UNIVERSITÉ DU QUÉBEC À MONTRÉAL

MOLECULAR CHARACTERIZATION OF RHEUMATOID ARTHRITIS BY MASS SPECTROMETRY-BASED PROTEOMICS AND METABOLOMICS TOWARDS TRANSLATIONAL BIOMARKERS

THESIS

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CARACTÉRISATION MOLÉCULAIRE DE LA POLYARTHRITE RHUMATOÏDE PAR DES APPROCHES DE PROTÉOMIQUE ET DE MÉTABOLOMIQUE FONDÉES SUR LA SPECTROMÉTRIE DE MASSE, EN VUE DE L'IDENTIFICATION DE BIOMARQUEURS À VISÉE TRANSLATIONNELLE

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“Science means constantly walking a tightrope between blind faith and curiosity; between expertise and creativity; between bias and openness; between experience and epiphany; between ambition and passion; and between arrogance and conviction — in short, between and old today and a new tomorrow.” — Henrich Rohrer, Nobel Prize in Physics (1986)

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LIST OF ABBREVIATIONS

2D-GE	Two-dimensional Gel Electrophoresis
2D-DIGE	Two-dimensional Difference in Gel Electrophoresis
ACN	Acetonitrile
ACPA	Anti-citrullinated protein antibodies
CCP	Cyclic citrullinated peptide
CNS	Central nervous system
CS	Cushing's Syndrome
DAP	Differentially abundant Protein
DAS	Disease Activity Score-28
DMA	Dimethylamine
DMARDs	Disease-modifying anti-rheumatic drugs
DTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray ionization
FDA	Food and Drug Administration
FDR	False discovery rate
FLS	Fibroblast-like Synoviocytes.
FTMS	Fourier Transform ion cyclotron Mass Spectrometer
GH	Growth hormone
GPR101	G protein-coupled receptor 101
GWAS	Genome-wide association
HAS	Human serum albumin
HLA	Human leucocyte antigen
IAA	Iodoacetamide
IgG	Immunoglobulin gamma
IR	Insulin resistance
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
m/z	Mass-to-charge ratios
MALDI	Matrix-assisted laser desorption ionization
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
NCD	Noncommunicable Disease
NETs	Neutrophil extracellular traps
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
PA	Pituitary adenomas
PCA	Principal Component Analysis
PMF	Peptide mass fingerprinting
PTM	Post-Translational Modification
PTMs	Posttranslational modifications
RA	Rheumatoid arthritis

RF	Rheumatoid factor
rhGH	Recombinant human growth hormone
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis
SF	Synovial fluid
TCR	T cell receptor
TFA	Trifluoroacetic Acid
TNFi	Tumour necrosis factor inhibitor
TOF	Time-Of-Flight
UPLC Q-TOF	Ultra-performance liquid chromatography quadrupole time of flight
WHO	World Health Organization

Résumé

La polyarthrite rhumatoïde (PR) est une maladie auto-immune chronique qui touche environ 1 % de la population mondiale, entraînant une destruction progressive des articulations, une inflammation systémique et un handicap. Malgré les avancées thérapeutiques, de nombreux patients, en particulier ceux présentant une maladie installée et de longue durée, continuent de souffrir d'une inflammation résiduelle et de douleurs chroniques. De plus, l'hétérogénéité clinique et la compréhension incomplète des mécanismes moléculaires contribuent à la variabilité des réponses thérapeutiques et aux résistances observées. Le diagnostic repose encore sur des marqueurs sérologiques indirects tels que le facteur rhumatoïde (FR) et les anticorps anti-protéines citrullinées (ACPA), dont la sensibilité et la spécificité sont limitées, ainsi que sur des marqueurs inflammatoires élevés tels que la vitesse de sédimentation érythrocytaire (VS) pour suivre l'évolution et la rémission de la maladie. Un obstacle majeur au diagnostic de la PR réside dans l'absence de biomarqueurs moléculaires validés, peu invasifs, permettant un diagnostic précis, une stratification des patients, un suivi efficace et une prédiction de la réponse thérapeutique. Malgré les progrès des biothérapies et des petites molécules, la PR est de plus en plus reconnue comme un trouble multifactoriel impliquant une signalisation immunitaire dysrégulée, un métabolisme cellulaire altéré et une rupture de l'homéostasie tissulaire. Ce processus inflammatoire sous-jacent découle d'interactions complexes entre cellules immunitaires et non immunitaires, gènes et environnement, qui perturbent l'équilibre physiologique et biochimique global de l'individu. Cette pathogenèse complexe et ses signatures moléculaires nécessitent encore d'être élucidées. Les technologies « omiques » à haut débit, notamment la protéomique et la métabolomique, offrent un potentiel considérable pour révéler des voies moléculaires, identifier des biomarqueurs fiables et orienter des thérapies ciblées grâce à une approche de biologie des systèmes permettant de décoder cette complexité. Toutefois, ces approches restent encore sous-exploitées dans le diagnostic et la pratique clinique de la PR.

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects approximately 1% of the global population, leading to progressive joint destruction, systemic inflammation, and disability. Despite advances in therapeutic strategies, many patients, particularly those with established and longstanding disease still continue to experience residual inflammation and chronic pain. Moreover, clinical heterogeneity and incomplete understanding of molecular mechanisms contribute to variable patient outcomes and therapeutic resistance. Diagnosis remains dependent on indirect serological markers such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), which lack sensitivity and specificity, and on elevated inflammatory markers such as erythrocyte sedimentation rate (ESR), to monitor the disease progression and remission. A major barrier to diagnosing RA is the lack of validated, minimally invasive molecular biomarkers that can accurately diagnose, stratify, and monitor patients, as well as predict therapeutic response. Despite advances in biological and small-molecule therapies, RA is increasingly recognized as a multifactorial disorder involving dysregulated immune signaling, altered cellular metabolism, and breakdown of tissue homeostasis. There exists an underlying inflammatory process that arises due to complex interactions between immune and nonimmune cells, the genes and the environment that affects the overall physiological and biochemical balance of an individual. This complex underlying pathogenesis of RA's interactions and molecular signatures need to be further elucidated. High-throughput omics technologies including proteomics and metabolomics have the potential to uncover molecular pathways, discover reliable biomarkers, and guide targeted therapies using a system biology approach that allows us to decode this complexity. However, these approaches remain underutilized in RA diagnostics and clinical practice.

1.1 Central Problem and Rationale

Traditional approaches to understanding RA pathogenesis have focused on single pathways or limited immune markers. These methods do not completely to capture the dynamic molecular landscape that underlies disease initiation, progression, and response to treatment. There is an urgent need to characterize RA using unbiased, high-dimensional analyses that can pinpoint novel proteins and metabolites driving inflammation and tissue damage. Furthermore, bridging omics discoveries with diagnostic utility remains a key challenge. While mass spectrometry can identify potential biomarkers, translation to point-of-care tools requires robust, sensitive methods for biomolecule capture and detection. We aim to achieve this by characterizing patients' circulating plasma proteome and metabolome in established RA through a hypothesis-generating strategy as opposed to a hypothesis testing one. This will allow us to carry out an exploratory, discovery-based omics investigation by employing an untargeted proteomic and metabolomic analysis. The significant proteins identified will be validated using the multiple reaction monitoring (MRM) liquid chromatography-mass spectrometry method (LC-MSMS). This thesis addresses these gaps through a two-pronged multiomics approach: (1) proteomic profiling of RA tissues to uncover dysregulated proteins and (2) metabolomic analysis to identify disease-associated metabolic pathways.

1.2 Thesis Objectives

The overarching goal of this research is to provide a molecularly informed view of rheumatoid arthritis using omics technologies and to enable translational applications. This PhD thesis is focused on the molecular characterization of RA through the application of mass spectrometry-based proteomics and metabolomics. The aim in this thesis is to identify reliable specific non-invasive and sensitive biomarkers for disease monitoring and stratification. Accordingly, this thesis is divided into two parts with three main objectives. The first part is dedicated to the proteomic profiling of dysregulated proteins in RA, with a focus on plasma that is known to contain the circulating markers reflecting the overall systemic changes. Using advanced liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), protein alterations linked to altered metabolic and signaling pathways are characterized. Findings from this will be used to carry out bioinformatics analysis and generate network pathway maps that relate to protein protein interactions and focus on dysregulation of pathways with disease.

The second part of the thesis focuses on metabolomic profiling, employing untargeted mass spectrometry to examine alterations in the different metabolic and biochemical pathways related immune system, to changes in amino acid metabolism, lipid remodeling, and energy pathways. These analyses will reveal key dysregulated metabolites, such as those related to glycolysis, the tricarboxylic acid cycle, and fatty acid oxidation, which not only differentiate RA patients from controls but also highlight mechanisms of persistent inflammation in chronic disease. The identified metabolites will then be used to develop network pathway maps using bioinformatics analysis highlighting the metabolite to metabolite interactions and metabolite to protein interactions.

The specific objectives are (i) to characterize the proteomic profile of established RA cases using high resolution mass spectrometry coupled to bioinformatics to identify dysregulated proteins linked to disease pathology, followed by validation of significant proteins using MRM, (ii) to investigate metabolic perturbations in RA patients using high resolution untargeted metabolomics, coupled to bioinformatics analyses to identify underlying pathophysiology of disease and (iii) to discuss the broader use of metabolomics in endocrine disease.

In Chapter 1 I will present a brief background on RA, summarizing the current knowledge, including its pathophysiology, clinical heterogeneity, diagnostic limitations, and the need for improved biomarkers. I will also introduce mass spectrometry-based proteomics and metabolomics as complementary approaches for molecular characterization of the disease that in turn will provide a rationale for the thesis and lay down the foundation for the work. In Chapter 2 I will present a comprehensive proteomic study using high-resolution mass spectrometry to compare protein expression between RA patients and matched healthy controls. Key proteins and pathways involved in immune dysregulation, cell signaling, and extracellular matrix remodeling are identified, shedding light on disease mechanisms and candidate biomarkers. In Chapter 3, I will next expand on the molecular characterization of the metabolome, using LC-MS to uncover dysregulated metabolic pathways in RA. Findings from this part of the work will highlight alterations in metabolites belonging to amino acid metabolism, lipid processing, and nucleic acid metabolism. Chapter 4 will describe the broader use of metabolomics in understanding endocrine-related rare diseases.

The present thesis is situated within this context. By applying mass spectrometry based proteomics and metabolomics to patients with longstanding RA, it aims to characterize the molecular landscape of chronic disease, where persistent inflammation, treatment resistance, and systemic complications often dominate. This work is not only intended to extend biomarker discovery beyond early RA, where most studies have concentrated, but also to generate insights into the biology of chronic disease. Ultimately, the goal is to identify translational biomarkers that bridge molecular pathophysiology with clinical utility, contributing to the broader vision of precision medicine in rheumatoid arthritis. It will also provide novel insights into the molecular architecture of longstanding RA and underscore the translational potential of omics-based approaches. By focusing on patients with chronic disease, this thesis expands biomarker discovery beyond early RA and contributes to the broader goal of precision medicine, where therapeutic strategies are guided by molecular profiles rather than broad clinical categories.

CHAPTER 1 — INTRODUCTION

TOWARD MOLECULAR PRECISION IN RHEUMATOID ARTHRITIS: OMICS-DRIVEN DISCOVERY AND FOR PRECISION DIAGNOSIS.

Abstract

Molecular profiling in chronic cases is both challenging and valuable. Rheumatoid arthritis is a chronic, systemic autoimmune disease with a global prevalence of 0.5–1%, imposing a significant individual and socioeconomic burden (Zhang *et al.*, 2025). The disease follows a heterogeneous clinical course, evolving from preclinical autoimmunity to early RA and subsequently to established and longstanding phases. While early intervention offers the best outcomes, many patients, particularly those with longstanding disease, continue to experience persistent pain, functional disability, and systemic complications despite intensive treatment. Discordance between clinical assessments, remission criteria, and biochemical markers such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) further complicate disease monitoring. Patients may meet remission definitions yet exhibit ongoing inflammation or radiographic progression, while others report chronic pain in the absence of objective inflammatory activity (Orr *et al.*, 2018).

Traditional biomarkers such as Rheumatoid factor (RF), anti-citrullinated protein antibodies **(**ACPA), ESR, and CRP, along with imaging modalities, have improved diagnosis and disease monitoring but remain insufficient. A major challenge persists in seronegative RA, where diagnosis is often delayed, and in treated patients who continue to show biochemical evidence of inflammation or residual disease activity. Emerging biomarkers such as calprotectin, 14-3-3η, and multi-biomarker disease activity scores provide proof-of-concept but lack widespread validation and clinical uptake (Soyfoo et Sarrand, 2026). This highlights the urgent need for translational biomarkers that can bridge the gap between molecular mechanisms and clinical decision-making. The pathophysiology of RA reflects a convergence of a genetic predisposition, environmental triggers, and immune dysregulation, resulting in chronic synovial inflammation, pannus formation, cartilage erosion, and systemic effects. This multifactorial biology is reflected not only in immune-mediated protein alterations but also in profound shifts in metabolic pathways, including glycolysis, lipid remodeling, and amino acid metabolism (McInnes et Schett, 2011). While proteomic studies have mapped inflammatory

proteins and autoantigens, and metabolomic studies have highlighted metabolic rewiring, each approach alone captures only a fragment of the disease process.

A multiomics approach, combining proteomics and metabolomics offers a systems-level perspective, connecting upstream immune dysregulation with downstream biochemical consequences. Such approaches have the potential to identify mechanistically coherent biomarkers that can aid in early diagnosis, stratification of disease trajectories, prediction of therapeutic responses, and sensitive monitoring of subclinical disease. Although challenges remain in terms of cohort heterogeneity, standardization, and clinical translation, advances in mass spectrometry technologies and computational biology now provide the tools necessary to overcome these barriers.

1.1 Prevalence of Rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease characterized primarily by persistent synovial inflammation and progressive joint destruction. It affects approximately 0.5–1% of the global population, making it one of the most common autoimmune diseases worldwide, with a higher prevalence in women than men (female-to-male ratio ~3:1). Typically, it presents between the ages of 30 and 60, though it may occur at any age, and its incidence has been shown to vary across geographic and ethnic groups. In North European and North American populations, the prevalence is 0.5–1.1% and in Canada, the prevalence was approximately 1.2% in individuals aged 16 and older who lived with diagnosed RA in 2016–2017. The overall burden of RA in Canada increased by 13% from 1990 to 2019 (Hassen *et al.*, 2024). Studies from developing countries report a lower prevalence (between 0.01–0.5%), although worldwide rheumatoid arthritis (RA) prevalence is rising, with 17.9 million cases in 2021 (Collaborators, 2023; Ma *et al.*, 2025). The disease is known to have a worldwide distribution affecting all races globally (Alamanos et Drosos, 2005). The rising prevalence of RA is driven by a combination of host and modifiable environmental factors. Among these increasing rates of aging population increasing rates of obesity, increased air pollution, smoking and improvements in diagnostic have contributed to the higher prevalence. Cigarette smoking and environmental pollution have been identified to trigger an immune response that precede clinical RA by years. Among these, smoking is the most well-established risk factor, with a clear gene-environment interaction. REF GBD 2021(Holers et al., 2018). Beyond inhalation exposures, unhealthy diet, obesity, low socioeconomic status, and physical inactivity

have all been linked to increased RA incidence and worse treatment outcomes (Schäfer & Keyßer, 2022).

1.2 Clinical phases and progression

The clinical course of RA is heterogeneous. Rheumatoid arthritis does not follow a uniform trajectory; instead, it evolves through distinct clinical and pathophysiological phases. The clinical course of RA is traditionally divided into phases: preclinical, early, established, and longstanding disease. Some patients present with aggressive early disease that leads to rapid joint destruction, whereas others experience milder forms with intermittent flares and remissions. Characterized by persistent synovial inflammation and progressive joint destruction, RA imposes a significant burden on quality of life, work productivity, and healthcare systems. The earliest phase, sometimes referred to as preclinical RA**,** is characterized by the presence of autoantibodies such as ACPA and RF in the absence of overt joint symptoms, reflecting a period of immune priming. This may progress to early RA**,** defined clinically within the first two years of symptom onset, when patients often present with polyarthritis and systemic inflammatory features. Early RA is considered a "window of opportunity," as the initiation of Disease-Modifying Anti-Rheumatic Drugs (DMARDs) during this phase is associated with the greatest chance of achieving sustained remission and preventing irreversible joint damage. Early disease is often defined as within the first two years of symptom onset, where intervention with DMARDs can substantially alter long-term outcomes. In contrast, established and longstanding RA, typically defined by disease duration beyond 10 years, is associated with cumulative joint damage, irreversible disability, and treatment resistance. Patients in these later phases often present with refractory pain, persistent inflammation, and comorbidities that complicate management (Scherer *et al.*, 2020).

Beyond the musculoskeletal system, RA exerts systemic effects, including an increased risk of cardiovascular disease, interstitial lung disease, osteoporosis, and premature mortality, contributing to a substantial socio-economic and healthcare burden globally. As the disease advances beyond this period, it enters the established phase**,** where persistent synovitis drives progressive joint damage, structural erosions, and functional disability. In this stage, treatment often requires escalation to biological or targeted synthetic DMARDs, yet remission remains difficult to sustain. These patients often face irreversible structural disability, higher comorbidity burden, and treatment-resistant pain, even when inflammation is apparently controlled (Scherer *et al.*, 2020). Without adequate treatment, chronic synovitis leads to pannus

formation, cartilage erosion, and bone destruction, resulting in functional disability and decreased quality of life. Despite advances in diagnostics and therapeutics, unmet challenges remain**.** Although advances in DMARDs, including biologics and targeted synthetic agents, have transformed management over the past two decades, the highly heterogeneous nature of the disease makes it incurable. The natural progression of disease in these patients may follow divergent clinical trajectories: some achieve remission with timely treatment, while others progress to severe disability and systemic complications despite intensive therapy (Wu *et al.*, 2022).

Early diagnosis remains a challenge, particularly in ACPA-negative or atypical presentations as well as in assessing prognosis or in effective treatment and management of disease. Furthermore, even after receiving treatment a significant proportion of patients fail to achieve sustained remission, highlighting the need for novel biomarkers that can aid in patient stratification, predict treatment response, and guide precision medicine approaches. Taken together, RA represents not only a clinically and immunologically complex disease but also one where molecular characterization is necessary to address critical gaps in understanding disease, provide reliable diagnosis, and assess prognosis. This provides the rationale for exploring high-throughput, unbiased approaches such as proteomics and metabolomics to unravel disease mechanisms and identify translational biomarkers.

1.3 Pathophysiological mechanisms

RA is a prototypical autoimmune disease characterized by persistent synovial inflammation, pannus formation, and systemic immune dysregulation. Although its precise etiology remains incompletely understood, it develops from the convergence of a genetic predisposition, environmental triggers, and aberrant immune responses, ultimately leading to chronic joint damage and extra-articular manifestations. The pathophysiology of RA is highly complex, involving multiple cellular compartments, cytokine networks, and metabolic reprogramming, all of which contribute to disease heterogeneity and therapeutic challenges.

1.3.1 Genetic and Genomic Basis of RA Pathophysiology

An increased risk for developing RA was associated with multiple genetic, transcriptomic, epigenetic and environmental factors that together form a complex association for development of disease. Although these factors have been studied extensively, several aspects still remain largely unknown and they remain to be clarified. The majority of the related genetic studies

have been conducted in Caucasians and there is a limited number of studies among other ethnicities (Kim *et al.*, 2022). From among the most commonly identified genetic predispositions, is belonging to the female gender with a positive family history of RA. The strongest of the genetic risk factors is a set of alleles within the Major Histocompatibility Complex (MHC) that encode amino acid sequences in the Human Leukocyte Antigen (HLA) peptide-binding groove. Genetic factors are known to account for up to 60% of RA susceptibility, with the strongest associations linked to the HLA-DRB1 loci within and outside the shared epitope motif. Genome-Wide Association Studies (GWAS) have identified risk of sequence variance for RA of approximately 4.6% in HLA-DRB1 and risk variance of other weaker variants that cumulatively account for approximately 18% (Stahl *et al.*, 2012), indicating an additional role of other genetic loci in disease pathology. With respect to HLA, three other statistically significant loci along with HLA-DRB1were noted in amino acid position 9 of HLA-DPB1 (another HLA class II gene) and two associations within HLA class I genes (amino acid position 9 of HLA-B and position 77 of HLA-A are known to be independently associated with RA susceptibility. The changes in these alleles alter the presentation of the citrullinated peptides on antigen presenting cells to influence CD4 and CD8 T cells to proliferate and increase autoimmunity. Beyond HLA, polymorphisms in genes encoding PTPN22, STAT4, TRAF1-C5, and CTLA4 have been implicated in modulating immune responses and RA risk (Sharma *et al.*, 2024). Transcriptomic studies, that measure mRNA levels were also investigated to discern their role in the pathogenesis of RA. Plasma transcriptome profile revealed significant changes in RNA levels of monocytes in patients who were resistant to treatment. This study identified an upregulation of Toll-like receptor 5 and interleukin-17 receptor A - hepatocyte growth factor axis in non-responders. Measuring changes in these markers before treatment could help predict response to Abtacept and its effectiveness in treating RA (Iwasaki *et al.*, 2024). Synovial fluid from RA was investigated to identify genes and pathways related to RA. Transmembrane protein adipocyte-associated 1, Huntingtin-interacting protein 1 and RP11-73E17.2.

