing monooxygenases, FMO3, forming trimethylamine-N oxide (TMAO), which is then released into circulation [164]. TMAO is a candidate biomarker for meat and fish intake, as can be seen in Table 2.

Despite being so well studied in relation to cardiovascular inflammation, we were not able to find studies evaluating the role of TMAO in RA. Our group found that serum TMAO was associated with measures of joint (tender joint count, swollen joint count, DAS28-CRP) and skin inflammation (body surface area affected by psoriasis) in a small cohort of patients with psoriasis and PsA [165]. The increased TMAO in patients with psoriasis and PsA, two diseases associated with metabolic syndrome [166], could be due either to an increased activity of FMO3, which has been described to be upregulated in obesity [167], but also to changes in the microbiome composition, which is an intermediate component of TMAO synthesis. TMAO, as well as choline, was found to be increased in serum samples in the murine K/BxN model of arthritis compared to control mice [168]. Choline is also a nutrient uptaken by the cells and metabolized via the Kennedy pathway, during which several phospholipids that function as signaling molecules are produced, such as glycerol-phosphocholine (GPC), phosphocholine, phosphatidylcholine (PC), lyso-PC, diacylglycerol, and lysophosphatidic acid [169]. Importantly, choline metabolism has been related to the RA FLS phenotype [170] and IL-1β secretion in macrophages [142].

BCAA (Branched-chain amino acids). Decreased levels of valine, leucine, and isoleucine were found in RA patients. Decreased levels of BCAA could be explained by low dietary consumption or by a higher intake of these amino acids by inflamed tissue. These are essential amino acids, so their source is the diet, and lately, they have been related to inflammation by inducing oxidative stress (via NADPH and Akt-mTOR signaling) and promoting the secretion of proinflammatory cytokines (IL-6, TNF) as well as the migration of peripheral blood mononuclear cells [171]. Branched chain aminotransferases1 (BCAT1), an enzyme that initiates BCAA metabolism, is the predominant isoform in human primary macrophages. Its action on leucine produces acetyl-CoA and glutamate, which enter the TCA cycle. Treatment of LPS and TNF stimulated human macrophages with ERG240, a leucine analogue that blocks BCAT1 activity, decreased oxygen consumption and glycolysis. Moreover, oral administration of ERG240 reduced the severity of collagen-induced arthritis in mice [172].

Glutamine is an amino acid used as a source to fuel metabolism. Glutaminase 1, the enzyme responsible of glutaminolysis, is upregulated in RA synovial fibroblasts [173], and inhibition of this enzyme decreased the aggressive phenotype of the FLS and improved the severity of arthritis in the SKG murine model of arthritis.

Glycolytic intermediates: The RA joint is characterized by a shift of the aerobic oxidative phosphorylation to a glycolytic state, in which less ATP is produced but at a faster rate, to be able to ensure the necessary energetic requirements for the highly active cells. Metabolites related to the glycolytic pathway have been detected in several studies on animal models, as well as human metabolomics studies.

Lactate is the end product of glycolysis, a metabolic pathway that is upregulated in activated FLS and macrophages. High concentrations of lactic acid are found in both blood and synovial fluid from inflamed joints in RA patients. Several studies have shown that lactate promotes the aggressive phenotype of FLS [174], the pro-inflammatory properties of macrophages [152–154], stimulates IL-17 secretion by CD4+ T cells and, at the same time, decrease CD4+T migration, which is related to the maintenance of a chronic inflammatory infiltrate [155,175]. Moreover, recently, a new lactate induced histone modification was described, lactylation, which correlates with the levels of lactate and is different from acetylation [176]. These findings require further study to evaluate their role in disease states, since altered epigenetic marks have been recently described in RA FLS [177].

Succinate is elevated in the synovial fluid of patients with RA [151]. The TCA metabolite promotes inflammation by stimulating IL-1β secretion in murine macrophages through HIF-1α [152]. Moreover, succinate activates NLRP3 inflammasome inducing IL-1β secretion by synovial fibroblasts in a rat model of RA [178]. It seems that succinate also plays a role in innate and adaptive immune responses.

Researchers found that the genetic deficiency of Sucnr1, a succinate receptor expressed by immune cells, decreases trafficking of dendritic cells and reduces expansion of Th17 cells in the lymph nodes, reducing the symptoms of arthritis in the mouse antigen-induced arthritis model [179].

Itaconate, a macrophage activation marker, is thought to play an anti-inflammatory role, since it inhibits the succinate dehydrogenase-mediated oxidation of succinate, and through this, exerts anti-inflammatory effects in activated macrophages, as shown in an in vivo model of ischemia-reperfusion injury [180]. However, in an animal model of RA, higher levels of itaconate were found to be associated with high disease activity [181].