Epigenetics, the study that deals with how genes are turned on or off, is influenced by factors like environment, diet, and stress, and is known to lead to gene dysregulation. The clinical course of RA in general and its level of aggressiveness are also determined by epigenetic changes. These modifications, including chromatin structural alterations, DNA methylation (hypo and hypermethylation), changes in small non-coding RNAs (miRNA) and histone acetylation have been shown to be associated with development of RA, with disease severity

and with the systemic complications arising from established or long-standing disease activity. (Bottini et Firestein, 2013). Among these Long Noncoding RNAs (LncRNAs), that are key components of the epigenetic machinery regulating chromatin remodeling and gene expression were found to be involved in the development of several autoimmune diseases. In a cohort of early-phase-RA patients 97 LncRNAs were identified of which six: FTX, HNRNPU-AS1, MIATNB, RP11-498C9.15, RP4-714D9.5 and RP11-73E17.2 were highly significant (Dolcino *et al.*, 2019). Methylation changes in several regions in the genome of fibroblast-like synoviocytes from RA joints were also noted to be associated with more aggressive forms of RA. Moreover, a plethora of research indicates that DNA methylation also plays a role in the onset and progression of RA. (Xing *et al.*, 2022). Both hypermethylation and hypomethylation are hypothesized to alter gene expression and increase disease activity in patients with RA, although it is not yet clear when in the course of disease these changes occur, and if they are considered as late changes and not related to initiation of disease (Maeshima *et al.*, 2016; Whitaker *et al.*, 2015).

Generally, results from GWAS data sets, epigenetic and differentially methylated gene analysis, consist of thousands of genes and correlating them specifically to disease is difficult. In a study this correlation was carried out where the three data sets were overlapped to identify the genes that specifically relate to RA. The authors identified seven genes in the three-way intersect that includes Autoimmune Regulator gene; Caspase-8 (an apoptosis-related cysteine peptidase), Colony Stimulating Factor 2 (CSF2), Engulfment and Cell Motility 1, ETS Proto-Oncogene 1 Transcription Factor, Major Histocompatibility Complex Class II DQ alpha 1 (HLA-DQA1), and Limb Bud and Heart genes, which were highly statistically significant. Some are already known to be relevant to RA, such as HLA-DQA1 and CSF2. (Whitaker *et al.*, 2015). The recurrence and continuation of numerous complicated diseases or biological processes are intimately linked to the DNA methylation control of gene expression (Brandt *et al.*, 2019). The precise role, however, of many variants remains yet to be elucidated so as to determine the underlying mechanisms behind genetic associations. Besides changes in DNA methylation in RA, RNA methylation is also known to play an important role in its pathogenesis. The most prevalent internal alteration found in eukaryotic mRNAs was in N6-methyladenosine (m6A). Transcriptional mapping of m6A in MH7A cells obtained from the synovium was performed that showed a correlation between the RNA methylation alteration and RA-related genes. Differential regulation of m6A methylation using m6Aseq was noted in

206 genes of which 118 had substantially elevated m6A methylation and 88 had significantly decreased m6A methylation (Jiang *et al.*, 2021).

Another potential genetic means of assessing drug responsiveness is through measuring specific miRNA (microRNA) levels. Alterations in specific miRNA levels following treatment with various DMARD showed several miRNAs were upregulated in the serum after TNFi therapy. Since miRNAs are stable in body fluids, they can be accurately quantified, and are known to regulate gene expression involved in drug metabolism and in numerous immunologic pathways. This emerging field of pharmaco epigenomics is an exciting field that may be of value if a validated biomarker can predict which specific drug responds best in an individual patient. Whole-genome microarrays were also utilized in RA to identify differential gene expression among the responders compared to the non-responders. Canet et al., investigated which single-nucleotide polymorphisms might be associated with Tumour necrosis factor inhibitor (TNFi) responsiveness using a registry of 1985 TNFi treated patients. They identified a significant correlation with improvement in Disease Activity Score-28 (DAS28) after TNFi with the CYP3A4 and CYP2CP variants. (Canet *et al.*, 2019).

RNA sequencing from blood samples and synovial fluid also identified 898 expression of quantitative trait loci (eQTL) in naive RA patients,of which 232 of which were common between blood and synovium compartments. An eQTL on HLA-DPB2 with the critical triad of single nucleotide polymorphisms rs3128921, correlated with increased disease severity and a higher risk of developing a lymphomyeloid phenotype. Nine genetic polymorphisms related to DNA repair genes were identified as potential triggers for the development of the disease, and their usefulness as biomarkers has been hypothesised. Their presence could indicate a genetic predisposition, leading to increase the risk of developing RA in individuals belonging to this specific population (Goldmann *et al.*, 2024). Methylation of a number of genes including ADAM Like Decysin 1, Immunoglobulin Heavy Constant Mu, osteoglycin, osteoprotegerin, Scavenger Receptor Class A Member 3, C-X-C Motif Chemokine Receptor 5, and Pleiotrophin were observed to be implicated in immune regulation, extracellular matrix remodeling, bone metabolism, and inflammatory signaling that in turn influenced disease activity in RA as well as osteoarthritis patients. Not only hypermethylation but also hypomethylation been recognized to be a predictor of disease development. Hypomethylation of the TNF-a gene, which plays an important role in RA, was noted to occur differently in subjects with RA compared to healthy controls. Hypomethylation has been found not only in the TNF-a, but also in the CDKN2A

promoter (Zheng *et al.*, 2023). Certain miRNAs were defined in RA as biomarkers of disease activity and made it possible to identify novel targets for further pharmacological (D'Orazio *et al.*, 2024). Although no specific methylation signatures have yet been identified in RA patients, there is a large body of evidence that methylation anomalies might be of significant etiological importance in RA.

Environmental, microbial and behavioural factors, especially related to dental hygiene, have also been documented as factors that trigger autoimmunity and predispose to RA. Among these cigarette smoking is the most established environmental risk factor which exerts a range of compound modulatory effects on both adaptive and innate immune systems promoting protein citrullination and the generation of ACPA This suggests that there may be biological interactions between these factors that drive the development of RA or at the very least RA-related autoimmunity. Other proposed triggers include microbial dysbiosis (notably in the gut and oral cavity), silica exposure, and hormonal influences, which may partly explain the higher incidence in women (Deane *et al.*, 2017; Zhao *et al.*, 2021). Emerging evidence indicated that the changes in the functions and composition of mucosal microbiota are very closely associated to RA. Bacterium responsible for many oral diseases Porphyromonas gingivalis present in the oral cavity and gut have a role in the advancement of RA. (Xie *et al.*, 2025).

Although GWAS have identified numerous genetic loci associated with RA, The genetic mechanisms cannot fully explain RA prevalence and neither the functional consequences of these variants, particularly their downstream molecular effects, remain largely elusive. Bridging this gap requires connecting genetic variation to intermediate molecular phenotypes, such as circulating proteins and metabolites, which more directly modulate disease pathophysiology (Aslam *et al.*, 2020).

1.3.2 Innate and Adaptive Immune Dysregulation

In RA a complex interplay exists between the adaptive and innate immune system that is the driving force behind the sustained chronic inflammation. The pathogenesis underlying the immune metabolic theory involves a breakdown of immune tolerance mediated by both these arms. T cells especially the CD4+ T helper cells are known to produce cytokines such as IL-17, IFN-γ, and GM-CSF that perpetuate inflammation and also infiltrate the synovium early in disease. CD 4^{+} T helper (Th) cells are detrimental in the initiation and progression of inflammation in RA, primarily by polarization from Th1 or Th2 cells. Th1 cells majorly secrete

tumor necrosis factor (TNF)-α and interferon (IFN)-γ while interleukin (IL); IL-4, IL-5, IL-10, and IL-13 are secreted by Th2 cells (Luo *et al.*, 2022). Activation of B-cell relies on T-cell activation. Proinflammatory mediators and some humoral response secretion is associated with Th1, whereas the secretions of the anti-inflammatory mediators that are principally responsible for the secretion of immunoglobulin E are associated with Th2 cells (Yap *et al.*, 2018). An imbalance of the Th1 and Th2 response exists in RA leading to INFγ overproduction and lower production of IL-4, IL-10, and IL-13 that are the Th2 mediators. The conflicting role of INFγ in RA has also been well reviewed. Additionally, in RA, the documentation of the T-regulatory (Treg) and Th17 lymphocytes clarified the imbalance in Th1/Th2. Th17 cells, in particular, promote neutrophil recruitment and osteoclastogenesis, linking adaptive immunity to bone destruction (Ruschpler et Stiehl, 2002). TNF-α is a potent osteoclastogenic cytokines which is crucial in the pathogenesis of RA.

B cells, on the other hand, are the antigen-presenting cells that interact with the T cells and are responsible for antibody production. They present antigens to the T cells and provide costimulatory signals for the activation of T-cell, enhancing effector functions and stimulating clonal expansion (Chimenti *et al.*, 2015). Typically, full T-cell activation requires binding of the T-cell receptor to the MHC molecule on the surface of the APC. The conventional costimulatory pathway for T-cell activation is the CD28-B7 pathway, where both cluster of differentiation costimulatory proteins, CD80 (B7-1) and CD86 (B7-2), interact with the CD28 receptor expressed on the T-cell surface. Cytotoxic T lymphocyte—associated protein 4 (CTLA-4) is another receptor on T-cells that binds to the same antigens, resulting in an adverse immune response and controlling the T-cell activity. (Uehara et McGrath, 2019; Walunas *et al.*, 1996). This costimulatory pathway existing between the T cells and B cells relies on secretion of various cytokines by T-cells such as interleukins and TNF-α that in turn stimulates B-cell differentiation and produces autoantibodies, such as RF and ACPA. The production of these autoantibodies contributes to the formation of immune complexes and enhances osteoclast activity.

Besides the adaptive immune system, the innate immune system comprising of neutrophils, macrophages and dendritic cells orchestrate both local joint inflammation and systemic acute-phase responses via production of producing TNF-α, IL-1β, and IL-6**.** Neutrophils accumulate in synovial fluid, releasing reactive oxygen species, proteolytic enzymes, and neutrophil extracellular traps that drive tissue damage and provide additional autoantigens. Circulating

monocytes differentiate into macrophages, and have a crucial role in tissues as apoptotic debris' scavengers and invading pathogens. Reduction in the macrophage population in arthritic synovium improves the symptoms of the disease. Macrophages on activation can cause rise in the expressions of cytokines (IL-6, IL-1, TNFα), chemokines (e.g., MCP-1/CCL2), reactive oxygen species, collagenases and prostaglandins, that can further intensify inflammation and in the synovium lead to deterioration of the extracellular matrix and normal tissues. Furthermore, the activated macrophages contribute to the stimulation and proliferation of the antigen-specific T cells while TNF-α secreted by them further prompts receptor acquisition by natural killer (NK) cells and plays a crucial role in NK-dependent maturation of dendritic cells from monocytes (Lin *et al.*, 2020).

1.3.3 Cytokine Networks and Signaling Pathways

Cytokines form a complex and intricate network of numerous mediators displaying diverse roles and influencing multiple cellular pathways in pathogenesis of RA. They contribute to the induction and maintenance of inflammation through a delicate balance between the proinflammatoy and anti-inflammatory mediators. Many cytokines are involved in RA, and among these a few critical ones are TNF, IL-6, IL-1, IL-17, and GM-CSF. TNF and IL-6 are both well-established proinflammatory cytokines that are known to directly influence chronic inflammation and contribute to joint damage and progressive disease. TNF-α is considered as the central driver of inflammation, that promotes cytokine cascades, leukocyte recruitment, and angiogenesis. IL-1 represents a more complex cytokine that acts as a secondary mediator and plays a crucial role in the propagation of joint inflammation and in cartilage and bone erosion. IL-1β on the other hand is involved in the amplification of local inflammation, cartilage destruction, and osteoclastogenesis. Similarly, IL-17 is also known to promote erosive arthritis by stimulating osteoclastic bone resorption. Besides the interleukins chemokines such as CXCL8, CCL2, CCL5) drive inflammation through leukocyte trafficking to inflamed joints. From among these mediators of inflammation IL-17 and GM-CSF are at the center of bridging the adaptive immune system with neutrophilic inflammation and bone resorption. Together, these cytokines sustain chronic inflammation and their uncontrolled actions lead to structural damage (McInnes et Schett, 2007).

In RA, many immune cells infiltrate the synovium in response to pro-inflammatory cytokines and chemokines, leading to inflammation and tissue destruction The RA synovium is a cytokine-rich environment where multiple pro-inflammatory mediators act in concert via

different signaling pathways. Signal transduction pathways regulate cellular responses to stress and play a critical role in inflammation. Binding of a cytokine to its receptor activates the receptor-associated JAKs leading to activation, dimerization, and translocation of STAT into the nucleus, causing an increase in the transcription of more and more inflammatory cytokines, which continues the loop of inflammatory signaling. Inhibiting this loop of inflammatory signaling may be mediated by inhibiting the JAK pathways IL-6 is known to signals through the JAK/STAT pathway and is implicated as the main cause for development of systemic manifestations of RA (Ibrahim et Huttunen, 2021)**.**. Even though these pathways have been studied extensively, the exact etiology and pathogenesis of RA are still unclear. Identifying these cytokines and understanding the interaction between cytokines and their signaling pathways are the basis for the development of new strategies with small molecules or bispecific antibodies. Although well-established, measuring the levels of these cytokines in RA proved unsatisfactory due to their non-specificity and also due to complexity of signaling pathways that represent major hurdles for developing effective, safe therapeutic interventions. This has a created a demand for identification of new more specific biomarkers of disease (Ibrahim et Huttunen, 2021; McInnes et Liew, 2005).

1.4 Systemic Manifestations of RA

RA extends far beyond the joints and is known to have numerous systemic manifestations (Wu *et al.*, 2022). Chronic systemic inflammation accelerates atherosclerosis**,** conferring a 50% higher risk of cardiovascular events compared to the general population. Pulmonary involvement manifests as interstitial lung disease and pleural effusions, while ocular complications include scleritis and keratoconjunctivitis sicca. The synovium, undergoes profound hyperplasia in RA. Fibroblast-like synoviocytes (FLS) adopt an aggressive, tumor-like phenotype, characterized by hyperproliferation, resistance to apoptosis, and secretion of matrix-degrading enzymes such as matrix metalloproteinases. These pathogenic FLS contribute to pannus formation, a hallmark of RA pathology in which inflamed synovial tissue invades cartilage and bone. The activation of osteoclasts, that are bone resorption cells due to RANKL signaling from activated T cells, B cells, and synovial fibroblasts is another key feature of RA. Increase in the number of osteoclasts results in progressive bone erosions and an increase in bone fragility. Bone fragility in RA is caused by a combination of systemic inflammation, autoantibodies circulation, and the secretion of pro-inflammatory cytokines (Fang *et al.*, 2020). Even though the systemic manifestations of RA have been documented and targeted therapies are in use. However, the underlying pathological mechanism of RA

complications remains still unclear and is an area of active research (Wu *et al.*, 2022). Metabolic comorbidities such as insulin resistance, cachexia, and osteoporosis reflect the systemic impact of inflammatory cytokines and altered metabolism. These systemic effects underscore RA as a multisystem disease**,** further highlighting the need for biomarkers that capture its complexity.

1.5 Current status of Clinical Diagnosis of RA

In clinical practice, the diagnosis of RA is mainly based on a combination of signs and symptoms in an individual at presentation, on the presence of positive family history of disease, serological markers and radiographic evidence. Being a highly heterogeneous, progressive disease that evolves over the years from the onset of pathophysiological mechanisms to the severe clinical manifestations, it is difficult to diagnose early on. In the RA continuum, the following stages can be distinguished: i) Preclinical stage characterized by the absence of clinical symptoms ii) a clinical phase characterized by the presence of initial symptoms but maintaining functionality in daily living activities and iii) an established stage where the symptoms are manifested.

Patients with RA typically present with pain and stiffness in multiple joints, especially the wrist joint, proximal interphalangeal joints, and metacarpophalangeal joints. That characteristic feature is, of course, morning stiffness lasting more than one hour suggests an inflammatory etiology. Systemic symptoms of fatigue, weight loss, and low-grade fever may occur with active disease. The diagnosis of RA is inherently complex due to its heterogeneous presentation. Current diagnostic approaches rely on a combination of clinical, serological, and imaging criteria. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria (ACR/EULAR) classification criteria emphasize early detection and include measures of joint involvement, acute phase reactants, serological markers, and symptom duration. Serologically, the presence of RF and ACPA are important indicators, though both suffer limitations. RF, while historically important, lacks specificity and can be elevated in other autoimmune conditions and even in healthy individuals. ACPA is more specific and predictive of erosive disease, yet approximately 30–40% of RA patients lack both RF and ACPA at disease onset. These patients frequently experience diagnostic delays, as the non-specific nature of their presentations can mimic other inflammatory arthritides such as psoriatic arthritis, systemic lupus erythematosus, or undifferentiated inflammatory arthritis. Currently, many laboratories and imaging modalities correlate with RA clinical disease

activity. The most widely used wet biomarkers include ESR and CRP levels, which are nonspecific markers of active inflammation and often correlate with active synovitis in many, but not all, RA patients. Serologic tests such as rheumatoid factor (RF) and the more specific ACPA are usually associated with more aggressive disease including deformities, erosions seen on imaging and extra-articular disease manifestations. Even more sensitive than the clinical examination of active joint inflammation, which makes up the DAS 28 score used to document disease activity are magnetic resonance imaging (MRI) and ultrasound examinations to detect active synovitis (Meehan *et al.*, 2021). Another area where these markers are lacking is in the diagnosis of seronegative RA where, regardless of the phase of the disease, both RF and ACPA remain negative. Alternative autoantibodies such as anti-carbamylated protein antibodies (anti-CarP), anti-acetylated peptide antibodies, and antibodies to heterogeneous nuclear ribonucleoproteins (hnRNPs) have been investigated as potential markers in seronegative cases. However, none has yet demonstrated sufficient sensitivity and specificity to warrant routine clinical adoption. Biomarkers such as ESR and CRP reflect systemic inflammation but do not always correlate with disease activity or progression, particularly in seronegative patients (Steiner *et al.,* 2024). As a result, RA remains a poorly defined representing a clear area where molecular profiling may provide much-needed diagnostic precision.

In addition to diagnosis, accurate monitoring of disease activity is fundamental to RA management. The goal of therapy is to achieve clinical remission or low disease activity, typically assessed using composite indices such as the DAS28. These indices incorporate tender and swollen joint counts, patient-reported outcomes, and laboratory markers (ESR, CRP). While widely used, these tools face important limitations. These include subjectivity and variability in the joint counts are examiner-dependent and prone to inter-observer variation. Differences are noted in the involvement of acute phase reactants levels, that is in the levels of ESR and CRP which are influenced by factors beyond RA activity, including infections, age, sex, and metabolic comorbidities. Musculoskeletal ultrasound and magnetic resonance imaging (MRI) detect synovitis, erosions, and bone marrow edema with higher sensitivity than clinical examination or plain radiography. Importantly, these imaging modalities can reveal subclinical disease activity even in patients classified as in remission by DAS28, suggesting persistent low-level inflammation. However, the widespread use of imaging for routine monitoring is constrained by cost, accessibility, and the need for trained operators.

1.5.1 Extended Autoantibody Profiling

The spectrum of autoantibodies that are associated with either the diagnosis of RA or disease progression has expanded beyond RF. The presence of ACPAs including anti-CCP is associated with more severe RA and articular destruction. While anti-CCP has long been used to evaluate disease severity and improve diagnostic accuracy, other auto antibodies against citrullinated fibrinogen, citrullinated collagen, mutated citrullinated vimentin and anti-citrullinated vimentin (Harre *et al.*, 2012; Kuhn *et al.*, 2006), have been identified along with anti-protein arginine methyltransferase (Huang *et al.*, 2025). These autoantibodies, as mentioned previously are known to induce pro-inflammatory mediators such as TNFα and enhance neutrophil-mediated inflammation and have been shown to correlate with disease severity. Analysis of these newer classes of autoantibodies is currently limited to understanding their role in the pathogenesis of RA, however, such studies may lead to their potential as biomarkers for disease staging and possible therapeutic decisions in the future. Other autoantibodies under investigation for their potential in diagnosis or monitoring include; anti-hinge antibodies proteolytic cleavage product of IgG molecules due to increased levels of endogenous proteases such as MMPs, antiacetylated vimentin antibodies in patients with early inflammatory arthritis and actylated lysine that may be the link between microbiome dysbiosis and the development of RA.

1.5.2 Peripheral Blood as a Source of Clinical Diagnostic Biomarkers

Serum proteins, cytokines, and chemokines have been measured for years in RA patients to better understand immunopathogenesis, prognosis, disease susceptibility and to document systemic disease activity. A large number of biomarkers were identified to be altered between RA and controls, but many of the studies focused on the early stages of disease and response to treatment with the different treatment modalities. Peripheral blood has been utilized as the main source of these markers as it is easily accessed, processed, and is routinely obtained from patients as part of their laboratory work up. These were identified using a targeted and an untargeted approach utilizing different methodologies, single or multiplexed ELISA. Serum levels of CRP, sICAM-1, sVCAM-1, serum amyloid A (SAA), matrix metalloproteinases (MMPs 1, 3 and 9) and metabolic markers such as active glucose-dependent insulinotropic polypeptide, active glucagon-like peptide-1, C-peptide, glucagon, insulin, leptin, pancreatic polypeptide (PP) were measured by Meso Scale Discovery multiplex analysis assay. Previous studies have shown that serum levels of sICAM-1, MMP1, MMP3, PP, c-Peptide, CRP and SAA were specifically upregulated in RA (Veale *et al.*, 2025)

1.5.3 Synovial Fluid and tissue as a Source of Biomarkers

Synovial fluid (SF) is a promising source of valuable biomarkers to predict drug responsiveness in RA since peripheral blood, contains tens of thousands of different proteins over a very large dynamic range, released from multiple extra-articular sites or even from the gut microbiome which contains 10^{14} organisms. Therefore, measuring peripheral blood proteins will not likely reflect changes in the synovial fluid or intra-articular structures. Prior clinical studies have also demonstrated a poor correlation between key regulatory cytokines in the peripheral blood compared to SF levels in RA, OA, and traumatic arthritis. In a study by Wright et al. from 42 RA patients, they found that 12 SF cytokines were much lower in the peripheral blood than when measured simultaneously in the SF (Wright *et al.*, 2012). Immune cells isolated from RA or healthy control synovial tissue have been informative in defining changes in specific inflammatory cell populations and mapping these cell populations such as pro-inflammatory monocytes or activated synovial fibroblasts to specific inflammatory mediators. The analyses of SF from the peripheral joints, being the primary site of RA disease, has also demonstrated the different phenotypes of RA based upon the infiltration of specific immune cell types. While synovial fluid and biopsy provides a means to study immunologic pathways, it requires arthroscopy or an ultrasound-guided needle biopsy that are both expensive and invasive techniques

1.6 Emerging but Non-Routine Biomarkers

Beyond traditional serological and inflammatory markers, several novel biomarkers have been investigated in RA to improve diagnosis, disease monitoring, and prediction. While promising, none has yet achieved widespread clinical implementation due to issues of cost, limited validation, or lack of standardization. Recently a serum based multi-biomarker panel was developed using 12 serum proteins, which included IL-6, serum amyloid A, tumor necrosis factor receptor I, and vascular cell adhesion molecule-1, epidermal growth factor, vascular endothelial growth factor-A, TNF-R1, MMP-1, MMP-3, YKL-40, leptin, resistin, SAA, CRP for assessing disease activity. These markers were then used to derive a multi-biomarker disease activity (MBDA) score for use in patients with RA. The use of MBDA, known commercially as Vectra DA, although approved was limited as it was only used to detect flares and sustained clinical remission after discontinuation of adalimumab (ADA) in RA. This score integrates 12 serum proteins, to provide a composite readout of disease activity. Studies suggest that MBDA may better reflect subclinical inflammation compared with CRP or DAS28, and

could aid in predicting radiographic progression. However, its performance across diverse patient populations with RA has been variable, and it remains costly and less accessible than standard markers (Meznerics *et al.*, 2023; Rech *et al.*, 2016).

Other markers of disease activity or remission that have been identified were Calprotectin, 14-3-3η protein, cartilage degradation products. Calprotectin a heterodimer of S100A8/A9 proteins is released by activated neutrophils and macrophages and has emerged as another candidate biomarker. Elevated calprotectin levels correlate strongly with synovial inflammation and may outperform CRP in detecting residual disease activity in patients in clinical remission. Despite this promise, standardization of assays and definition of clinical thresholds remain challenges. Similarly, 14-3-3η protein is a signaling molecule implicated in joint inflammation and has been proposed as both a diagnostic and prognostic biomarker in RA. Elevated serum levels have been associated with increased evidence of radiographic progression of bone disease and worse functional outcomes. Yet, its diagnostic performance in distinguishing RA from other inflammatory arthritides is inconsistent, and large-scale validation is needed. Other exploratory biomarkers include cartilage degradation products such as cartilage oligomeric matrix protein, C-terminal cross-linked telopeptide of type II collagen, circulating cytokine panels, and autoantibodies against novel post-translational modifications (e.g., acetylated or carbamylated peptides). While each of these adds incremental insights into disease biology, none has yet achieved the sensitivity, specificity, or reproducibility required for integration into routine clinical algorithms (Martins et Fonseca, 2019).

To date the current RA diagnostics are based on and limited to serology (RF, ACPA) and inflammatory markers (ESR, CRP), and imaging modalities that are supported by composite indices such as DAS28. While useful, these tools fail to capture the full heterogeneity of RA, leaving major gaps in diagnosis, in monitoring of subclinical disease, and the prediction of therapeutic response. Emerging markers like MBDA, calprotectin, and 14-3-3η provide proof-of-concept that molecular biomarkers can refine disease management, but their limited validation underscores the need for more robust and unbiased approaches. This unmet clinical need provides the rationale for applying mass spectrometry-based proteomics and metabolomics. These approaches offer the potential to identify translational biomarkers that are not only statistically robust but also mechanistically informative and can prove to be potentially clinically actionable.

1.7 Need for New Biomarkers and New Avenues for Diagnosis

The limitations in the current methods for diagnosing disease present a situation with a need for sensitive and specific biomarkers for not just diagnosis, but also for monitoring the disease and assessing the impact of treatment. Disease-specific molecular markers could provide earlier and more accurate classification, guiding therapy by aligning patients with the most effective treatments. It will also facilitate the timely initiation of therapy and improve the assessment of remission, thereby reducing the need for excessive medications and promoting a more personalized approach to medicine in RA from the outset.

Despite major advances in our understanding of RA and the development of highly effective therapies, biomarkers that can reliably guide diagnosis, prognosis, and treatment decisions remain elusive. The complex interplay of immune, stromal, and metabolic pathways described in RA pathophysiology highlights why traditional serological and inflammatory markers provide only a partial picture of disease biology. Bridging these gaps with translational biomarkers molecular signatures that can move from discovery to real-world clinical application represents a critical unmet need in RA research. The limitations of existing biomarkers such as RF, ACPA, ESR, and CRP are well established. RF lacks specificity, while ACPA, though highly specific, is absent in up to 40% of patients, particularly those with seronegative RA. A variety of laboratory and imaging techniques are used to assess disease activity in RA in accordance with clinical findings. Prominent biomarkers include CRP and ESR, reflecting non-specific markers for inflammation, frequently correlating with active synovitis in patients of RA. Serologic tests, including more focused ACPA and RF are associated with severe disease outcomes, including extra-articular manifestations, erosions and deformities. Ultrasound and MRI are more sensitive than clinical assessments for detecting active synovitis ESR and CRP, while useful in monitoring systemic inflammation, are nonspecific and frequently discordant with actual synovial pathology. Imaging techniques such as ultrasound and MRI provide detailed insights into joint inflammation but are resource-intensive and not standardized for routine use.

These shortcomings translate into several critical challenges in clinical practice including delayed diagnosis, inaccurate assessment of disease activity, with some patients experiencing ongoing subclinical inflammation despite “remission” state based on DAS28. There is also an uncertainty in predicting disease progression**,** as current markers do not reliably stratify patients by risk of erosive disease or extra-articular complications. Lack of predictive tools for

treatment response**,** forcing reliance on trial-and-error strategies for biological and targeted synthetic DMARDs.

The ideal test or biomarker would need to be relatively inexpensive, available as a point-of-care test in the physician's office as a blood-based assay, and require minimal sample preparation or manipulation. A validated biomarker would revolutionize the care of patients with autoimmune illnesses by allowing the judicious use of these very expensive biologics and small molecule drugs to enhance a personalized medicine approach and allow for the prescription of the best drug for an individual patient early in the disease process.

1.8 Molecular Complexity for RA and the rationale for using Omics Approaches

Disease processes for RA unfold across multiple biological scales: immune activation, stromal cell transformation, cytokine signaling, metabolic reprogramming, and systemic inflammation**.** Each of these layers contributes unique signatures at the protein and metabolite level. The multifaceted pathophysiology of RA demonstrates the reason why single biomarkers rarely suffice for accurate diagnosis, prognosis, or monitoring. As a result, conventional biomarkers such as RF, ACPA, ESR, and CRP capture only limited aspects of disease biology and do not fully reflect the heterogeneity of RA across patients or across disease stages. The heterogeneity of RA is illustrated not only in its pathophysiology but also in its clinical presentation. This provides a strong biological rationale for applying multi-omics approaches to unravel disease mechanisms and identify translational biomarkers that can improve patient care. Mass spectrometry-based proteomics and metabolomics are uniquely suited to address these challenges. These techniques when used along with clinical information, allow us to obtain a systems-level perspective to unravel disease mechanisms. They can also provide opportunities to identify molecules with diagnostic and predictive capabilities that can provide a foundation for precision therapy. Proteins, are often targeted by therapeutics, and metabolites, which sensitively reflect metabolic disturbances and early pathological changes, offer promising avenues for biomarker discovery and disease monitoring.

1.9 Application of Omics in Rheumatoid Arthritis for Precision Medicine

To overcome these limitations, approaches capable of profiling the molecular landscape of RA in an unbiased, high-throughput manner are needed. Mass spectrometry-based techniques provide the sensitivity, specificity, and molecular resolution needed to examine complex biological samples such as plasma. It works by converting molecules in a sample into charged ion species (positive and negative) that are next separated according to their mass-to-charge

ratio (m/z) within a mass analyzer and then detected. Detection and identification is done based on the relative abundances of the various ionic species, as a function of their *m/z* value, that are recorded as mass spectra. Mass spectrometry-based proteomics and metabolomics techniques offer precisely this capability. The widespread use of MS in proteomics is primarily due to its exceptional sensitivity and specificity, allowing the detection and identification of proteins at very low concentrations, making it invaluable in proteomics and clinical research. By interrogating thousands of proteins and metabolites simultaneously, these technologies can identify novel biomarkers reflective of immune activation, stromal transformation, metabolic remodeling, and systemic inflammation. This capability is further enhanced by the use of advanced technologies like tandem mass spectrometry (MS/MS).

Proteomics can uncover differentially expressed proteins in serum, synovial fluid, and tissue, including cytokines, acute-phase proteins, and tissue-degrading enzymes. Such markers may provide insights into disease mechanisms and reveal candidates for diagnosis or therapy monitoring. Metabolomics capture alterations in amino acids, lipids, energy intermediates, and oxidative stress markers that reflect the metabolic rewiring characteristic of RA. These signatures may serve as sensitive indicators of disease activity or treatment response. When integrated, proteomics and metabolomics provide complementary perspectives that align closely with the multifactorial nature of RA pathophysiology. Importantly, omics-derived biomarkers are not limited to statistical associations: they often map directly onto biological pathways, thereby offering mechanistic insights alongside diagnostic potential.

1.9.1 Proteomics:

The proteome refers to the entire set of proteins produced by a cell or organism and proteomics is the large-scale study of these proteins. In broad terms, proteomics can be divided in three distinct types, namely expression proteomics, functional proteomics and structural proteomics. Expression proteomics is aimed at the study of the expression of proteins, both quantitatively and qualitatively. It includes techniques such as two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS)-based technologies. Structural proteomics has the objective of understanding protein interaction and function through a structural basis; techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) are utilised at the protein level together with electron microscopy and electron tomography for visualisation of complexes and cellular context. Ultimately, the goal of functional proteomics is to study protein function and molecular mechanisms. Because proteomics investigates the overall picture of intracellular

protein composition, structure, and activity, it is capable of identifying biomarkers and improving the understanding of pathogenesis (Mun *et al.*, 2021). Proteomics deals with a comprehensive analysis of changes in the expression of proteins within different sample matrices. It allows the identification of the proteins that are changed with disease, or treatment and between various stages of the disease. Along with identifying the altered expression of the proteins, proteomics associated with bioinformatics allows them to be characterized based on their function, biological process, location and interactions within the different metabolic pathways. Different methodologies for protein separation including electrophoretic and chromatographic methods coupled to mass spectrometry have been utilized for this purpose. Research methods are diverse and efficient, covering microarray-based technologies as well as the latest single-cell and high-sensitivity protein analysis methods. First, although proteins are by far the most utilised class of molecules in clinical use, proteomics has not been amply utilised for biomarker discovery (Mun *et al.*, 2021).

Proteomic analysis has been utilized in RA to identify peptide mediators and key proteins, and also demonstrates unique value in the detection and quantification of cytokines. These technologies not only facilitate early diagnosis of RA but also provide potential biomarkers for monitoring disease progression and treatment response. Methodologies using MS and LC enable not just quantification of large numbers of proteins and peptides, but also analysis of their structure, function, modifications, and interactions in a variety of sample types. MS, LC-MS, and Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) are some of the methods that have been utilized for proteomic studies in RA. Samples utilized for analysis have included serum, plasma, synovial fibroblasts, fluid or tissue, peripheral blood mononuclear cells, monocyte/macrophages, and neutrophils. The focus of proteomic studies has been largely to identify proteins or peptides that are unique to RA patients compared with healthy individuals.

In another study, proteomic analysis of T-cell phenotypes, Researchers believe that metabolic dysregulation driving the transition of T-cell phenotypes may induce the development of autoimmune diseases was determined highlighting the importance of Th1 and Th2 phenotypes in the metabolic network. Th1 cells exhibit a stronger glycolytic flux compared to Th2 cells, suggesting that glycolysis in Th1 cells could be a predictive target for RA. Furthermore, IL-6 inhibits Transforming Growth Factor β, impairing Treg phenotype and thus reducing the inhibition of adaptive T-cell responses, promoting the formation of Th17 cells, which are

associated with the incidence and severity of RA (Vyas *et al.*, 2019). Segigawa and colleagues using 2D LC-MS/MS analysis, investigated serum or plasma proteins differentially expressed after anti-TNFα therapy. They identified FAM62A/MBC2 proteins related to the TNF-α-mediated pathway for nuclear factor kappa B (NF-κB) activation and/or CTGF protein related to the metabolism (including regeneration) of articular cartilage (Sekigawa *et al.*, 2008). Researchers utilizing MS analysis of RA synovial tissues have been able to identify specific citrullination sites on fibrinogen (Hermansson *et al.*, 2010). Through differential profiling of proteins in the <40-kd portion of the SF proteome, we selected 33 prospective candidate biomarkers from a total of 418 identified proteins. Among the proteins that were elevated in the SF of patients with erosive RA were CRP and 6 members of the S100 protein family of calcium-binding proteins. Significantly, levels of CRP, S100A8 (calgranulin A), S100A9 (calgranulin B), and S100A12 (calgranulin C) proteins were also elevated in the serum of patients with erosive disease compared with patients with nonerosive RA or healthy individuals. (Liao *et al.*, 2004). Seven biomarker candidates were verified through multiple reaction monitoring-based quantitative analysis, of which angiotensinogen, SAA4, vitamin D-binding protein, and retinol-binding protein-4 had an area under the curve over 0.8, thus distinguishing RA patients, including seronegative (RF- and anti-CCP-negative) RA patients, from healthy controls (Mun *et al.*, 2021).

The proteomics of SF from SpA and RA patients showed a marked difference in the amounts of proteins from the innate immune system, primarily originating from neutrophil granulocytes. The presence of these proteins was more pronounced in the RA patient group. These proteins were also correlated to SF circulating cell free DNA indicating NETosis. Neutrophils produce IL-6 that induces acute phase reactants. Still, surprisingly, little correlation between NETs proteins/cell free DNA and acute phase reactants/CRP was seen, indicating that two different inflammatory mechanisms are used for increase in CRP and cfDNA. Proteomic-protein interaction networks analysis in RA has identified fibronectin 1, Acetylcholinesterase , and Aquaporin 1 Dysregulation of proteins were linked to changes in the metabolic products of the glycerophospholipid pathway in RA.(Jian *et al.*, 2023).

Several groups have published data from proteomic studies performed on FLS and have identified proteins that are unique or are differentially expressed in RA samples. These proteins include structural and enzymatic proteins, some of which have been identified to be autoantigens. Although proteomic studies have largely focused on diagnostic markers for RA

and monitoring disease activity and severity, they have also been utilized for profiling to identify proteins and peptides that are capable of assessing response to treatment using different DMARDS. 51 differentially expressed peptides in the serum of responders that correlated with response to TNFi, 3 of which were predictive of a good response in RA patients treated with triple therapy MTX, leflunomide, and TNFi (Chen *et al.*, 2019). In a smaller study of 10 RA patients, Dwivedi et al. compared peptide profiling of pre-treatment and Infliximab treated serum samples and showed that a robust response to infliximab was associated with downregulation of several TNFα—regulated proteins (Dwivedi *et al.*, 2009). Protein microarray technology is capable of detecting up to 10 cell equivalents. Combined with the high quality accuracy of mass spectrometry (<10 ppm), it is capable of identifying any protein in the sample (Kunz et Ibrahim, 2009). 2D gel electrophoresis combined with mass spectrometry allows for the efficient separation and identification of specific proteins in a sample. Despite the high resolution, the number of proteins that can be identified by current techniques is typically less than 10,000, whereas the number of known proteins is approximately one million.

In most previous proteomics studies, blood samples were pooled for MS analysis. However, it is important to analyze individual serum samples to reflect individual alterations in serum protein levels, but it is difficult to analyze individual serum samples. First, it is difficult to obtain an adequate volume of individual serum samples. Second, individual MS analysis is costly and time-consuming. Third, clinical data interpretation is challenging owing to the complexity of the status of RA patients. However, most studies are inadequate in allowing reliable conclusions. The analysis of RA may be more complicated than other inflammatory diseases because of its combination of inflammatory processes, including synovial inflammation and angiogenesis. Data obtained from proteomic analysis of studies including a larger number of patients must be considered a fundamental requirement for more targeted progress

1.9.2 Metabolomics

Metabolomics is the scientific study of all small molecules or metabolites in biological samples that aids in revealing the functional state of cells and organisms through the analysis of molecular changes. Metabolomics provides novel opportunities for discovering biomarkers that enable fast, accurate, and reliable diagnosis by identifying metabolic alterations in cases of complex diseases. Metabolite expression is associated with the genetic blueprint, sequence

variation, RNA transcription, and protein translational processes interacting with environmental exposure and reflecting genotype, especially for multifactorial diseases. Among genomics and proteomics, metabolomics perhaps is closely linked to the phenotype (Vitale *et al.*, 2024). By analyzing low-molecular-weight metabolites, metabolomics contributes to precision medicine by providing a more comprehensive picture of disease and individual variability, enabling tailored interventions and transforming healthcare from a one-size-fits-all model to a personalized approach. Correlating the metabolic phenotype of individuals into subgroups that respond differently is also becoming a reality through metabolomics. This is the perception of the metabotype that has emerged and is now playing a crucial role in developing a personalized healthcare system.

The application of metabolomics not only reveals the responses of cells and organisms in various disease states, growth stages, or under environmental stimuli, but also provides unique insights into biological systems. This aids in disease diagnosis and the discovery of new therapeutic targets. Recent advances in metabolomics technologies and methodologies have identified significant metabolites related to rare endocrine disease conditions of the adrenal gland (hyperaldosteronism, primary adrenal insufficiency), parathyroid (hypoparathyroidism), and cystic fibrosis (Masood *et al.*, 2023). Metabolomics profiling combined with genomics is increasingly being employed for improving understanding, clinical diagnosis, and management of these clinically challenging conditions. Advances in gas and liquid chromatography combined with tandem mass spectrometry (GC/LC MS/MS) techniques have improved the profiling of steroid metabolites. Significant alterations in levels of these metabolites demonstrate the potential to serve as specific markers of disease, help in their stratification, and contribute toward moving to personalized medicine (Zhang *et al.*, 2015).

In RA, the majority of the studies have looked at changes in the synovial metabolome and in the metabolome from FLS, while a few have looked at changes in the serum metabolome in patients with early disease and others in determining the treatment responses with different DMARD. These studies have also used different methods to carry out the analysis, including using LC-MS, gas chromatography-MS (GC-MS), or nuclear magnetic resonance spectroscopy (NMR). In a study, using GC/MS eighteen significantly altered metabolites in RA were identified and among them L-cysteine, citric acid and L-glutamine were proposed to predict RA with high accuracy. Additionally, the dysregulated taurine biosynthesis (He *et al.*, 2021). In a recent study, targeted metabolomics was carried out by UHPLC-QTOF-MS in RA patients

with active disease and those that were in remission. Levels of the metabolite 1-oleoyl-sn-glycero-3-phosphocholine were found to be lower compared with controls prompting the conclusion that glycerophospholipid metabolism is the main metabolic pathway associated with the differential IL-6 expression in RA patients (Su *et al.*, 2022). Metabolomics analysis of human serum using GC-MS also identified pyruvate and glucose, increased levels of fatty acids and cholesterol, which were primarily associated with glycolytic pathway, fatty acid and amino acid metabolism, and other related pathways including TCA cycle and the urea cycle (Zhou *et al.*, 2016). These changes are significantly reflected in the dysregulation of histidine, glycerophospholipid metabolism, and the metabolism of serine, glycine, and threonine. Nineteen active, seropositive patients with RA, on concomitant methotrexate, were studied. Synovial fluid metabolome was analysed using NMR that showed alterations in serine, glycine, phenylalanine metabolism and aminoacyl-tRNA biosynthesis (Narasimhan *et al.*, 2018) while in another study acetate, acetylated-saccharides, glycine, branched chain amino acids, methionine, sarcosine, threonine, 3-hydroxybutyrate, 2-hydroxybutyrate, 3-hydroxyisovaleratecitrate, acetylcholine, adenosine, among others (Anderson *et al.*, 2018). On the other hand LCMS analysis of synovial fluid between RA and controls identified 30 metabolites as putative rheumatoid arthritis biomarkers including various phospholipids, diol and its derivatives, arsonoacetate, oleananoic acid acetate, docosahexaenoic acid methyl ester, and linolenic acid and eicosatrienoic acid derivatives (Carlson *et al.*, 2019). In another study synovial fluid metabolome identified the presence of glucose and lactic acid using GC-MS (Yang *et al.*, 2015). Utilizing UPHLC-QTOF-quadrupole-time of flight mass spectrometry, researchers have demonstrated that the levels of tryptophan metabolites in RA synovial fluid are lower compared to those in patients with osteoarthritis. Concurrently, the β-oxidation pathways of cholesterol esters, taurine, and linoleic, oleic, and sphingolipids are more intensely activated in RA patients (Kang *et al.*, 2015). These significant changes that occur in plasma metabolites and metabolic pathways can help to identify biomarkers of RA, which in turn can lead to the development of new diagnostic tools and therapeutic strategies. Glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway (PPP), the arachidonic acid (AA) metabolic pathway, and amino acid metabolism have been widely studied for their roles in RA (Srivastava *et al.*, 2018). The levels of intermediate metabolites in these metabolic pathways were shown to be increased or decreased compared with those in healthy controls. Metabolomics An additional prospective study involving industry partners and 140 investigators out of the UK, (RA-MAP Consortium), is an additional ongoing study to more accurately stratify patients based upon clinical features and drug responsiveness using a multi-

omics approach (Cope *et al.*, 2018). This information may provide insight into various immunometabolic profiles, or pathways in different RA phenotypes, which might have predictive value in drug responsiveness. However, metabolomics faces some challenges such as difficulty in identifying many compounds, lack of comprehensive enzyme kinetic data, and complex data processing. Moreover, the heterogeneity of RA makes it difficult to generalize the findings and the study design for these studies were limited by their small sample size.