Cholesterol comes from the diet and its levels are increased in RA patients [2,6]; this was found to be predictive of RA in women, but not men [182]. Lipid metabolism is altered in RA, but cholesterol metabolism in RA has not been specifically studied. Interestingly, cholesterol was recently found to be high in OA chondrocytes, due to an increased uptake, upregulation of cholesterol hydroxylases, and increased production of oxysterol metabolites [183].

Free fatty acids (FFA) can be either taken from the diet (essential FA, alpha-linolenic acid -an omega-3 FA- and linoleic acid -an omega-6 FA-) or synthesized in the organism. it was suggested that they were proinflammatory, since they contribute to low-level inflammation in obese patients. FFA levels were higher in the serum of RA patients than in control subjects, and correlated with disease activity [184]. Frommer et al. showed that FFA contribute to the pathogenesis and damage in RA, OA, and PsA, since stimulation of FLS with oleic, palmitic, and linoleic acid induced the secretion of proinflammatory cytokine IL-6, the chemokines IL-8 and MCP-1, as well as the matrix metalloproteinases pro-MMP1 and MMP3 [185]. Arachidonic acid (ARA) is the precursor of classically described prostaglandins (PGE2), which are known to be involved in inflammation in general, but also in arthritis [186,187].

3.2. Anti-Inflammatory Metabolites

Polyunsaturated Fatty Acids (PUFA) Related Metabolites. Docosahexaenoic acid, DHA, and eicosapentaenoic acid, EPA have anti-inflammatory properties, mainly because they compete with ARA for the action of the enzymes (cyclooxygenase-COX, lypooxigenase-LOX, cytochrome P450) which results in a decreased production of ARA derived proinflammatory oxylipins and an increased production of DHA and EPA derived anti-inflammatory oxylipins (Figure 5) [188,189]. Several studies have described improved outcomes in RA patients after dietary intervention [188,190,191]. Decreased levels of EPA and DHA were described in Spanish RA patients, and were associated with higher disease duration, positivity for rheumatoid factor, erosive disease and with a worse response to TNF inhibitors [192]. Gene variants of the enzymes involved in the PUFA metabolism can determine the metabolic and clinical response to dietary intake of PUFA. For example, 5-lipoxygenase (ALOX5) gene variants were found to influence response to fish oil supplementation, changing the oxylipin profile and, consequently, having a different effect on cardiovascular risk [193]. Another study checked the association between genetic variants of ALOX5, ALOX12, ALOX12B, and ALOX15, and type-2 diabetes mellitus (T2D), and found that ALOX12 and ALOX12B genetic variants increased susceptibility to T2D development, possibly though alterations in PUFA/ARA metabolism.