1.10 Future perspectives Towards Clinical Translation—Why Translational Biomarkers Matter

The concept of translational biomarkers extends beyond simple association with disease. For a biomarker to be clinically useful, it must demonstrate robust reproducibility, mechanistic relevance, and clear utilities in guiding clinical decisions. In RA, this translates into four major areas of potential impact, diagnosis and early detection, that is in identifying molecular signatures that precede clinical onset or enable the recognition of seronegative disease. In prognosis and risk stratification where in predicting which patients will develop aggressive erosive disease versus those with milder trajectories. In treatment selection and response prediction which aims to align patients with therapies most likely to be effective, reducing the need for sequential treatment failures and lastly in assessment of monitoring and remission by providing objective readouts of synovial inflammation and subclinical disease activity to guide treatment escalation or de-escalation.

The desired outcome of identifying new biomarkers is to take them forward towards clinical implementation that is from discovery to translation improve disease diagnosis, prognosis, and treatment through personalized medicine (Ivanisevic et Sewduth, 2023; Molla et Bitew, 2024)**.** This requires that the identified biomarkers must be validated in larger cohorts to assess their specificity, sensitivity and reproducibility in diagnosing the disease. The advances in computational biology and in machine learning have now made this possible as they can now provide powerful frameworks for the analysis of big data like the omics datasets, thereby accelerating the transition from biomarker discovery to clinical applications. Moreover, these have to be standardized and conform to the guidelines. Additionally, these biomarkers can be integrated to existing clinical, serological, radiological data and diagnostic tools or criteria to improve the diagnostic predictive power (Ivanisevic et Sewduth, 2023).

By linking together diverse datasets to uncover previously unknown causal pathways and correlations, big data enables far greater precision and customization than was ever achievable

before. Recent scientific advancements in high-throughput, high-resolution data-generating technologies allow cost-effective analysis of big datasets on individual health. However, analyzing and integrating such vast amounts of information requires new computational approaches, including faster and more integrated processors, larger computer memories, improved sensors, sophisticated algorithms, methodologies, and cloud computing. These advancements have the potential to guide future clinical practice by delivering clinically useful information.

The present thesis was designed to address an important gap in the rheumatoid arthritis literature. Although omics-based studies have provided valuable insights into RA pathogenesis, many previous investigations have focused on early disease, synovial tissue, treatment response, or single classes of biomarkers. In contrast, this thesis focuses specifically on established, long-standing rheumatoid arthritis and examines circulating plasma as a clinically accessible and minimally invasive source of molecular information. By applying untargeted mass spectrometry-based proteomic and metabolomic approaches, this work seeks to characterize RA at two complementary molecular levels and thereby provide a broader view of the proteomic, and metabolic disturbances associated with chronic disease. The novelty of this approach lies not in a single methodological novelty, but in the combined and translational application of complementary omics platforms to a clinically defined cohort of established RA. In this way, the thesis extends beyond conventional serological markers and beyond single-omics descriptions to generate candidate circulating biomarkers and pathway-level insights relevant to disease characterization, patient stratification, and future validation studies.

Table 1-1 Summary of RA Pathophysiology, Clinical Diagnosis, and Biomarkers

	Clinical / Diagnostic Relevance	Key References
RA Pathophysiology		
Genetic Risk Factors	• HLA risk alleles inform understanding of autoantigen (ACPA) generation. • Non-HLA polymorphisms guide drug target discovery (JAK/STAT, co-stimulatory pathways). • Genetic risk does not fully explain prevalence and molecular phenotyping is required.	Sharma et al., 2024 Stahl et al., 2012 Goldmann et al., 2024 Aslam et al., 2020
Epigenetic Regulation	• Methylation signatures as biomarkers of disease aggressiveness and treatment response. • miRNA panels: predictive tools for DMARDs / biologic response (CYP3A4, CYP2CP variants). • m6A machinery linked to RA pathogenesis and a potential epigenetic therapeutic target.	Brandt et al., 2019 Dolcino et al., 2019 D'Orazio et al., 2024 Canet et al., 2019 Jiang et al., 2021 Zheng et al., 2023
Environmental & Microbial Triggers	• Smoking cessation and dental hygiene as modifiable RA risk reduction strategies. • Microbiome-based biomarkers and therapeutics under investigation. • Environmental risk profiling for pre-clinical RA identification.	Deane et al., 2017 Zhao et al., 2021 Xie et al., 2025
Immune Dysregulation (T cells, B cells, Innate)	• Th17/Treg ratio: disease activity biomarker; IL-17 inhibitors in clinical use. • B-cell depletion and co-stimulatory blockade. • NETosis signature: emerging SF/plasma biomarker distinct from CRP-mediated inflammation.	Luo et al., 2022 Ruschpler & Stiehl, 2002 Chimenti et al., 2015 Uehara & McGrath, 2019 Jian et al., 2023 Lin et al., 2020
Cytokine Networks & Signalling	• Established therapeutic targets: TNFi, IL-6R inhibitors, IL-1 blockers, IL-17 inhibitors, JAK inhibitors. • Cytokine measurement alone is non-specific; complexity of networks demands unbiased multi-omics approaches. • JAK-STAT pathway profiling used to predict treatment response.	McInnes & Schett, 2007 McInnes & Liew, 2005 Ibrahim & Huttunen, 2021
Systemic Manifestations & Pannus Formation	• Anti-RANKL for bone protection; MMP inhibition as therapeutic strategy.	Wu et al., 2022 Fang et al., 2020

	• Cardiovascular risk management integral to RA care. • Systemic metabolic markers (insulin, adipokines) as comorbidity monitors.	
Current Diagnostic Biomarkers & Their Limitations		
Established Serological Markers	• RF + ACPA: part of ACR/EULAR 2010 classification criteria. • Seronegative RA (~30–40%): diagnostic delays; neither RF nor ACPA present at onset. • ESR/CRP: fail to distinguish biological from clinical remission; inadequate for monitoring subclinical inflammation.	Meehan et al., 2021 Steiner et al., 2024 van Delft & Huizinga, 2020
Extended Autoantibodies & Peripheral Blood Biomarkers	• Anti-MCV / anti-CarP: potential role in seronegative RA sub-classification. • MBDA: FDA-approved; useful for relapse prediction after biologic tapering; not yet standard of care. • Peripheral blood preferred for clinical scalability over synovial fluid.	Harre et al., 2012, Huang et al., 2025, Veale et al., 2025, Meznerics et al., 2023 Rech et al., 2016
Synovial Fluid & Tissue Biomarkers	• SF biomarkers provide a mechanistically richer signal than peripheral blood but require invasive collection (arthroscopy / US-guided biopsy) and limits scalability. • Calprotectin: promising non-invasive surrogate for synovial inflammation; assay standardisation still needed. • Drives need for non-invasive plasma-based multi-omics to capture equivalent molecular information.	Liao et al., 2004 Wright et al., 2012 Mun et al., 2021 Martins & Fonseca, 2019

Abbreviations: ACPA = anti-citrullinated protein antibodies; APC = antigen-presenting cell; CRP = C-reactive protein; DAS28 = Disease Activity Score 28; DMARD = disease-modifying anti-rheumatic drug; ESR = erythrocyte sedimentation rate; FLS = fibroblast-like synoviocytes; GWAS = genome-wide association study; HLA = human leukocyte antigen; IL = interleukin; IFN-γ = interferon-gamma; JAK = Janus kinase; lncRNA = long non-coding RNA; MBDA = multi-biomarker disease activity; MHC = major histocompatibility complex; miRNA = microRNA; MMP = matrix metalloproteinase; m6A = N6-methyladenosine; NETs = neutrophil extracellular traps; NF-κB = nuclear factor kappa B; PAD = protein arginine deiminase; RANKL = receptor activator of NF-κB ligand; RBP-4 = retinol-binding protein 4; RF = rheumatoid factor; SF = synovial fluid; STAT = signal transducer and activator of transcription; Th = T helper; TNF-α = tumour necrosis factor alpha; Treg = regulatory T cells.

1.11 Conclusion

Rheumatoid arthritis remains a chronic, heterogeneous autoimmune disease in which current diagnostic and monitoring tools provide only partial insight into disease biology. While biomarkers such as RF, ACPA, ESR, and CRP, along with composite indices, have improved clinical care, they remain insufficient for early diagnosis, accurate monitoring, or prediction of therapeutic response. Even patients deemed to be in remission often experience residual pain, persistently elevated inflammatory markers, or subclinical synovitis detectable only on imaging. These limitations are particularly evident in patients with longstanding disease, where decades of inflammation, structural damage, and cumulative treatment exposure shape a complex clinical phenotype that is poorly captured by conventional measures.

Advances in mass spectrometry-based proteomics and metabolomics provide an opportunity to address these gaps by enabling high-resolution characterization of the molecular landscape of RA. Proteomics reveals alterations in immune and inflammatory proteins, while metabolomics reflects the metabolic rewiring that accompanies chronic inflammation. Together, these complementary approaches offer a systems-level view of RA that can generate mechanistically grounded and clinically relevant biomarkers.

CHAPTER 2 (SCIENTIFIC ARTICLE)

PROTEOMIC PROFILING REVEALS NOVEL MOLECULAR INSIGHTS INTO DYSREGULATED PROTEINS IN ESTABLISHED CASES OF RHEUMATOID ARTHRITIS

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CONTRIBUTION OF THE MAIN AUTHOR AND CO-AUTHORS

Afshan Masood, Dr. Anas Abdel Rahman, and Prof. Mohamed Siaj conceived the idea of this Project. Afshan Masood designed the experiment and developed the mass spectrometry method and run the validation and the patient samples and drafted the manuscript. Dr Assim Alfadda, and Dr. Abdulrahman S. Alarfaj designed the clinical project, recruited the study patients from their clinic. Ms. Maha Almogren and Dr. Hicham Benabdelkamel helped in the mass spectrometry setup and in MRM validation. Dr. Abdel Rahman and Prof. Siaj have critically reviewed the manuscript. All authors approved the current version of the manuscript for publication.

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that predominantly affects synovial joints, leading to inflammation, pain, and progressive joint damage. Despite therapeutic advancements, the molecular basis of established RA remains poorly defined. In this study, we conducted an untargeted plasma proteomic analysis using two-dimensional differential gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in samples from RA patients and healthy controls in the discovery phase. Significantly (ANOVA, $p \leq 0.05$, fold change > 1.5) differentially abundant proteins (DAPs) were identified. Notably, upregulated proteins included mitochondrial dicarboxylate carrier, hemopexin, and 28S ribosomal protein S18c, while CCDC124, osteocalcin, apolipoproteins A-I and A-IV, and haptoglobin were downregulated. Receiver operating characteristic (ROC) analysis identified CCDC124, osteocalcin, and metallothionein-2 with high diagnostic potential (AUC = 0.98). Proteins with the highest selected frequency were quantitatively verified by multiple reaction monitoring (MRM) analysis in the validation cohort. Bioinformatic analysis using Ingenuity Pathway Analysis (IPA) revealed the underlying molecular pathways and key interaction networks involved STAT1, TNF, and CD40. These central nodes were associated with immune regulation, cell-to-cell signaling, and hematological system development. Our combined proteomic and bioinformatic approaches underscore the involvement of dysregulated immune pathways in RA pathogenesis and highlight potential diagnostic biomarkers. The utility of these markers needs to be evaluated in further studies and in a larger cohort of patients.

Keywords: rheumatoid arthritis; plasma proteomics; osteocalcin; metallothionein-2; 2D DIGE; MALDI-TOF; biomarker

2.1 Introduction

Rheumatoid arthritis (RA) is a complex and challenging disease characterized by a chronic autoimmune inflammatory state mainly involving the synovial joints, leading to articular damage, pain, and disability. The global prevalence of RA has been estimated to affect approximately 0.1–2.0% of the population, affecting females more than their male counterparts (Almutairi *et al.*, 2021). The etiology of RA is multifactorial, arising from complex interactions between genetic predisposition, immune dysregulation, and environmental triggers. Its development is strongly influenced by T cells and genetic susceptibility linked to the major histocompatibility complex (MHC), particularly HLA class II genes. Polymorphisms within the HLA-DR, DP, and DQ sub regions—especially the HLA-DRB1 locus—have been consistently associated with increased RA susceptibility (Huang *et al.*, 2021). The onset of RA is hypothesized to involve dysregulation of immune signaling pathways, leading to heightened local and systemic inflammation. To this effect, immune cells of both the innate and adaptive immune systems, including monocytes, macrophages, as well as the B and T lymphocytes acting through several mediators, have been implicated in the pathogenesis of RA (Petrelli *et al.*, 2022).

Patients with RA generally present clinically with swelling and pain in the joints that, in severe cases, result in the erosion of bones and deformity of the joints (Schett et Gravallese, 2012). This can be accompanied by complications such as anemia, osteoporosis, cardiovascular disease, and lung disorders [5] in prolonged cases of the disease. The early diagnosis and treatment of RA can ameliorate these conditions, but many reports show that the underlying pathology is not resolved. Routine laboratory diagnosis of RA is primarily based on assessing disease activity through detecting high levels of autoantibodies such as rheumatoid factor, ACPAs, and anti-carbamylated protein antibodies that have been used, although with low sensitivity and limited reliability (Sokolova *et al.*, 2022; van Delft et Huizinga, 2020).

Proteomics, a powerful branch of molecular biology, has emerged as a promising tool for investigating the complex and dynamic changes occurring within cells and tissues during disease through a systematic study of the entire proteome. The high-throughput nature of proteomic technologies allows for the simultaneous assessment of numerous proteins, making it well-suited for the comprehensive profiling of the molecular alterations associated with RA. Proteomics approaches have used different biological fluids, including serum, plasma, and synovial fluid, to elucidate the molecular heterogeneity of RA to study markers for the early

diagnosis of disease (Mun *et al.*, 2019) and to identify those that can indicate remissions and avoid disease flare-ups (Birkelund *et al.*, 2020; Park *et al.*, 2015; Ren *et al.*, 2021). In recent years, significant progress has been made in RA proteomic research aiming to yield novel biomarkers and potential therapeutic targets and in ascertaining potential pathways involved in RA pathogenesis (Hu *et al.*, 2022; Jung *et al.*, 2022; O'Neil *et al.*, 2021; Onuora, 2020). Recently, two studies by Hu et al. and Cheng et al. using proteomics proposed that changes in the serum levels of ORM1 and FCN-2, respectively, were correlated with disease activity (Cheng *et al.*, 2014; Hu *et al.*, 2022). Proteomic analysis of the synovial fluid by Liao et al. showed changes in S100A9 and S100A12 proteins as prognostic markers of erosive forms of RA (Liao *et al.*, 2004). Recently, a targeted proteomic analysis of plasma samples using LC-MS/MS in MRM mode was carried out to accurately quantify soluble co-stimulatory molecules as potential RA diagnostic biomarkers (Malkawi *et al.*, 2023).

Despite the significant steps towards understanding RA, the underlying pathophysiology and molecular mechanisms underlying disease remain elusive. In the present study, we aimed to use 2D-DIGE coupled with MALDI-TOF-MS to identify changes in the plasma proteome between the RA and control groups and relate them to the affected metabolic pathways using bioinformatics analysis. Exploring the untargeted plasma proteome will provide the ability to gain a better perception of changes in molecular pathways and provide potential biomarkers for disease diagnosis, prognosis, or management.

2.2 Materials and Methods

2.2.1 Ethical Approval, Patient Recruitment, and Consent to Participate

The study procedures and protocols were reviewed and obtained from the institutional review board of the College of Medicine, King Saud University (IRB number: E-21-6341). The primary physician confirmed the RA diagnosis based on ACR/EULAR 2010 criteria (Aletaha *et al.*, 2010; Rosenfeld *et al.*, 1999). The patients in the present study were recruited from those attending the rheumatology outpatient clinics at King Khalid University Hospital, College of Medicine, King Saud University, in the age group of 36–65 years. A total of 12 patients with known RA for a mean duration of 20 years in clinical remission (DAS28 < 2.6), mostly women, were selected from a cohort for the discovery phase, and 60 patients were selected for the validation phase. Control plasma samples were obtained from the blood bank and age-matched volunteers who were otherwise healthy and used in the discovery and validation phases. Their

attending physician carried out the clinical assessment, and those willing to participate consented. The sample size was determined by conducting a power analysis using the Progenesis SameSpots non-linear dynamics statistical software to determine the minimum number of biological replicates required. The baseline demographic information and clinical laboratory parameters, including complete blood count with an erythrocyte sedimentation rate (ESR), lipid markers, liver function tests, fasting glucose, glycated hemoglobin, and C-reactive protein (CRP), were collected from the patient's medical records (Table 1). The study procedures were conducted according to the ethical standards of the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines. Blood samples were drawn in EDTA-containing vacutainers, and plasma was separated based on the standard protocol. The plasma samples were aliquoted and stored at −80 °C until further processing.

2.2.2 Sample Processing by Depletion Followed by Precipitation of Proteins

All plasma samples were processed with Top-20 Depletion ProteoPrep spin columns (Sigma-Aldrich, Saint Louis, MO, USA) to deplete the abundant proteins to decrease the sample complexity. Due to their abundance, these plasma proteins, including immunoglobulins, albumin, alpha-1 antitrypsin, and transferrin, mask the underlying low DAPs and are depleted before precipitation. Protein precipitation was carried out using the TCA/acetone (10% *w*/*v*) method, added to the depleted protein in a ratio of 1 to 4, vortexed for 15 s, and left overnight at −20 °C. The following day, sample mixtures were centrifuged (2000× *g* for 15 min at 4 °C), and the protein pellets obtained were re-suspended in labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris). All the samples were checked for their pH, and it was adjusted to 8.5 where required. Following this, the protein concentration of all the samples was estimated by the 2D-Quantkit (GE Healthcare, Danderyd, Sweden) in duplicate.

2.2.3 2D-DIGE Gel Electrophoresis for Protein Separation and Image Scanning

Cye dye labeling was performed for the samples using CyDye™ DIGE Fluor minimal dye. Briefly, 400 pmol (GE Healthcare, Danderyd, Sweden) of the cye dye, freshly dissolved in anhydrous dimethyl formamide (DMF), was used for the fluorescent labeling of 50 μg of protein taken from each sample using the dye switching technique between the control and RA patient samples using either Cy3 or Cy5 (Alfadda *et al.*, 2018; Alfadda, Assim A *et al.*, 2013; Benabdelkamel *et al.*, 2015; Masood *et al.*, 2020). A pooled internal standard of 50 μg of total

protein from each of the 24 samples was labeled with Cy2. After the addition of the cye dye, the samples were incubated in the dark on ice for the reaction, covalently linking with a fluorophore. Subsequently, the reaction was quenched by adding 1.0 μL of lysine (10 mM) and ice for 10 min in the dark. The samples were combined according to the experimental design and run on the same gel for comparison. Twelve Immobiline Dry Strips (24 cm, pH 3–11; GE Healthcare, Danderyd, Sweden) were rehydrated passively (30 V, 12 h) and were used to carry out the first-dimensional analytical gel electrophoresis. The isoelectric focusing using an Ettan IPGphor IEF unit (GE Healthcare) was performed at 20 °C and 50 μA per strip, according to the following steps and hold sequence: (1) 500 V for 1 h, (2) 1000 V for 1 h, (3) 8000 V for 3 h, and (4) 8000 V for 45,000 Vh. The IPG strips were next equilibrated in the equilibration solution (5 mM Tris—HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) at room temperature for 15 min each, with a solution containing 65 mM DTT, followed by a solution containing 250 mM iodoacetamide. The second, using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 5–20%) prepared using low-fluorescent glass, was performed (six vertical units, Ettan DALT, GE Healthcare, Danderyd, Sweden; 15 °C, 1 W per gel for 1 h, and then 2 W per gel until the bromophenol blue dye front reached the bottom of the gel). After SDS PAGE electrophoresis, the gels were scanned with the Sapphire Biomolecular Imager (Azure Biosystems, Dublin, OH, USA) and digitalized via the image analysis software Sapphire Capture system (version 1.12.0921.0) (Azure Biosystems, Dublin, OH, USA). Preparative gels were prepared using total protein (1 mg) obtained from a pool of equal protein amounts from the 24 depleted plasma samples. Gels were stained for 5 days, and the stained gels were briefly rinsed with Milli-Q water and stored until the spots could be picked and identified using MS, as previously described (Alfadda *et al.*, 2018; Alfadda, Assim A *et al.*, 2013; Benabdelkamel *et al.*, 2015; Masood *et al.*, 2020).

2.2.4 Statistical Analysis for Determining Differentially Abundant Proteins (DAPs) Spots

2D-DIGE gel images were analyzed for differences in spot intensities using an automated spot detection method, Progenesis SameSpots software v.3.3 (Nonlinear Dynamics, Newcastle upon Tyne, UK), which included modules for gel warping, DIGE normalization, and comparison. The gel images from individual analytical gels were imported into the software, aligned to the reference gel, and overlaid to ensure that no data were lost. The normalized volume (NV) of each gel from the ratio of Cy3 (or Cy5) to Cy2 spot volume was calculated,

and the spot volumes were log-transformed to generate normally distributed data. Log-normalized volume (LNV) was used to quantify the differential expression by directly comparing patient and control groups. All spots were pre-filtered and manually checked before applying statistical criteria using two cutoffs (analysis of variance (ANOVA), FDR $p \leq 0.05$, and fold change (FC) > 1.5). Instead of spot intensities, normalized spot volumes were used for statistical processing. An additional univariate analysis using a volcano plot analysis was performed for each binary comparison to identify significantly differentially expressed proteins based on a fold-change criterion > 1.5, with a false discovery rate (FDR)-adjusted p-value less than 0.05. The x-axis on the volcano plot represents the fold change (FC) between the two comparison groups, while the y-axis represents the p-value.

2.2.5 MALDI-TOF-Based Mass Spectrometric Analysis for Protein Identification

Coomassie-stained gel spots were excised manually, washed, and digested according to previously described methods. A mixture of tryptic peptides (0.8 μL) derived from each protein was spotted onto a MALDI target (384 MTP Anchorchip; 800 μm of Anchorchip; Bruker Daltonics, Bremen, Germany). MALDI-MS/MS spectra were obtained using an UltraflexTerm TOF mass spectrometer equipped with a LIFT-MS/MS device (Bruker Daltonics) at reflector and detector voltages of 21 kV and 17 kV, respectively, as described before. Peptide mass fingerprints (PMFs) were calibrated against a standard (peptide calibration standard II, Bruker Daltonics). The PMFs were assessed using the Flex Analysis software (version 2.4, Bruker Daltonics). MS data were interpreted using BioTools v3.2 (Bruker Daltonics). The peptide masses were searched using the Mascot search algorithm (v2.0.04, updated on 9 May 2021; Matrix Science Ltd., London, UK). The Mascot significance score was calculated using the formula Protein score = $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event; a Mascot score greater than 56 was considered significant ($p \leq 0.05$) and was accepted. ID proteins with low scores were excluded, as they were mostly random matches and insignificant ($p > 0.05$). The Mascot parameters were as follows: fixed cysteine modification with propionamide, variable modification due to methionine oxidation, one missed cleavage site in case of incomplete trypsin hydrolysis, and a mass tolerance of 100 ppm. Identified proteins were accepted as correct if they showed a Mascot score greater than 65. Not all spots of interest could be identified because some proteins were low-abundance and did not yield sufficiently intense mass fingerprints; other spots were mixtures of multiple proteins.

2.2.6 Biomarker and Bioinformatics Analysis

Biomarker analysis of the proteomics expression profiles was performed using MetaboAnalyst version 6.0 (McGill University, Montreal, QC, Canada). The raw data were normalized to the sample's total median to ensure that all samples were distributed normally, and they were corrected to make individual features more comparable by using log-transformation and Pareto-scaling, respectively. Because the data were Gaussian-distributed, the unpaired two-tailed Student's t-test was used for binary comparisons between any two study groups, where the significance levels for the protein data were considered at a false discovery rate (FDR)-corrected $p < 0.05$ with a 1.5-fold cutoff change; the values are presented as the mean ± SEM. Potential biomarkers were assessed using receiver operating characteristic (ROC) curve analysis performed via MetaboAnalyst (version 6.0, McGill University, Montreal, QC, Canada). ROC curves were generated based on an orthogonal partial least squares-discriminant analysis (OPLS-DA) model. Model robustness was evaluated through the fitness-of-model (R^2Y) and predictive ability (Q^2) metrics, where R^2Y values approaching 1 and Q^2 values above 0.5 indicate strong model performance. The DAPs were classified into different categories according to their molecular function and biological process using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (http://www.pantherdb.org (accessed on 23 July 2024)). The interaction between the statistically significant differentially abundant proteins—the protein–protein interaction (PPI) network—was also constructed using Ingenuity Pathway Analysis (IPA) version 9.0 (Ingenuity Systems, Redwood, CA, USA). The IPA software maps the UniProt IDs into the ingenuity knowledge base, a manually curated resource containing data from all published scientific studies and considered the world's largest. The list of identified proteins, with the FC and FDR p-values, was entered into the online software to define the interactions, key biological pathways, and molecular roles of the identified proteins between RA and control groups. The software-generated network maps provide a means of visualizing both direct and indirect protein—protein interactions and relate them to known biological and canonical pathways (Alfadda *et al.*, 2018; Alfadda, Assim A *et al.*, 2013; Benabdelkamel *et al.*, 2015; Masood *et al.*, 2020).

2.2.7 Validation of Results Using LC—MS/MS (MRM)

Four different proteins were selected from the proteomics profile, where at least one signature peptide per protein was identified using the criteria described previously, using Skyline Software v21 (MacCross Lab, Seattle, WA, USA) . The suggested MRM transitions were

exported to a Triple-Quadrupole-Tandem Mass spectrometer (XEVO TQmicro, Waters Corporation, Milford, MA, USA). A total of 120 samples were used for validation. A control-extracted sample (n = 60) was used to evaluate the calculated transitions and to optimize the collision energy and column retention time. The patient samples (n = 60) were digested with trypsin and solid-phase-extracted using the standard protocol reported by Galal et al., 2021 (Galal *et al.*, 2021). The validation batch of these samples was started with a calibration curve (7 points) and the 3 levels of QCs (low, medium, and high); all of these QCs and the calibration curves were evaluated with accuracy 85–115% and CV% < 15, with overall success > 67% of all QCs based on CLIA and FDA Guidelines. A pool of QC samples was also added as an extra control level to ensure a whole process of sample analysis control with <20 CV%. The same is now included in the manuscript. The extracted tryptic peptides were separated using an Acquity Ultra-Performance Liquid Chromatography (UPLC) (AQUITY BEH C18, 1.7 μm, 2.1 mm × 100 mm) column (at 25 °C) at a mobile phase flow rate of 0.3 mL/min over a total run time of 12 min (solvent A: 0.1% formic acid in H2O; solvent B: 0.1% formic acid in acetonitrile). The gradient profile for solvent A (0.1% formic acid in H2O) was 90% for 1 min, followed by a linear gradient to 10% over 10 min, which was then held at 10% for 1 min before returning to 90% in 2 min. For positive-mode mass spectrometric resolution, the eluted peptides were subjected to electrospray ionization (ESI). The source desolvation temperature was set to 450 °C, the desolvation gas flow was set to 700 L/Hr, the cone gas flow was set to 50 L/Hr, the MS capillary source voltage was set to 1.98 KV, and the cone source was set to 47 V. The total run time for each sample was 12 min at a mobile phase flow rate of 0.3 mL/min following the gradient table. The samples were stored in an autosampler at 4 °C, with an injection volume of 5 μL. During the run, frequent intermediate washing steps were performed to minimize the sample carryover. The experimental conditions are summarized in Supplementary Table S4

2.3 Results

2.3.1 Clinical and Biochemical Data for the Study Participants

The demographic and biochemical characteristics of the study participants in the discovery and validation phases are presented in Table 2- 1. The mean ages of the study participants in the RA and control groups were 46.8 ± 12.4 and 41.5 ± 6.2 years, respectively, and were matched in body weight, BMI, and other laboratory markers. Significant differences were noted in the ESR and CRP levels, which were higher in the RA group than in the control group.

Table 2-1. Baseline demographic and clinical characteristics of patients with rheumatoid arthritis and control groups in the discovery and validation phases.

Characteristics	Cases (Mean ± SD) (n = 12)	Controls (Mean ± SD) (n = 12)	p-Value	Cases (Mean ± SD) (n = 60)	Controls (Mean ± SD) (n = 63)	p-Value
	Discovery Phase			Validation Phase		
Duration (yrs)	14 ± 9			16 ± 7		
Age	46.8 ± 12.4	41.5 ± 6.2	0.19	49.8 ± 12.045	47.5 ± 13.86	0.32
BMI (Kg/m^2)	24.2 ± 3.8	22.8 ± 4.2	0.40	29.1034 ± 7.2	29.9 ± 6.21	0.52
HB (gm/L)	126.8 ± 8.7	132.3 ± 14	0.26	127.2 ± 9.3	129.7 ± 9.6	0.44
WBC 10^9/L	6.5 ± 1.8	6.9 ± 2.7	0.60	6.8 ± 2.2	6.6 ± 2.4	0.59
RBC (10^{12}/L)	4.4 ± 0.3	4.6 ± 0.6	0.15	4.8 ± 0.6	5.1 ± 1.3	0.07
Lymphocyte (10^9/L)	2.4 ± 0.8	2.5 ± 0.7	0.61	2.2 ± 0.7	2.4 ± 0.5	0.55
Neutrophil (10^9/L)	3.3 ± 1.3	3.6 ± 2.1	0.62	3.2 ± 1.3	3.1 ± 1.5	0.09
ESR (mm/hr)	48.5 ± 26.8	5.8 ± 2.1	<0.001 *	43.0 ± 23.8	5.6± 2.8	<0.001 *
ALT (IU/L)	20.5 ± 4.2	19.7 ± 9.3	0.7	18.99 ± 7.06	18.04 ± 9.01	0.08
AST (IU/L)	17.2 ± 6.7	17.4 ± 4.1	0.9	19.0 ± 7.0	16.7 ± 7.3	0.08
Albumin (gm/L)	38.4 ± 7.6	40.2 ± 3.3	0.3	39.9 ± 6.5	41.3 ± 4.9	0.22
Glucose(mmol/L)	4.9 ± 0.5	4.6 ± 0.4	0.6	5.48 ± 1.12	5.15 ± 0.85	0.07
Chol (mmol/L)	4.4 ± 1.2	4.3 ± 0.7	0.7	4.63 ± 0.78	4.77 ± 0.94	0.36
HDL (mmol/L)	1.4 ± 0.3	1.4 ± 0.4	0.5	1.47 ± 0.410	1.49 ± 0.37	0.316
LDL (mmol/L)	2.8 ± 1.0	2.4 ± 0.5	0.2	2.99 ± 0.81	2.70 ± 0.80	0.18

Triglycerides (mmol/L)	1.2 ± 1.2	1.1 ± 0.3	0.7	1.19 ± 0.74	1.27 ± 0.54	0.507
HbA1c	5.4 ± 0.5	5.2 ± 0.4	0.1	5.74 ± 0.54	5.71 ± 0.84	0.916
CRP (mg/L)	10.0 ± 7.8	2.4 ± 1.7	<0.001 *	9.70 ± 7.40	1.9 ± 3.4	<0.001 *

Values are expressed as mean ± standard deviation (SD) in the parenthesis. A significant difference between RA and control (independent t-test, p-value < 0.05 for parametric tests). BMI: body mass index, HB: hemoglobin, WBC: white blood cells, RBC: red blood cells, PLT: platelets, ESR: erythrocyte sedimentation rate, ALT: alanine aminotransferase, AST: aspartate transaminase, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HbA1c: glycated hemoglobin, CRP: C-reactive protein.*p-Value <0.05 is considered as statistically significant.

2.3.2 Proteomic Analysis and Identification of Differentially Expressed Proteins

The DAPs spots between the RA and control groups (24 samples from 12 gels) were assessed using 2D-DIGE coupled with MALDI-TOF mass spectrometry. Figure2- 1 shows the representative fluorescent protein profiles of the 2D-DIGE of control samples labeled with Cy5 (Figure 2-1A), RA samples labeled with Cy3 (Figure 2-1B), and a pooled internal control labeled with Cy2 (Figure 1D). A total of 1200 spots were detected, matching across all 24 gel images. These spot patterns were reproducible across all 24 gel images, leading to alignment and further analysis. Normalization across the complete gels and quantitative differential analysis of the protein levels were achieved using an internal standard with Cy2 labeling. Figure S1 shows a total of 89 spots that were statistically significant on the gels (cutoff: ANOVA, FDR $p \leq 0.05$; fold change of 1.5) between the RA and control groups.

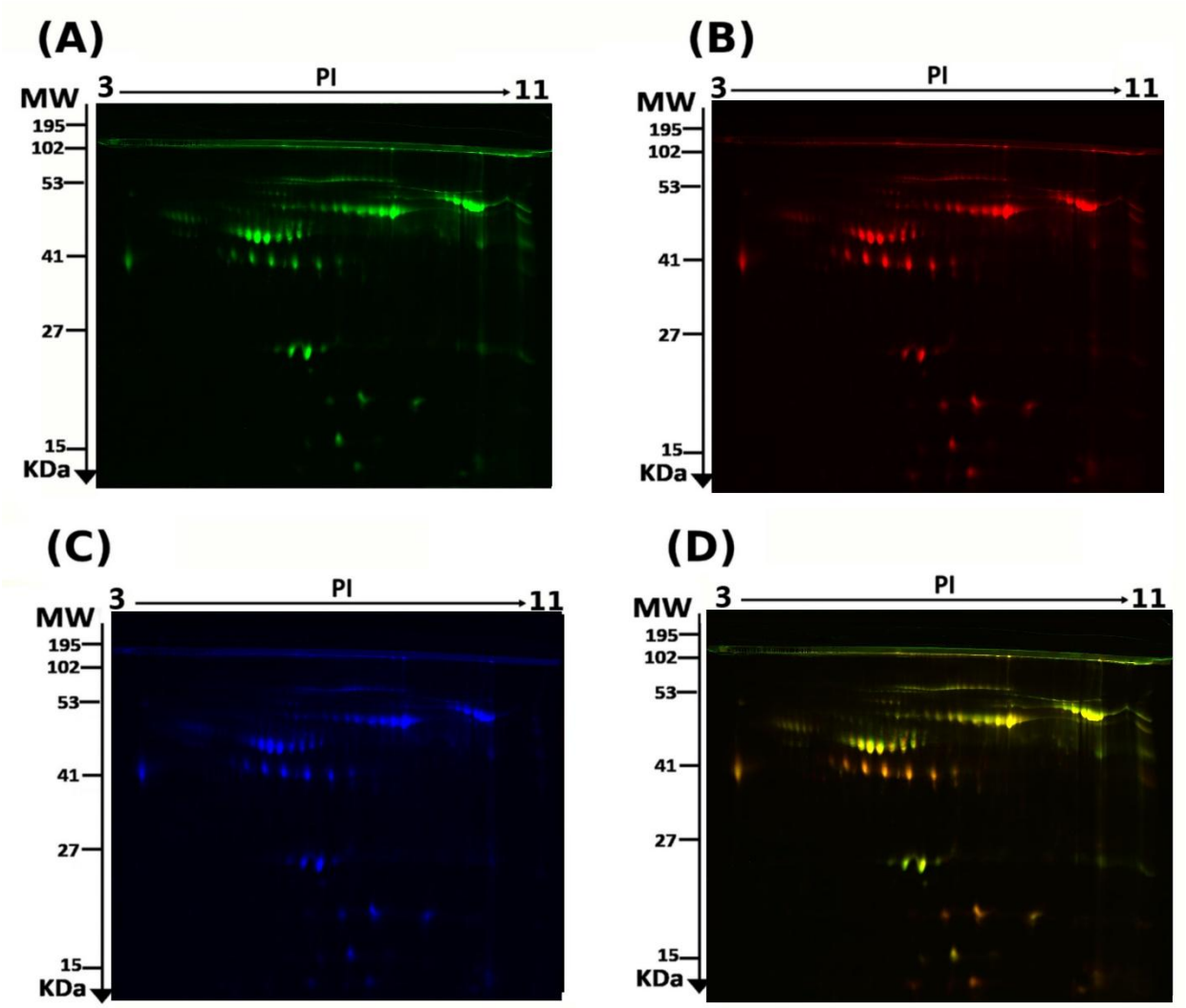

Figure 2-1 Representative images of two-dimensional difference in gel electrophoresis (2D-DIGE) gels depicting the separation of the fluorescent cye-dye-labeled proteins. RA samples labeled with Cy3 (A), controls labeled with Cy5 (B), pooled internal controls labeled with Cy2 (C), and the merged image (D).

An additional univariate analysis using the MetaboAnalyst software package (MetaboAnalyst version 6.0, McGill University, Montreal, Canada; http://www.metaboanalyst.ca) was conducted, where the data were further normalized via log transformation and Pareto scaling. The significant and dysregulated proteins in this study were further analyzed using PCA (Figure 2-2A) and a volcano plot, in which the x- and y-axis indicate the fold change (cutoff > 1.5) and the y axis t-test (FDR $p < 0.05$), respectively (Figure 2-2B). This rigorous analysis combining the expression fold change and FDR-corrected p-value (FDRp) showed that only 28 and 61 proteins were up- and downregulated in the RA patients compared with the control patients, respectively. The spots showing a statistical significance between the two conditions were then manually excised from the preparative gel for protein identification by MALDI TOF-MS.

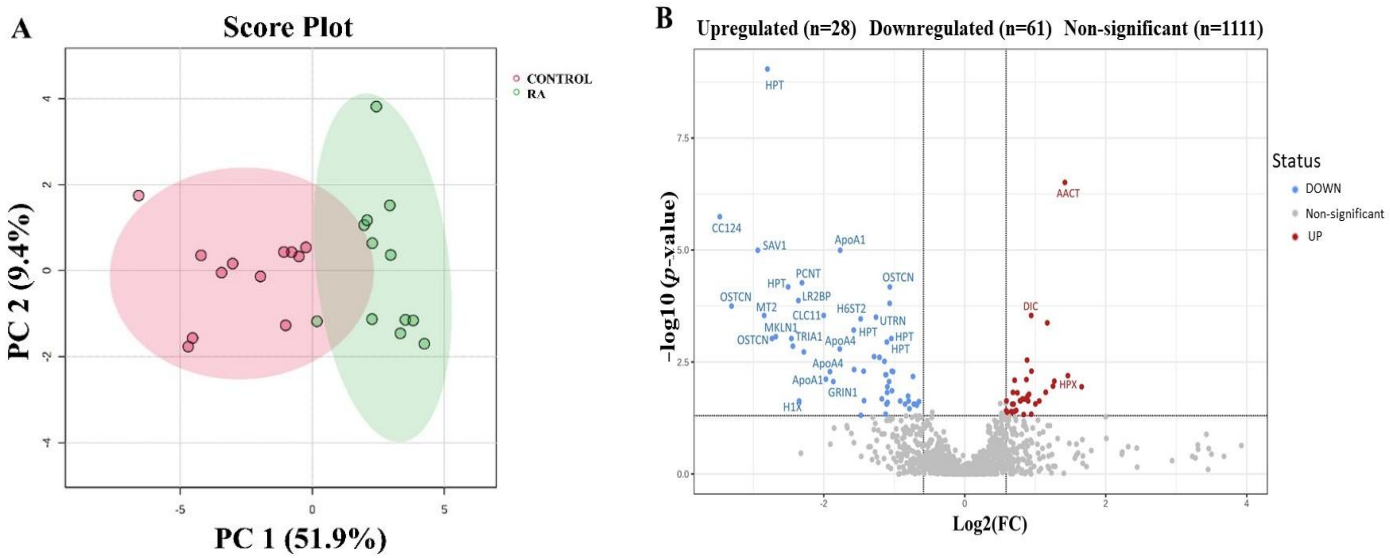

Figure 2-2 Unsupervised univariate analysis of the DAPs identified between RA and control groups. (A) Principal component analysis of the proteomic dataset demonstrating the separation between the RA and control groups. The green dots indicate the RA patients, and the red dots represent the controls. The PC1 components explained 41.9% of the selected spot's variability values. The colored dots and numbers represent the gels and spots, respectively. (B) The volcano plot represents the gene names corresponding to the protein spots between the RA and control groups, which revealed significant dysregulation in 89 protein spots based on the cutoffs (cutoff: ANOVA, p-value ≤ 0.05, and FC ≥ 1.5). From these, 28 (red) and 61 (blue) protein spots were upregulated and downregulated, respectively. The gray circled (Non-significant) protein spots (1111) failed to pass both cutoffs and were excluded from further analysis.

2.3.3 Mass Spectrometry Analysis and Protein Identification Using MALDI-TOF

Peptide mass fingerprints (PMFs) successfully identified 69 of the 89 protein spots excised from the preparative gel that were successfully matched to entries in the SWISS-PROT database by Mascot with high confidence scores. The sequence coverage of the proteins identified by PMF ranged from 8 to 75% (Table S1). In a few cases, variants of the same protein were found at several locations on the gel (Table S2, Figure S1). Among the differentially abundant proteins (DAPs) identified in RA vs. controls, 12 proteins demonstrated an increased abundance, while 57 had a decreased abundance (Table S2). The proteins with significantly increased abundance included mitochondrial dicarboxylate carrier (1.59-fold, $p < 0.001$), hemopexin (1.9-fold, $p < 0.001$), 28S ribosomal protein S18c (mitochondrial) (1.58-fold, $p = 0.017$), coiled-coil domain-containing protein 151 (2.87-fold, $p = 0.022$), and nucleolar and spindle-associated protein 1 (1.65-fold, $p = 0.01$); the full list is provided in Table S2. The significantly DAPs with decreased abundance included coiled-coil domain-containing

protein 124 (1.5-fold, <0.001), osteocalcin (2.87-fold, <0.001), apolipoprotein A-I (2.87-fold, <0.001), apolipoprotein A-IV (2.87-fold, <0.001), haptoglobin (HPT) (2.87-fold, p = 0.001), muskelin (1.92-fold, <0.001), heparan-sulfate 6-O-sulfotransferase 2 (1.6-fold, p = 0.002), branched-chain-amino-acid aminotransferase (1.66-fold, p = 0.029), and mitochondrial metallothionein-2 (1.5-fold, <0.001), with the full list presented in Table S2. Several proteins, 27 in all, including osteocalcin, haptoglobin, alpha-1-antitrypsin, and albumin, were found in more than one spot on the gels and appeared as multiple proteoforms with shifts in both pI or MW, which could be associated with their post-translational modifications for example (glycosylation and phosphorylation) or degradation, cleavage by enzymes (Table S2).