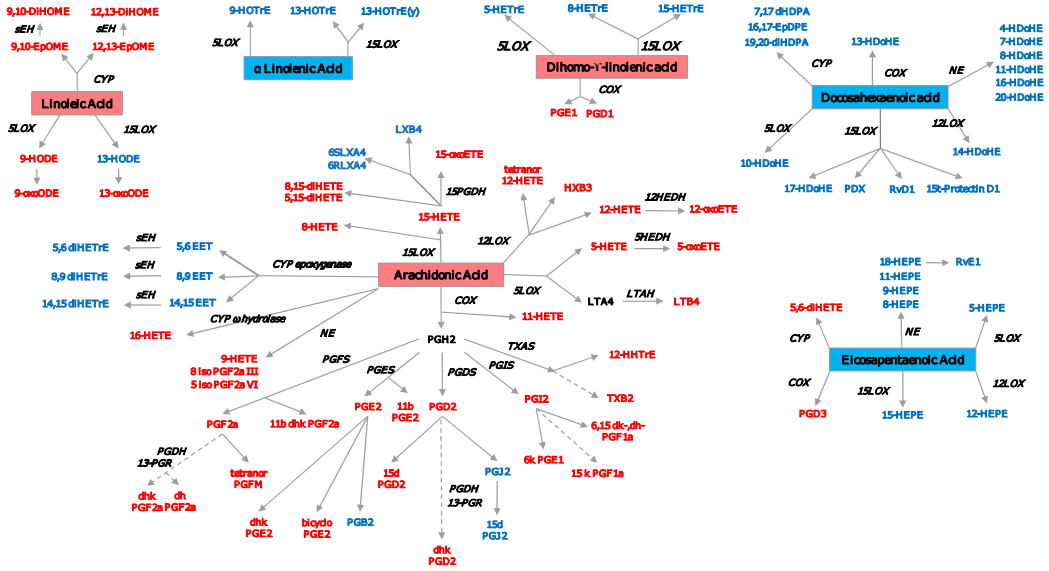

Figure 5. Oxylipin derived from PUFA. Pro-inflammatory oxylipins are marked in red, while anti-inflammatory ones are marked in blue. The precursor n3-PUFAs are marked in a red square, while the n6-PUFAs are marked in a blue square. COX—cyclooxygenase; LOX—lypooxigenase; CYP—cytochrome P450; NE—nonenzymatic; PGFS—prostaglandin F synthase; PGES—prostaglandin E synthase; PGDS—prostaglandin D synthase; PGIS—prostaglandin I synthase; TXAS—thromboxane A2 synthase; LTAH—leukotriene A4 hydrolase; MDB—membrane dipeptidase; HEDH—hydroxyeicosanoid dehydrogenase; PGDH—hydroxyprostaglandin dehydrogenase; 13-PGR—15-ketoprostaglandinΔ13 reductase; sEH—soluble epoxide hydrolase. A list with all the oxylipins can be found in Supplementary Table S1.

Oxylipin Related Pathways. Prostaglandins, thromboxanes and leukotrienes are the classically described oxylipins involved in the pathogenesis of RA. The newer methods of LC/MS and NMR make it possible to identify several other oxylipins, e.g., 8-HETE, 12-HETE, and 12-HEPE are products of the 12-lypoxygenase pathway. Liagre et al. demonstrated the presence of 12-LOX in type B synoviocytes and found that IL-1β and TNF stimulation increased 12-HETE production, while IL-6 and IL-4 did not have the same effect [194]. This pathway was also studied by Kronke et al., who showed that the deletion of 12/15-LOX in two models of arthritis (the K/BxN serum-transfer and a TNF transgenic mouse model) led to uncontrolled inflammation and tissue damage [195]. LTB4 and 5-HETE are products of ARA via 5-LOX pathway; 5- and 15-LOX are expressed in both OA and RA synovium in the lining and sublining macrophages, neutrophils, and mast cells, and have been shown to be involved in RA pathogenesis, promoting inflammation [196].

Short Chain Fatty Acids (SCFA) are byproducts of the metabolism of dietary fiber by the gut microbiome. They modulate immune and inflammatory responses via the activation of free fatty acid (FFA) receptors type 2 and 3 (FFA2 and FFA3 receptors) and G protein-coupled receptor 109A (GPR109A), and via inhibition of histone deacetylases (HDACs). A metabolomic study performed on a CIA rat model found decreased levels of acetate, propionate, butyrate, and valerate in fecal samples of arthritic rats compared to controls [197]. The administration of butyrate inhibited collagen-induced arthritis via Treg/IL10/Th17 axis [198].

Bile acids (BA) seem to have anti-inflammatory properties. Primary BAs are synthesized in the liver and are liberated in the gastrointestinal tract to help with lipid digestion. Gut bacteria metabolize primary bile acids and can deconjugate them, synthesizing secondary BAs. BAs have been detected in the systemic circulation, where their concentrations vary with diet, and have been related to insulin resistance [199]. High concentrations of BA can actually kill intestinal bacteria to prevent colonization, but they also regulate the mucosal immune functions through several receptors. A recent study showed

the role of BA in the maintenance of the homeostasis of the mucosal immune function in the gut, through the vitamin D receptor [126]. An in vitro study found that taurolithocholic acid suppressed the expression of genes involved in mediating pro-inflammatory effects, phagocytosis, interactions with pathogens and autophagy, as well as the recruitment of immune cells, such as NK cells, neutrophils and T cells [200]. BA exert their actions through both specific and nonspecific receptors. The activation of TGR5 receptor by endogenous BA suppressed the production of LPS induced inflammatory cytokines in macrophages, while no effect was seen in macrophages that lacked this receptor [201,202]. Of interest, a study described an anti-inflammatory role of taurochenodeoxycholic acid in RA FLS [203]. Most studies focused on the effects of the BA on the gut mucosal immunity, and hence, future studies are needed to elucidate the roles of circulating BAs in disease states.

Tryptophan metabolism. Tryptophan is an essential amino acid that must be provided in the diet. It has been described that microbes-derived tryptophan metabolites can exert systemic and anti-inflammatory effects [124]. Moreover, tryptophan and its catabolic metabolites generated through the kynurenine pathway are involved in inflammation. Kynurenine has known anti-inflammatory effects that are toxic to T cells and induce cell death by apoptosis. Kynurenine is formed from tryptophan by the activity of indoleamine 2, 3-dioxygenase (IDO). The activation of IDO is actively involved in the resolution of arthritis in mice associated with an increase in kynurenine metabolites [204]. Kynurenine itself has been identified as a ligand for the aryl hydrocarbon receptor, which is important in the maturation of immune cells, and its addition promotes the differentiation of regulatory T cells and suppresses the differentiation of pathogenic Th17 cells [205].