2.3.4 Orthogonal Partial Least Squares-Discriminant Analysis and Heat Map for the Proteomics Profile Between RA and Control Groups

A multivariate supervised analysis using the OPLS-DA model was used to analyze the DAPs identified between RA and control groups. The OPLS-DA showed a clear and distinct separation between the two groups, highlighting that the proteomic profile of patients with RA significantly differed from that of the control group. Model robustness was assessed through a permutation-based approach involving 100 iterations using MetboAnalyst that yielded an R2Y value of 0.987 and a Q2 value of 0.93, reflecting excellent fit and predictive capability (Figure 2- 3 A,B). The top 10 discriminant proteins with a variable influence on projection (VIP) value of >1 were responsible for the proposed separation (Figure 2- 3 C). A heat map and hierarchical clustering analysis of the differentially abundant proteins identified via MALDI-TOF-MS showed clusters of different expression patterns (Figure 2-3D) between the two groups. The clustering pattern demonstrates that the changes in the protein intensity of selected spots in RA compared to the control samples were significantly different (Figure 3- 3D). Shades of red indicate high levels of expression, and shades of green indicate low levels of expression.

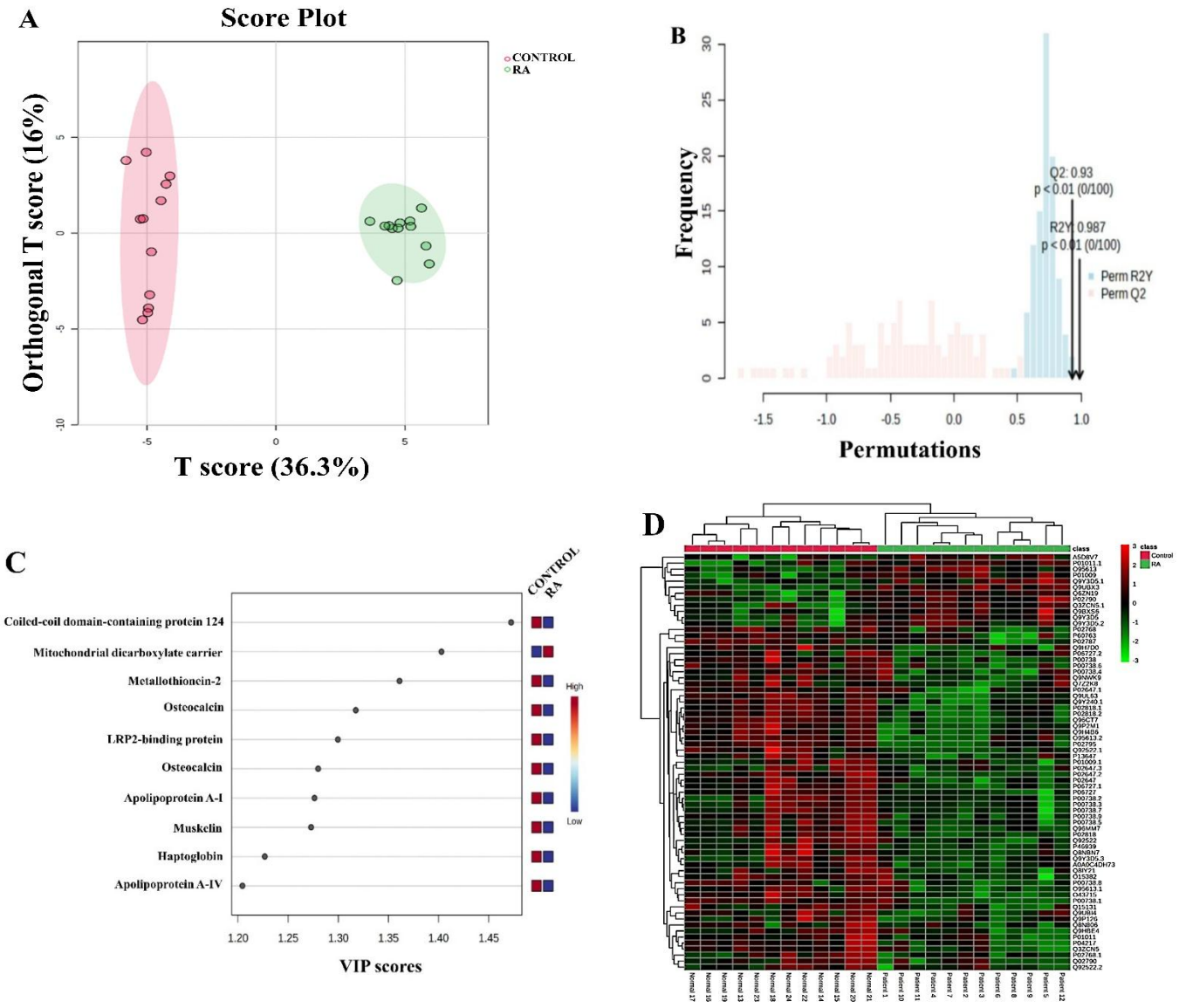

Figure 2-3 Proteomics profiling between the RA and control groups. (A) The orthogonal partial least squares-discriminant analysis (OPLS-DA) score plot showed evident separation between the two groups (RA and controls). The control and RA samples are represented as red and green circles, respectively. (B) The robustness of the created models was evaluated by the fitness of the model (R2Y = 0.987) and predictive ability (Q2 = 0.93) values. (C) The top 10 features of variable importance identified between the RA and control groups. (D) The heat map and hierarchical clustering analysis of the 69 identified proteins that were significantly altered between the RA and control groups based on the Pearson measure and average clustering method with the t-test/ANOVA using MetaboAnalyst Software V6 (Montreal, QC, Canada).

2.3.5 Functional Enrichment of DAPs in RA Group Compared to Control Group

The DAPs were classified using the Protein Analysis Through Evolutionary Relationships (PANTHER) system into their molecular and biological functions and cellular components (Figure 2- 4). Gene ontology analysis showed that the differentially expressed proteins were primarily associated with binding and catalytic activity as their main molecular functions (Figure 2-4A). These proteins were involved in cellular and metabolic processes and biological regulation and localized mainly within cellular anatomical structures and protein-containing complexes with corresponding FDR-adjusted *p*-values presented in Table S3; Figure 2-4B, C).

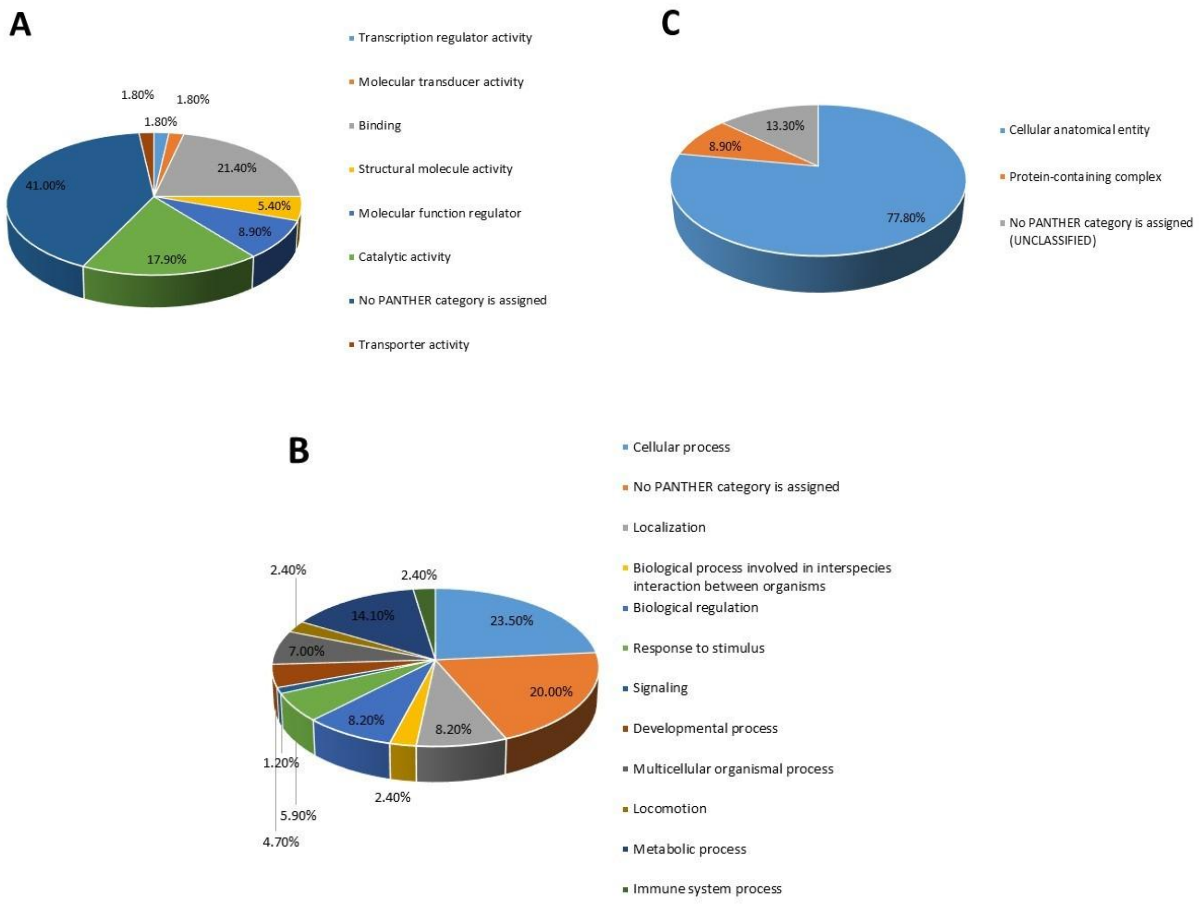

Figure 2-4 Pie charts showing the protein classes based on (A) molecular function, (B) biological process, and (C) cellular component of the significantly differentially abundant proteins between the RA and control groups.

2.3.6 Biomarker and Network Pathway Analysis of the DAPs Using Bioinformatics

We further analyzed the proteins using multivariate exploratory ROC analysis based on the identified common and significantly dysregulated proteins between the RA and control (n = 69) groups. The ROC curves were generated using PLS-DA as a classification and feature ranking method. The highest AUC of the exploratory ROC curve for a model with three proteins was 0.984 (Figure 2-5A). The frequency plot of the top 13 significantly dysregulated identified protein biomarkers in the RA and control groups showed that coiled-coil domain-containing protein 124, osteocalcin (OC), metallothionein-2, muskelin, etc., were downregulated in the RA group (Figure 2-5B). Four proteins showing the highest AUCs are shown, namely coiled-coil domain-containing protein 124 (AUC of 1, Figure 2-5 C), OC (AUC of 1, Figure 2-5D), metallothionein-2 (AUC of 0.933, Figure 2- 5E), and mitochondrial dicarboxylate carrier (AUC of 1, Figure 2-5 F).

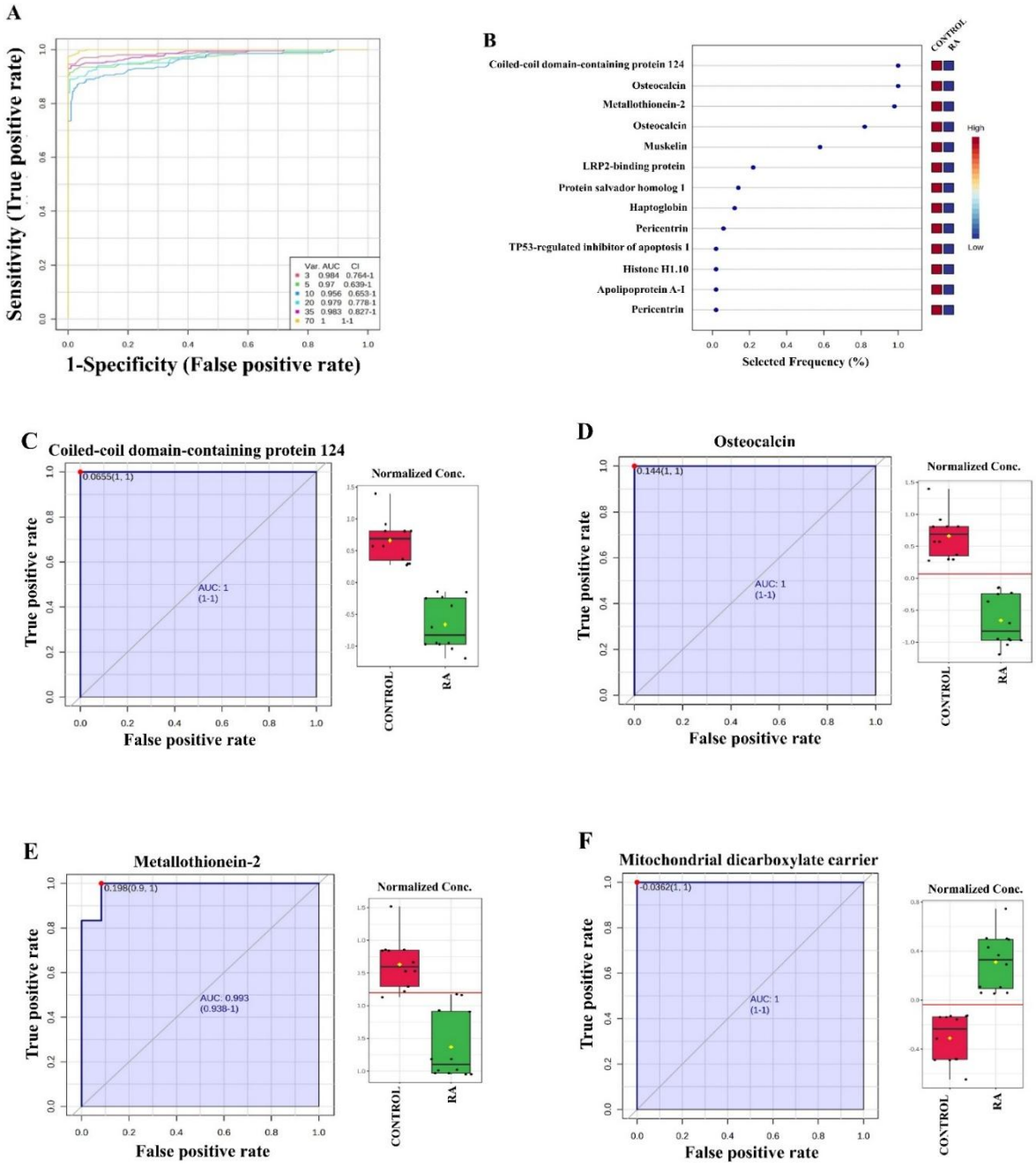

Figure 2-5 Multivariate biomarker analysis using the receiver operating characteristic (ROC) curve in patients with rheumatoid arthritis (RA) and control groups, using PLSDA as the feature ranking method. Multiple ROCs were generated using the biomarker analysis module of the MetaboAnalyst software (Montreal, QC, Canada) (www.metaboanalyst.ca). (A) The values of area under the curve (AUC between different models using 3, 5, 10, 20, 35, and 70 of the identified proteins). (B) The top 10 proteins with the highest selected frequency. The individual ROC for (C–E) down—and (F) upregulated proteins in RA along with the Box plot (FDR $p \leq 0.05$ and fold change ≥ 1.5), where red represents the control, and green represents RA.

The significant DAPs identified between the RA and control groups in our study were uploaded into the IPA software. IPA maps the proteins in our dataset to known proteins and their biological pathways. The DAPs were mapped to three major network pathways. The network with the highest score (score = 37) showed that these proteins were focused around the dysregulation of STAT1, TNF, and IL4 signaling pathways and were related to cell-to-cell signaling and interaction, hematological system development and function, and immune cell trafficking with a score of 37. The top canonical pathways identified were related to LXR/RXR activation (p-value of 2.76×10^{-9} and overlap of 5.7%, 7/122), the DHCR24 signaling pathway (p-value of 7.60×10^{-9} and overlap of 5.0%, 7/141), acute-phase response signaling (p-value of 4.79×10^{-8} and overlap of 3.8%, 7/184), response to elevated platelet cytosolic Ca^{2+} (p-value of 2.00×10^{-7} and overlap of 4.4%, 6/136), and binding and uptake of ligands by scavenger receptors (p-value of 2.25×10^{-6} and overlap of 4.4%, 5/113) (Figure 2-6 A,B).

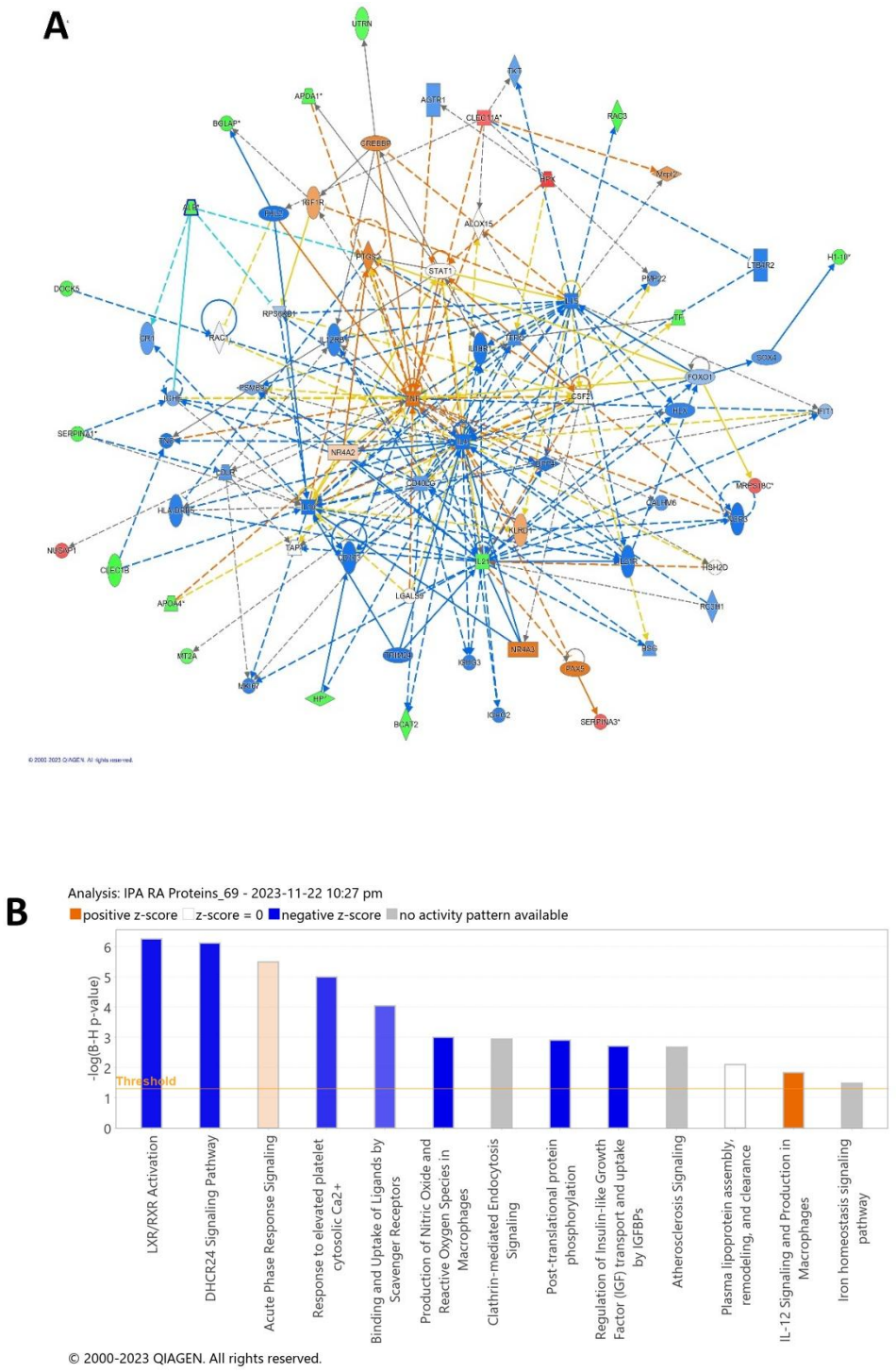

Figure 2-6 Network and biological pathway analysis relating to the significantly identified proteins in the study. (A) Network pathway analysis of the significantly dysregulated proteins identified in the RA group compared to the control group revealed dysregulation in pathways related to cell-to-cell signaling and interaction, hematological system development and function, and immune cell trafficking. The pathway identified TNF and IFNG as the central nodes. (B) The top canonical pathways related to the DAPs were LXR/RXR activation, DHCR24 signaling pathway, acute-phase response signaling, and response to elevated platelet cytosolic Ca2+ and IL-12 signaling. The blue color indicates an inhibition, while the orange color indicates an activation of these pathways between the RA and control groups.

2.3.7 Multiple Reaction Monitoring (MRM) Mass Spectrometry

Four significantly dysregulated proteins from the 2D-DIGE proteomic profile were selected for validation. Signature peptides for the selected proteins were identified using criteria described previously. Proteins were selected based on the highest selected frequency and on their involvement in the protein—protein interaction network pathway. Proteins with a higher number of interactions and fold changes showing significant changes in abundance (CC124, APOA1, OC, and HPT) were used to confirm the findings. The uniqueness and reliability of these signature peptides were confirmed using Skyline Software V3 and PeptideAtlas (Human 2025-01, https://peptideatlas.org/)(Benabdelkamel *et al.*, 2020). The MRM method was optimized using triple-quadrupole mass spectrometry (LC-MS/MS). Representative chromatograms for each protein signature peptide are shown in Figure 2- 7 and Table S4. This validation experiment shows that these four selected proteins have similar expression trends compared to the MALDI-TOF results, as shown in Figure 2- 7, with a different fold-change value. The expression profiles of these proteins were statistically evaluated using unpaired *t*-tests with PrismPad Software version 8 (Dotmatics, Boston, MA, USA).