4. Studies of Beneficial Effect of Diet in RA

In spite of the growing evidence of the relationship between diet and RA symptoms, research in the field is still limited to mostly observational studies; however, there are quite a few interventional studies in which diet has been evaluated as a strategy to improve RA symptoms. A detailed review of the studies can be found here [206–208]. Most interventions combine a diet with high intake of vegetables, fruit, and antioxidants with periods of fasting. The outcomes used in the majority of the studies include tender and swollen joint, disability index score, general health assessment scores, and a few inflammatory markers that can be quantified in blood, i.e., C reactive protein, erythrocyte sedimentation rate and proinflammatory cytokine (IL-6, TNF alpha).

These dietary intervention studies don't shed any light on the specific metabolites that are responsible for the effect, or their mechanism of action. One study evaluated the anti-inflammatory effects of a low ARA diet and fish oil in patients with RA. Besides the usual outcomes, this study also quantified fatty acids and eicosanoids, using radioimmunoassay and gas chromatography coupled with MS [209]. They found that the diet improved RA clinical signs. In terms of fatty acid changes, they observed an enrichment of eicosapentaenoic acid in erythrocyte lipids and lower formation of urinary leukotriene B(4), 11-dehydro-thromboxane B(2) and prostaglandin metabolites in patients receiving the fish oil diet, especially when fish oil was given for a longer period of time (up to 8 months).

The concentrations of plasma phospholipid related fatty acids were evaluated after a vegan and lacto-vegetarian diet intervention in RA patients. It was found that 20:3n-6 and 20:4n-6 were significantly reduced after 3.5 months of vegan diet, but the concentration increased to baseline values with a lactovegetarian diet. Also, 20:5n-3 was significantly reduced after both vegan and lactovegetarian diet periods. However, no significant difference in fatty acid concentrations was detected between diet responders and diet nonresponders after both diet periods, which suggests that the changes in the fatty acid concentrations were not in response to diet [210].

Another study in an RA Swedish population found that EPA and DHA were both increased in erythrocytes after a blue mussel diet compared to a control diet, resulting in a decrease of n-6 PUFA ARA (20:4 n-6) and dihomo-gamma-linolenic acid (DGLA; 20:3 n−6), as well as a small decrease in saturated fatty acids and the monounsaturated fatty acid palmitoleic acid (16:1 n-7). Baseline EPA and DHA levels in this population were higher than in healthy men and women, but this group had

already reported that the RA population from the studied area had a higher fish and shellfish intake compared to the general population of Sweden [211]. In contrast to these findings, erythrocyte levels of α-linolenic acid (ALA; 18:3n3), EPA 20:5n3, and the omega-3 index (EPA plus DHA) were found to be significantly lower in RA patients compared to healthy controls in a Korean population, although diet was not accounted for in this study. In addition, EPA and ALA were negatively associated with the risk of RA in Korean women [212].

Additionally, the study of other autoimmune diseases does not help in establishing a link between diet and metabolites with their pro or anti-inflammatory effect. In Crohn's disease, for instance, animal studies suggest the potential beneficial effect of short chain fatty acids, tryptophan, arginine, and glutamine due to their roles in the modulation of the immune system, but no clinical studies have been performed to date [213]. A cross-sectional metabolomics study also found decreased levels of essential PUFA in patients with lupus, but diet was not taken into account in this study [214]. Further studies are needed before we can make conclusions about the role of diet in the levels of circulating and local metabolites and their relationship with clinical outcomes in RA and other autoimmune diseases.

5. Conclusions

Metabolomics studies have clearly shown that there is an alteration of the metabolic profile in patients with RA which may be related to the pathogenesis of the disease, but also to exposure to external factors, since the levels of the metabolites are influenced by several factors, including genetic ones, diet, sex, drugs, comorbidities, and microbiome (Figure 1). The possibility of altering some of these factors represents an attractive approach for future therapeutically interventions. Diet is a modifiable factor, and studies have shown that it can be effective in improving RA symptoms. Understanding the complex relation between diet, metabolites, microbiome, and disease status is still an ongoing process, but existing studies are promising. The field of RA needs more studies, including mendelian randomization studies and randomized clinical trials, combining the use of metabolomics, transcriptomics, and the microbiome to help understand how these elements interact with each other, identify patients who would benefit from a dietary intervention, and design the correct intervention.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/4/827/s1. Table S1. Oxylipin abbreviations and names.

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