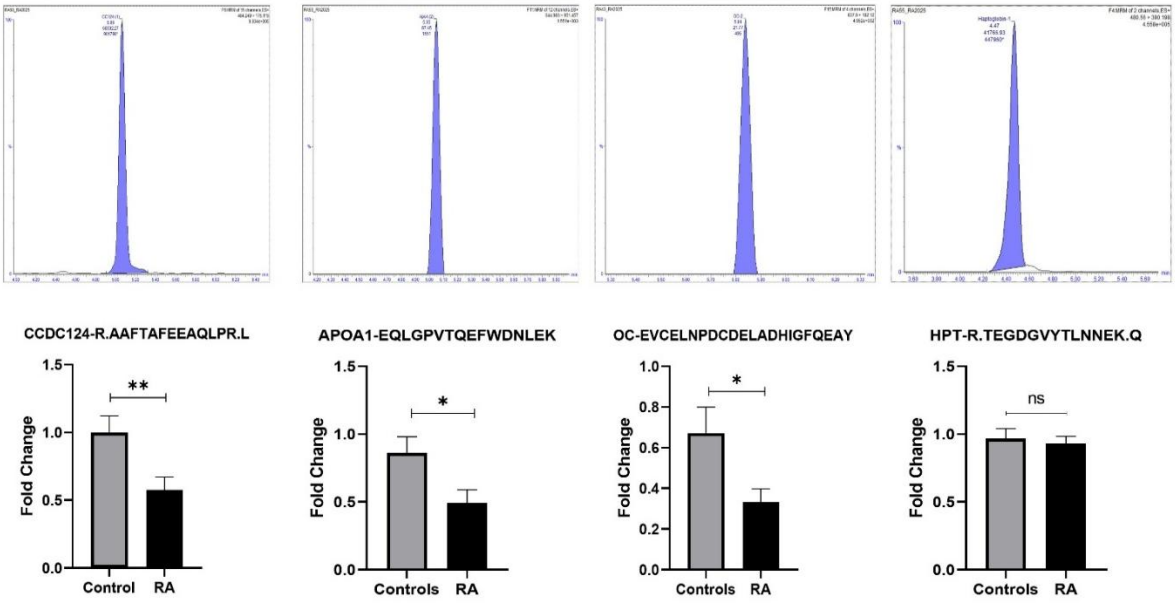

Figure 2-7 Multiple reaction monitoring (MRM) mass spectrometry for validating study findings. The MRM method based on signature peptides was developed to validate the expression of four proteins, namely CCDC124, Apo-A1, OC, and HPT, found in the proteomics approach (2D-DIGE MALDI-TOF-MS). The top panel shows representative MRM chromatograms of selected peptides asterisk (*) indicates the extrapolated concentration value. The bottom panel shows expression of these four proteins presented as fold changes between the RA and control groups. Statistical significance was evaluated using an unpaired t-test (n = 120), in which * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and ns represent non-significant.

2.4 Discussion

The present study identified the changes in the proteome between established cases of RA and the controls. The pathophysiology of RA is multifactorial and involves multiple different immunological and inflammatory pathways. Routine laboratory measurements of acute-phase reactants, such as ESR and levels of CRP, are commonly used to assess disease activity when following patients with RA. The levels of both these markers were significantly elevated in the RA group. Our plasma proteomic analysis revealed significant dysregulation of numerous proteins involved in extracellular matrix remodeling, bone metabolism, immune response, cell signaling, glycosaminoglycan synthesis, and acute-phase response proteins in patients with RA compared to the controls. The majority of the DAPs had a decreased abundance when compared to the controls.

2.4.1 Significantly Increased Proteins in RA Compared to Controls

Among the DAPs that showed an increased abundance was mitochondrial dicarboxylate (DIC) carrier protein. Mitochondria are major energy-producing organelles that have central roles in cellular metabolism. Mitochondrial activity affects the differentiation, activation, and survival of immune and non-immune cells that contribute to the pathogenesis of this disease (Clayton *et al.*, 2021; Palmieri, 2013). DIC belongs to the solute carrier family 25 (SLC25) transport proteins involved in the transport of dicarboxylates, participating in the TCA cycle within the immune and non-immune cells, regulating cellular energy production and influencing immune responses in the context of RA (An *et al.*, 2021). It is also known to regulate mitochondrial glutathione transport intracellular ROS levels. The knockdown of SLC25A10 induces sensitivity to glutamine deprivation, decreases NADP and NAPDH levels, and is associated with a low expression of key genes involved in the antioxidant system, including thioredoxin 2 (TXN2) and thioredoxin reductase 2 (TXNRD2), thereby increasing sensitivity to oxidative stress (Zhou *et al.*, 2015). Although the precise mechanisms remain an area of ongoing research, the involvement of DIC in cellular metabolism may have relevance to the altered proteomic profile observed in RA. Targeting mitochondrial metabolism has emerged as a promising avenue for developing potential treatments in RA, as it may offer new opportunities to modulate immune responses and attenuate inflammatory processes. Dysfunctional mitochondrial metabolism has been demonstrated to lead to increased oxidative stress and inflammation, contributing to the perpetuation of RA pathology. Understanding the specific

role of the mitochondrial dicarboxylate carrier in RA could unveil novel targets for therapeutic intervention.

Another notable protein with increased abundance in our study was hemopexin. Hemopexin is an acute-phase protein that is involved in modulating the inflammatory and immune response and also acts as an antioxidant by scavenging free heme. In animal models, hemopexin was demonstrated to downregulate the secretion of TNF and IL-6 from murine bone macrophages, following stimulation with TLR4-dependent agonists, and inhibit cytokine secretion, thereby decreasing inflammation. In another study, a deficiency in HPT and hemopexin was shown to have a negative regulatory role in Th17-mediated inflammation by favoring the differentiation of naïve CD4+ T cells towards the Th17 lineage and enhancing the stabilization and expansion of memory Th17 cells by IL-23 (Fink, 2009; Liang *et al.*, 2009; Rolla *et al.*, 2013). We identified multiple spots related to hemopexin in our study that can represent different proteoforms. Multiple proteoforms of hemopexin, primarily resulting from oxidation, differential glycosylation, and limited proteolysis, have been identified in different conditions, including fibromyalgia (Hahl *et al.*, 2017; Wahlen *et al.*, 2020). These may differ in their heme-binding efficiency and immunomodulatory properties. A recent study identified carbamylated hemopexin as a biomarker in patients with early RA (Sidiras *et al.*, 2022). An increase in hemopexin levels in our group of patients may indicate a decrease in differentiation of T cells and a decrease in inflammation (Galicia *et al.*, 2009; Liang *et al.*, 2009).

2.4.2 Significantly Decreased Proteins in RA Compared to Controls

A significant decrease was noted in the protein OC in patients with RA compared to the controls. OC, also known as bone GLA, is a bone matrix component representing non-collagenous proteins released into the circulation during bone formation. Although generally considered a surrogate marker for evaluating ongoing bone remodeling and bone formation and resorption (Coiffier *et al.*, 2013; Fardellone *et al.*, 2014), it is now known to have a much broader role that involves the regulation of whole-body metabolism, insulin regulation, and even cognition (Zoch *et al.*, 2016). RA is a commonly considered a form of inflammatory polyarthritis, which, in established cases, is associated with a greater risk of bone loss and osteoporosis (Sparks, 2019). The deterioration of the bone is considered to be due to the actions of polymorphonuclear leukocytes that act in concert with osteoclasts under the influence of cytokines, including TNF and IL1, as well as other cytokines, including IL-17 (Sakthiswary *et al.*, 2022). While T and B cells represent the immunological aspects of RA, most of the damage

from the disease is driven through effector cells and their products, including cytokines and other mediators. The fate of immune cells, besides immune metabolism, is regulated by diverse metabolic processes. Alterations in cellular metabolic states can influence immune cell behavior, contributing to susceptibility to diseases such as type 2 diabetes mellitus, cardiovascular disease, obesity, and cancer. Osteoporosis represents a well-known extra-articular manifestation of RA, and treatment with disease-modifying antirheumatic drugs is known to decrease erosive bone loss. Clinical studies addressing bone loss in RA have yielded conflicting results. Seriolo et al. found that serum osteocalcin concentrations in patients with active RA were found to be significantly lower than those in patients with remission of RA and in healthy controls (Seriolo *et al.*, 2002), while other authors found the levels to be similar to those in controls (Pietschmann *et al.*, 1989) or increased (Gevers *et al.*, 1986). Decreased levels of circulating OC may not only indicate a decrease in bone remodeling but also an overall decrease in body metabolism. A recent study showed that B cells from RA patients express molecules that inhibit osteoblast differentiation and inhibit bone formation. A significant decrease in the levels of OC in these patients may indicate a much broader role of osteoimmunology in RA. OC undergoes post-translational gamma-carboxylation at three residues, 17, 21, and 24. Based on the degree of carboxylation, it is known to exist in the carboxylated, undercarboxylated, and uncarboxylated forms, with each having distinct functions. In this regard, we found multiple spots of OC with a significant decrease in abundance on the 2D-DIGE gels. Uncarboxylated OC, unlike the carboxylated fraction, is known to have an important role in the modulation of glucose, insulin, and energy metabolism (Alfadda, A. A. *et al.*, 2013; Zhang *et al.*, 2020) and could have a potential impact on metabolic homeostasis in patients with RA. The decrease in OC levels was validated through MRM analysis and was statistically significant.

The levels of HPT, an acute phase reactant protein, were observed to be decreased in patients with RA compared to controls. Additionally, HPT acts as an antioxidant by scavenging free hemoglobin, thereby protecting tissues from oxidative stress and through its binding to neutrophils. It also modulates the inflammatory and immune responses by controlling cytokine production and binding to different immunologic cells, including CD8 T cells and human B cells that are known to be dysregulated in autoimmunity, including RA. Characteristically, HPT is known to have various proteoforms derived from genetic polymorphisms (HP1-1, Hp2-2 proteoforms) and post-translational modifications, especially glycosylation. Among its proteoforms, the Hp 2-2 genotype has been previously identified as an independent risk factor

for CVD in patients with RA (Xu *et al.*, 2024). Different glycosylated HPT proteoforms, characterized by increased fucosylation and decreased mannosylation, have also been previously identified in the serum of patients with alcoholic cirrhosis, cancer, and RA (Saroha *et al.*, 2011). In our study, we identified multiple spots with a decreased abundance related to HPT, which could indicate the presence of these different proteoforms in RA. MRM validation for HP was carried out between the cases and controls but was not statistically significant and warrants further studies.

A significant decrease in abundance was noted in a novel coiled-coil domain-containing protein-124 (CCDC124). CCDC124 is a putative mRNA-binding protein containing a coiled-coil domain motif involved in protein—protein interaction. These proteins are expressed in a wide range of tissues, with diverse localization, thereby exhibiting functional roles in most of the physiological processes (Sokolova *et al.*, 2022). Previous studies have shown its important role in cellular physiology and RNA metabolism, in regulating cytokinesis, and in the process of translation during mitotic cell division (Palmieri, 2013). Upregulation of the CCDC124 gene was noted in cases of cancer, and its mRNA levels are considered molecular prognostic signatures in breast, ovarian, endometrial, and urinary bladder cancers (Arslan *et al.*, 2021). Although CCDC124 has primarily been studied in the context of cancer, its roles in regulating cytokinesis and translation during mitosis may be critically relevant in autoimmune diseases such as RA, where aberrant immune function and cell division are observed. The decrease in levels of CCDC124 in patients with RA was confirmed through MRM in our validation cohort.

The levels of two apolipoproteins (APOA1 and APOA IV) were observed to have a decreased abundance in patients with RA compared to controls. Our findings are in line with other studies that have also shown a decrease in circulating levels of Apoa1 (Doherty *et al.*, 1998; Wilhelm *et al.*, 2010). Apolipoproteins are well-known for their roles in the regulation of lipid metabolism. Besides this role, they have been recently implicated in the regulation of immune cells and as negative acute-phase proteins that inhibit TNF-α and IL-1β production induced by stimulated T cells in monocytes or monocytic cells (Hyka *et al.*, 2001; Knowlton *et al.*, 2012; Rossol *et al.*, 2005; Weber *et al.*, 2021). ApoA 1 also regulates macrophage activation sites by T lymphocytes through its binding to surface factors on stimulated T cells. It acts by limiting contact-mediated cytokine induction in monocyte—macrophages and inhibits critical pathways associated with disease exacerbation (Bresnihan *et al.*, 2004). MRM validation confirmed the decrease in levels of ApoA-1. These findings indicate that ApoA-I has anti-inflammatory

properties and may have important implications for the treatment of chronic inflammatory diseases. In addition to ApoA1, a decrease in abundance of Apo AIV was also noted in RA compared to the controls. ApoA-IV is a multifunctional protein with anti-inflammatory properties. In mice models, Apo AIV was shown to reduce the secretion of pro-inflammatory cytokines and regulate immune cell infiltration (Recalde *et al.*, 2004). Aside from inflammation, it is involved in other physiological processes, including lipid metabolism, as an antioxidant, and in coagulation and glucose homeostasis, while its deficiency has been associated with atherosclerosis and diabetes. A decrease in the levels of this protein in RA may predispose these patients to continued inflammation, along with the dysregulation of lipid and glucose metabolism and increased levels of resting mast cells, CD8 T cells, and follicular helper T cells (Li *et al.*, 2022), a characteristic feature of RA.

Similarly, there was a decrease in the abundance of the protein spots related to the enzyme heparan-sulfate 6-O-sulfotransferase 2 in the RA group compared to the control group. HS6ST2 is a member of a family of enzymes involved in the sulfation of heparan sulfate, which is critical for its synthesis and functioning (Wang *et al.*, 2015). Heparin/heparan sulfate (HS) is a member of the glycosaminoglycan family of polysaccharides involved in leukocyte transmigration, an essential feature of the inflammatory response, in the recruitment of leukocytes from the blood, and in the activation of chemokines (Sabol *et al.*, 2014). 6-O-sulfation alters the affinity of HS to bind ligands, including signaling factors and molecules. Increased levels promote cartilage damage and were seen to be a feature in osteoarthritis (Chanalaris *et al.*, 2019). It is to be noted that patients with RA show an alteration in the transmembrane glycoproteins or lipids expressed on the surface of different cells. Many of these cell surface proteins or lipids are responsible for propagating the inflammatory immune response, including the cluster of differentiation (CD) molecules, such as CD24, CD28, and CD40, that act as potent T-cell co-stimulatory factors. CD40 leads to the occurrence and progression of RA (Huang *et al.*, 2021; Jang *et al.*, 2022). Measuring the changes in the levels of this enzyme could be an indicator for the glycosylation of glycosaminoglycans and also a potential marker for RA.

It is known that both cell proliferation and apoptosis were dysregulated in RA, and lymphocyte auto-reactivity and cell cycle interruptions are causes of autoimmunity. In RA, the dysregulation of cell proliferation and apoptosis have been reported (Ebrahimian *et al.*, 2023). This could be contributed to by the decrease in proteins muskelin and TP53-regulated inhibitor

of apoptosis 1, which are involved in regulating the proteasomal degradation and apoptotic pathways, as seen in proteomic analysis. Muskelin is a component of the CTLH E3 ubiquitin-protein ligase complex that selectively mediates ubiquitination and subsequent proteasomal degradation (Kobayashi *et al.*, 2007). It plays a role in regulating pathways controlling homeostasis and development and in the migration of cells, including lymphocytes (Maitland *et al.*, 2022). TP53-regulated inhibitor of apoptosis 1 is involved in the modulation of the mitochondrial apoptotic pathway. Impaired apoptosis and proliferation resulted in autoreactive lymphocyte development and inflammation in RA. Our findings are in line with Ebrahimian et al., who showed that downregulated expression in RA PBMCs could be correlated with RA pathogenesis by regulating apoptosis, cell survival, inflammatory mediator production, and proliferation (Ebrahimian *et al.*, 2023). Bioinformatic analysis using IPA highlighted the role of STAT1, TNF, and IL4 as the central nodes dysregulated in RA. These signaling nodes are key mediators of pro- and anti-inflammatory pathways that govern immune cell activation, differentiation, and trafficking. STAT1 is known to be involved in macrophage activation and interferon signaling, often contributing to sustained inflammatory responses in RA, and in some cases, is used to predict response to treatment (Balendran *et al.*, 2023). On the other hand, TNF is a well-established pro-inflammatory cytokine that drives joint inflammation, cartilage degradation, and systemic immune activation. IL-4, while classically anti-inflammatory, also modulates immune responses, especially Th2 responses, and may influence the balance between regulatory and effector T cells in the RA microenvironment. The identification of cell-to-cell signaling, hematopoietic system development, and immune cell trafficking network as the highest scoring pathway associated with the DAPs highlights its relevance to the chronic inflammatory milieu that characterizes RA. Future studies need to be carried out to confirm our findings within a larger cohort and by using other techniques such as LC-MS/MS.

2.5 Conclusions

A comprehensive plasma proteomic profiling of RA unveiled significant differences in proteins, underscoring the multifaceted nature of RA. The identified proteins were associated with inflammation, immune regulation, metabolism, and cellular homeostasis. The identification of differentially abundant proteins—such as the upregulation of mitochondrial dicarboxylate carrier and hemopexin and the downregulation of osteocalcin, apolipoproteins (ApoA1 and ApoA-IV), and CCDC124—reflects the complex interplay between metabolic dysfunction, immune cell dysregulation, impaired bone remodeling, and defective apoptotic

pathways in RA pathophysiology. These proteins not only contribute to underlying disease pathology but also hold promise as potential biomarkers or therapeutic targets.

CHAPTER 3 (SCIENTIFIC ARTICLE)

UNTARGETED METABOLOMICS REVEALS IMMUNE-METABOLIC SIGNATURES IN ESTABLISHED CASES OF RHEUMATOID ARTHRITIS

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Abstract

Rheumatoid arthritis (RA) is a complex progressive autoimmune disorder in which chronic inflammation is tightly coupled to metabolic reprogramming. The known diagnostic markers are not sensitive and specific enough to reflect disease activity. Finding a metabolomics-based biomarker specific for established cases of RA is important. This study aimed to investigate the metabolomic profiles of patients with established RA compared to those of controls. An untargeted mass spectrometry (MS)-based metabolomics approach was used to analyze 123 plasma samples, patients (n=60), and controls (n=63).

Results from the OPLSDA model showed a clear separation between patients with RA and controls (Q 2 = 0.736, R 2 = 0.988), indicating significant metabolic differences between the groups. A total of 300 putatively identified metabolites were significantly associated with RA. Among these dysregulated metabolites, 60 were endogenous (36 upregulated and 24 downregulated) in RA patients compared to controls. The plasma metabolomics profile revealed statistically significant disturbances in lipid (including Succinyladenosine FC = 62. 9, P value = 0, CDP— DG (PGE 2/i- 19: 0) FC = 18. 1, P value = 0, PGP(i- 24: 0/PGD 2) FC = 5. 5.2, P value = 0, Octadecenoylcarnitine FC =, P value = 0.04), amino acid (including L-Isoleucine, Cysteinyl- Serine FC = 6. 6.8, P value = 0), and nucleotide (Inosine FC = 2. 7, P value = 0.04, N 6—Methyladenosine FC = 4. 1, P value = 0), metabolism in RA patients, consistent with integrated immune—metabolic dysregulation. Bioinformatics and network pathway analysis using IPA showed interconnectedness between the metabolites centered around IL-6, IL-2, IL-1, MAPK, and kininogen. The pathways most affected in the comparison between RA and controls included humoral immune response, inflammatory response, hematological system development, and function, with scores of 19 and 8 focus molecules. The identified metabolites influenced eicosanoid/kinin signaling, nucleic acid—mediated innate immune activation, mitochondrial dysfunction, and altered glycosylation. Subgroup analysis based on erythrocyte sedimentation rate (ESR) above 35 mm/hr identified 68 metabolites that differentiated patients with high versus low ESR. Among these, three metabolites, namely cysteinyl-serine (upregulated), tyrosyl-arginine and N2-acetyl,N6-methyllysine (downregulated) overlapped with the comparison between RA and controls, suggesting links between specific metabolic changes and systemic inflammation. Our findings support the translational potential of plasma metabolomics for phenotyping and highlight potential candidate biomarkers for disease activity and discovery in RA.

Keywords: Rheumatoid arthritis; metabolomics; plasma biomarkers; immune–metabolic dysregulation; nucleic acids; glycerophospholipids; N2-acetyl,N6-methyllysine

3.1 Introduction

Rheumatoid arthritis (RA) is a complex systemic autoimmune disorder that extends beyond being a joint disease. RA is characterized by joint inflammation with pain and swelling, which can lead to irreversible cartilage and bone damage. The etiology is still unknown, but environmental factors, a genetic predisposition, and autoimmunity are involved with the disease's susceptibility and severity (Scott *et al.*, 2010). The pathophysiology of RA results from the activation of self-reactive T and B cells, which leads to synovitis, cellular infiltration, and a disorganized process of bone destruction and remodeling. The joint space is lined by a synovial membrane that suffers a tumor-like enlargement, called pannus, with local destructive effects (Choy, 2012). While joint inflammation (articular manifestations) is a hallmark of RA, it is also known to be a systemic disease with extrarticular manifestations affecting various organs, leading to a range of complications and comorbidities. In clinical practice, it is often noted that patients, even when in remission, experience joint pains, the underlying factor for which is still unclear (Motyl *et al.*, 2024). Patients with RA are at increased risk of developing other comorbid conditions, such as osteoporosis, cardiovascular disease, diabetes, infections, and certain types of cancer (van Onna et Boonen, 2016).

Although established serological markers of diagnosis, such as rheumatoid factor and anti-cyclic citrullinated peptide antibodies, are commonly used in clinical practice for early diagnosis, disease stratification, and long-term monitoring, these remain challenging. These limitations are evident in patients who achieve clinical remission based on joint scores but continue to show elevated ESR, indicating ongoing subclinical inflammation and increasing the risk of relapse or joint damage (Morales-Ivorra *et al.*, 2022).

Omics studies, including metabolomics, have emerged as a powerful tool that helps in unravelling the biochemical basis of different diseases, including RA. Unlike genomics and proteomics, which capture partially dynamic layers of disease biology, metabolomic profiling reflects the real-time biochemical consequences of cellular activity, immune activation, and therapeutic intervention (Qiu *et al.*, 2023). In our previous study we identified significant changes in proteins between Ra and control groups (Masood *et al.*, 2025). At the molecular level, metabolomics gives dynamic insights into inflammatory, immunologic, and energy-related perturbations. It also provides information that can be predictive of distinctive phenotypes within the disease. In systemic diseases such as RA, alterations in the circulating metabolomic profile may arise from an interplay of genetic susceptibility, localized

inflammatory activity, comorbid conditions, and environmental influences (Coras *et al.*, 2020). Recent metabolomics studies have identified altered pathways in RA, including elevated levels of amino acids like glutamine, tryptophan, and arginine; perturbed tricarboxylic acid cycle intermediates; dysregulated lipid mediators such as prostaglandins, lysophospholipids, and nucleotides (Kishikawa *et al.*, 2021; Xu *et al.*, 2022). Over the last decade, untargeted and targeted metabolomics have consistently shown broad biochemical dysregulation in RA and links to clinical activity and outcomes. In plasma, Hur *et al.* 2021 identified metabolite signatures that correlated strongly with DAS28-CRP and improved quantitative disease-activity prediction using multivariable models, establishing metabolomics as a viable tool for precision phenotyping (Hur *et al.*, 2021). Koh *et al.* 2022 demonstrated that synovial fluid lipidomes are profoundly perturbed and that serum lipid profiles distinguish active RA from remission and even preclinical RA, tying lipid remodeling to disease evolution (Koh *et al.*, 2022). Integrative studies have combined plasma metabolomics with the gut microbiome, revealing alterations in glycerophospholipid and amino-acid pathways that discriminate RA from controls and highlight gut—joint metabolic crosstalk (Zhu *et al.*, 2022). In early RA, large-cohort serum profiling has linked baseline metabolites (organic acids and nucleosides) to subsequent disease activity and treatment response, enabling predictive modeling, albeit with moderate performance (Fatima *et al.*, 2025). Metabolomics was also used to reveal the changes within the synovial fluid that are uniquely present in the joint environment and not observed in circulation (serum or plasma) (Carlson *et al.*, 2019). However, most of these studies focus on early or active RA stages, with limited emphasis on patients in clinical remission who may still harbor low-grade inflammation.

Recent advances in metabolomics have shifted the paradigm of RA research, enabling the identification of disease-specific metabolomic patterns and signatures across different phenotypes. Therefore, we conducted an untargeted, high-resolution plasma metabolomics study with bioinformatics and network pathway analysis comparing established RA patients to controls to define the systemic metabolic signature of the disease. As a secondary analysis, we stratified patients by ESR to investigate whether specific metabolite patterns correlate with residual inflammatory burden despite clinical remission.

3.2 Materials and Methods

3.2.1 Ethical approval, patient recruitment, and consent to participate

The study procedures and protocols were reviewed and approved by the institutional review board of the College of Medicine, King Saud University. Written informed consent was obtained from all the participants (IRB number: E-21-6341). The primary treating physician determined the diagnosis for RA based on the American College of Rheumatology and the European League Against Rheumatism (ACR/EULAR) 2010 criteria. The patients in the present study were recruited from those attending the rheumatology outpatient clinics at King Khalid University Hospital, College of Medicine, King Saud University, in the age group of 36-65 years. A total of 60 patients with known RA for a mean duration of 20 years in clinical remission (DAS28) <2.6), mostly women, were recruited. Control plasma from age-matched volunteers who were otherwise healthy was obtained from the blood bank after informed consent was obtained. The baseline demographic information and clinical laboratory parameters, including complete blood count with an ESR, lipid markers, liver function tests, fasting glucose, glycated hemoglobin, and C-reactive protein (CRP), were collected from the patient's medical records (Table 3-1). Patients were also stratified according to their ESR levels as high (>35 mm/hr) and others as low. The study procedures were conducted according to the ethical standards of the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines.

3.2.2 Sample Collection, Storage, and Metabolite Extraction

The blood samples were collected by venipuncture into EDTA-containing tubes from all participants and processed (centrifuged at 1,500×g for 10 min at 4°C) to obtain clear plasma. The plasma was aliquoted and stored at −80 °C until the day of analysis. Metabolites were extracted from plasma using our previously established protocol (Alfadda *et al.*, 2024). Briefly, cold methanol and chloroform were added to 50 µL of plasma, followed by water and shaking. Equal volumes of chloroform and water were added before centrifugation at 10,000×g for 5 min. All samples were dried using a vacuum centrifugal evaporator and stored at − 80 °C until further analysis.

3.2.3 Metabolomics Mass Spectrometric Analysis

Metabolomics profiling was carried out utilizing untargeted metabolomics analysis via LC-HRMS, as previously reported (Sebaa *et al.*, 2023). Metabolites were analyzed using a Waters ACQUITY UPLC system and an Xevo G2-S QTOF mass spectrometer with an electrospray ionization source (ESI) in positive and negative modes (ESI+, ESI−). The metabolites were chromatographed using an ACQUITY UPLC XSelect column (100 × 2.1 mm, 2.5 μm) from Waters Ltd., Elstree, UK. The mobile phases A and B were pumped to the column in a gradient mode (0-16 min 95-5% A, 16-19 min 5% A, 19-20 min 5-95% A, 20-22 min 5-95% A) at a flow rate of 300 μL per minute. The MS settings were as follows: the source temperature was 150 °C, the desolvation temperature was 500 °C for both modes, the capillary voltages were 3.20 kV (ESI+) or 3 kV (ESI−), the cone voltage was 40 V, the desolvation gas flow was 800.0 L/h, and the cone gas flow was 50 L/h. In MS^E mode, the collision energy of low and high functions was set to 10-50 V, respectively. As indicated by the seller, the mass spectrometer was calibrated with sodium formate in the 100-1200 Da range in both ionization modes. Data-independent acquisition (DIA) was performed in continuum mode using a Masslynx™ V4.1 workstation (Waters Inc., Milford, MA, USA).

3.2.4 Data Handling and Processing

The raw mass spectrometry data underwent processing through a standard pipeline involving m/z and retention time alignment, peak selection, and quality-based signal filtering, using Progenesis QI v.3.0 (Waters). Multivariate analysis was performed with MetaboAnalyst v.5.0 https://www.metaboanalyst.ca/ accessed on 10 Apr 2025, applying median, Pareto scaling, and log transformation to normalize the data. Models such as PLS-DA and OPLS-DA were constructed, with model fitness evaluated by R2Y and Q2 metrics. Univariate differences were assessed via (unpaired t-test, FDR, p-value ≤ 0.05, Fold change (FC) 1.5) using Mass Profiler Professional (MPP) v.15.0. Venn diagrams were generated with MPP, and key features were annotated against the Human Metabolome Database with emphasis on precursor mass and fragmentation; the tolerance was 5 ppm. Exogenous substances, including drugs and food additives, were manually excluded from the final feature list.

3.2.5 Bioinformatic and Network Pathway Analysis

Two independent and complementary bioinformatics approaches, MetaboAnalyst 5.0 https://www.metaboanalyst.ca/ accessed on 10 Apr 2025, and Ingenuity Pathway Analysis (IPA, QIAGEN), were used to analyze the metabolomic data to ensure robust interpretation.

MetaboAnalyst v5 was employed for statistical analysis, visualization, and pathway-based interpretation of the metabolite data using the Metabolomics Pathway Analysis (MetPA) module based on the KEGG database. Meanwhile, IPA network pathway analysis was applied to identify connections between different metabolites and provide a biological and mechanistic perspective by integrating the metabolomic data with curated biological knowledge bases.

3.3 Results

3.3.1 Clinical and Biochemical Data for the Study Participants

The demographic and biochemical characteristics of the study participants, in the discovery and validation phases, are presented in Table 3- 1. The mean ages of the study participants in the RA and control groups were 49.88 ± 12.05 and 47.52 ± 13.86 years, respectively, and were matched in body weight, BMI, and other laboratory markers. Significant differences were noted in the ESR and CRP levels, which were significantly higher in the RA group than in the control group.

Table 3-1. Baseline demographic and clinical characteristics of patients with rheumatoid arthritis and the control groups

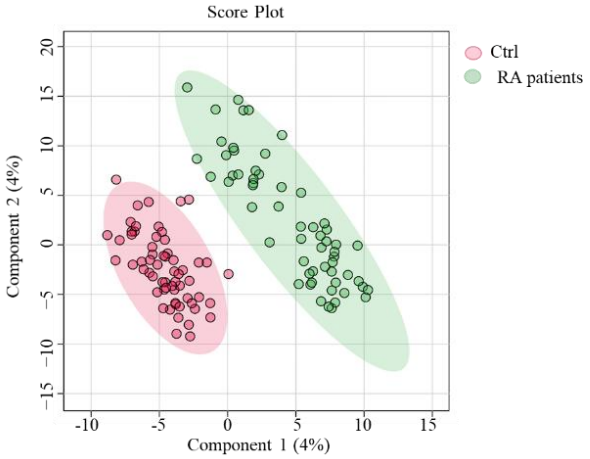
Characteristics	Patients (mean ± Std)(n=60)	Controls (mean ± Std) (n=63)	P-value
Duration (yrs)	15 ± 7	na	na
AGE	49.88 ± 12.05	47.52 ± 13.86	0.31
Gender (F/M)	55/5	57/6	0.38
BMI (Kg/m^2)	28.76 ± 8.09	29.89 ± 6.21	0.38
ALT (IU/L)	18.99 ± 7.06	18.04 ± 9.01	0.51
AST (IU/L)	17.07 ± 5.03	20.14 ± 10.12	0.06
Albumin (gm/L)	42.99 ± 4.14	44.03 ± 2.70	0.10
Alk Phs (IU/L)	76.09 ± 20.37	77.57 ± 30.55	0.90
Glucose(mmol/L)	5.56 ± 2.50	5.51 ± 1.28	0.90
Chol (mmol/L)	4.63 ± 0.78	4.77 ± 0.94	0.36
HDL (mmol/L)	1.47 ± 0.41	1.48 ± 0.37	0.89
LDL (mmol/L)	2.70 ± 0.80	2.99 ± 0.81	0.06
Triglycerides (mmol/L)	1.19 ± 0.74	1.27 ± 0.54	0.50
HbA1c (%)	5.74 ± 0.54	5.71 ± 0.84	0.87
ESR (mm/hr)	43.04 ± 20.6	5.64 ± 2.80	0.00
CRP (mg/L)	9.70 ± 7.40	1.9 ± 3.4	0.00
RF	277.70 ± 75.46		

Values are expressed as mean ± standard deviation (SD) in parentheses. A significant difference between RA and control (independent t-test, p-value < 0.05 for parametric tests). BMI: body mass index, HB: hemoglobin, WBC: white blood cells, RBC: red blood cells, PLT: platelets, ESR: erythrocyte sedimentation rate, ALT: alanine aminotransferase, AST: aspartate transaminase, Chol: Cholesterol, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HbA1c: glycated hemoglobin, ESR: Erythrocyte Sedimentation Rate, CRP: C-reactive protein.RF: rheumatoid factor* p-Value < 0.05 is considered statistically significant.

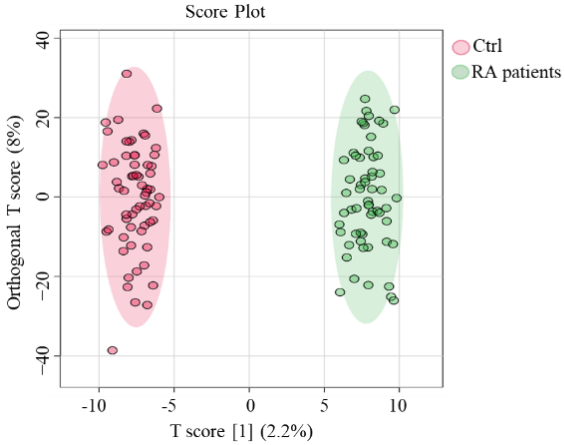
3.3.2 **Detection of Mass Ion Features and Metabolomics Profiling between RA and Control**

An initial untargeted metabolomics analysis detected a total of 18,065 mass ion features, including 11,000 in positive ion mode and 7,065 in negative ion mode. Following data preprocessing steps—such as alignment, peak picking, and removal of missing values—a filtering criterion requiring a cut-off percentage of 50% of all samples in at least one condition was applied, reducing the dataset to 12,212 features. To ensure data normality and minimize technical variation, the dataset was median-centered, log-transformed, normalized, and processed using Pareto scaling. The metabolites that distinguished between RA and control are displayed in Figure 3- 1. The OPLS-DA, a supervised multivariate approach between the two groups, RA patients and controls, is shown in Figure 3- 1A. The distinct separation of the RA group from the control suggests that plasma metabolites may be useful for identifying RA. The PLSDA plot also shows separation between study groups, as shown in Figure 3- 1B.

(A)



(B)



(C)

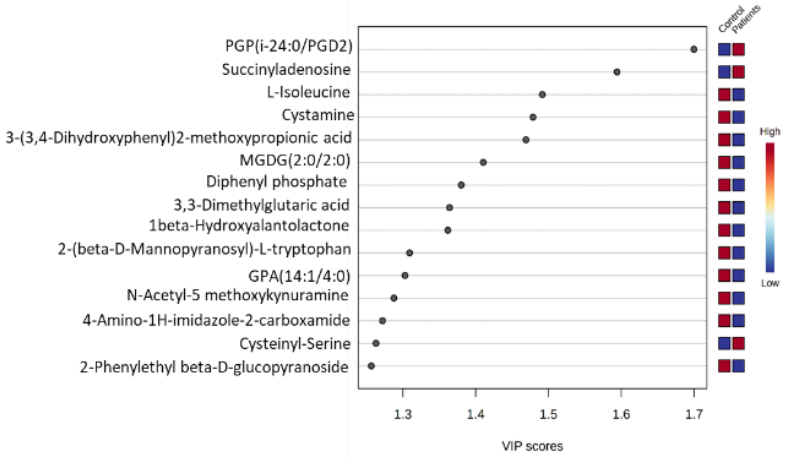


Figure 3-1 Comparison of metabolite features between the RA and control groups. (A): Partial least squares discriminant analysis (PLS-DA) shows separation between groups. n represents the number of metabolite features identified with a significant p-value ($p < 0.05$). (B): Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot showing evident separation between RA and control groups. The robustness of the created models was evaluated by the model's fitness ($R2Y = 0.988$) and predictive ability ($Q2 = 0.736$) values. (C): A variable importance in projection (VIP) score plot of discriminative metabolites in rheumatoid arthritis. Metabolites with the highest VIP scores are shown, indicating their contribution to group separation.

The statistical evaluation between two groups (RA and controls) was performed on 12,212 features using a volcano plot (unpaired T-test, FDR $p<0.05$, FC 1.5), which revealed 300 significantly dysregulated metabolites between the study groups, with 147 and 153 metabolites up- and down-regulated, respectively (Figure 3-2). Among these, these182 were successfully annotated using Human Metabolome Database (HMDB), Metlin MS/MS, LipidBlast, lipidMap, and KEGG. After excluding the exogenous molecules (i.e., drugs, drug metabolites, environmental exposures, etc.), 60 endogenous metabolites were identified between the RA and control groups (Supplementary Table S1). The identified endogenous metabolites in the data set were cross-checked independently with our in-house library, and inosine, cytidine, l-isoleucine, mandelic acid, L-Acetylcarnitine, and 4-Hydroxyphenylpyruvic acid were confirmed. Additionally, subgroup analysis revealed that 509 metabolites were significantly dysregulated in RA with high ESR compared to RA with low ESR, with 199 and 310 metabolites being up- and down-regulated, respectively. 67 endogenous metabolites were identified between patients with high and low ESR (Supplementary table S2). Of these three, two endogenous metabolites overlapped with the metabolites identified between the RA and control groups. (Figure 3- 3)

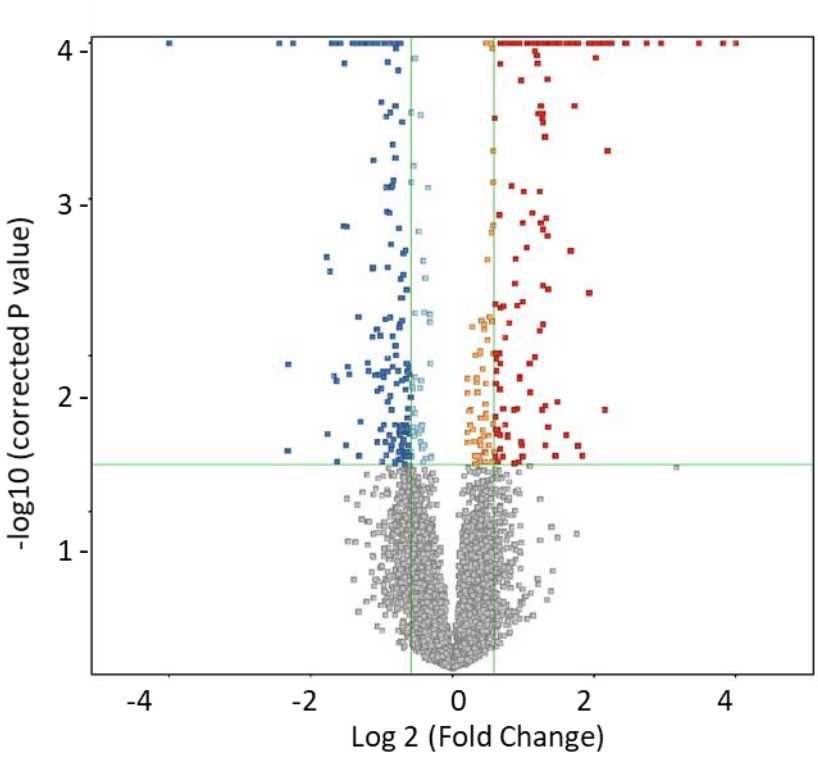


Figure 3-2 Metabolomics profiling between the RA and control groups. The volcano plot shows a significant change in the levels of several metabolites, of which red represents upregulated and blue represents downregulated tissue metabolites in RA and control groups (unpaired t-test, FDR p-value ≤ 0.05, fold change ≥ 1.5).

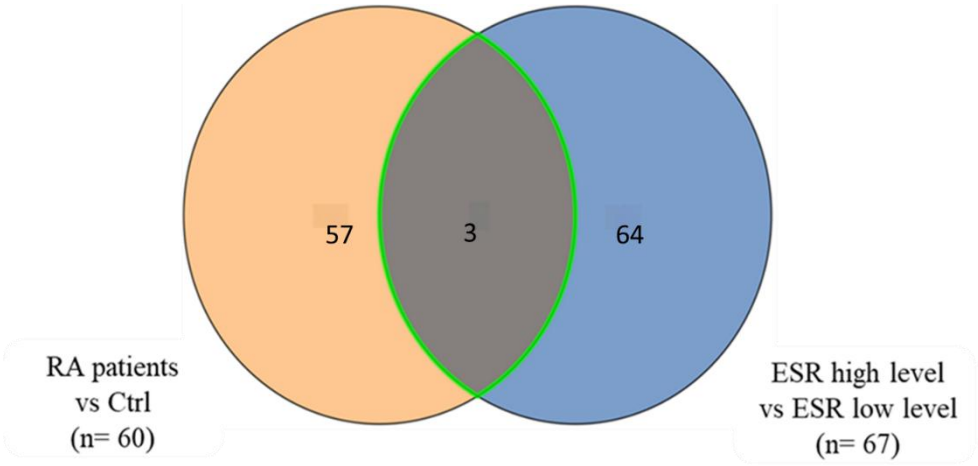


Figure 3-3 Venn diagram illustrating the overlap between significantly dysregulated metabolites identified in two independent comparative analyses: rheumatoid arthritis (RA) patients versus healthy controls, and RA patients with high erythrocyte sedimentation rate (ESR) versus RA patients with low ESR. In the RA versus control comparison, 60 metabolites were significantly altered, of which 57 were unique to this comparison while in the high-ESR versus low-ESR comparison, 67 metabolites were significantly altered, including 64 unique metabolites with 3 metabolites common to both comparisons. The three shared metabolites common to both comparisons, suggest a potential overlap between disease-associated and inflammation-associated metabolic signatures.

3.3.3 Evaluation of Metabolite Biomarkers between RA and controls and Bioinformatic Network Pathway analysis

To elucidate metabolic biomarkers and pathways related to RA patients, an unpaired t-test with FDR p value 0.1, FC 1.5 was conducted and revealed 410 significantly dysregulated metabolites, where 178 and 232 metabolites were up (red)and down (blue)-regulated between the two groups. Among these, only 77 endogenous metabolites were identified. The potential biomarkers that differed between RA and controls are shown in Figure 3- 4. OPLS-DA, a multivariate supervised method, is displayed in Figure 3- 1A. A clear separation in the identified metabolites was observed between the groups, indicating that plasma metabolomics can be used to identify RA-related metabolites that serve as biomarkers for distinguishing RA.

A multivariate exploratory ROC analysis was conducted using OPLS-DA as a feature-ranking and classification approach based on the identified common and significantly dysregulated metabolites between the RA and control groups. The AUC of the exploratory ROC curve for the top 3 metabolites was 0.981, as shown in Figure 3- 4A. The frequency plot shows the significantly dysregulated endogenous metabolites between the RA and control groups. OPLS-DA was used as a classification and feature ranking approach for the multivariate exploratory ROC analysis based on the common and significantly dysregulated metabolites identified between the two groups (Figure 3- 4B). From the top 15 metabolites, two metabolites, PGP(i-24:0/PGD2) (AUC=.912 and 0.953), and succinyladenosine were upregulated, while expression of L-isoleucine (AUC=0.931) was downregulated in the RA group, as shown in Figure 3- 4 C and D, respectively.

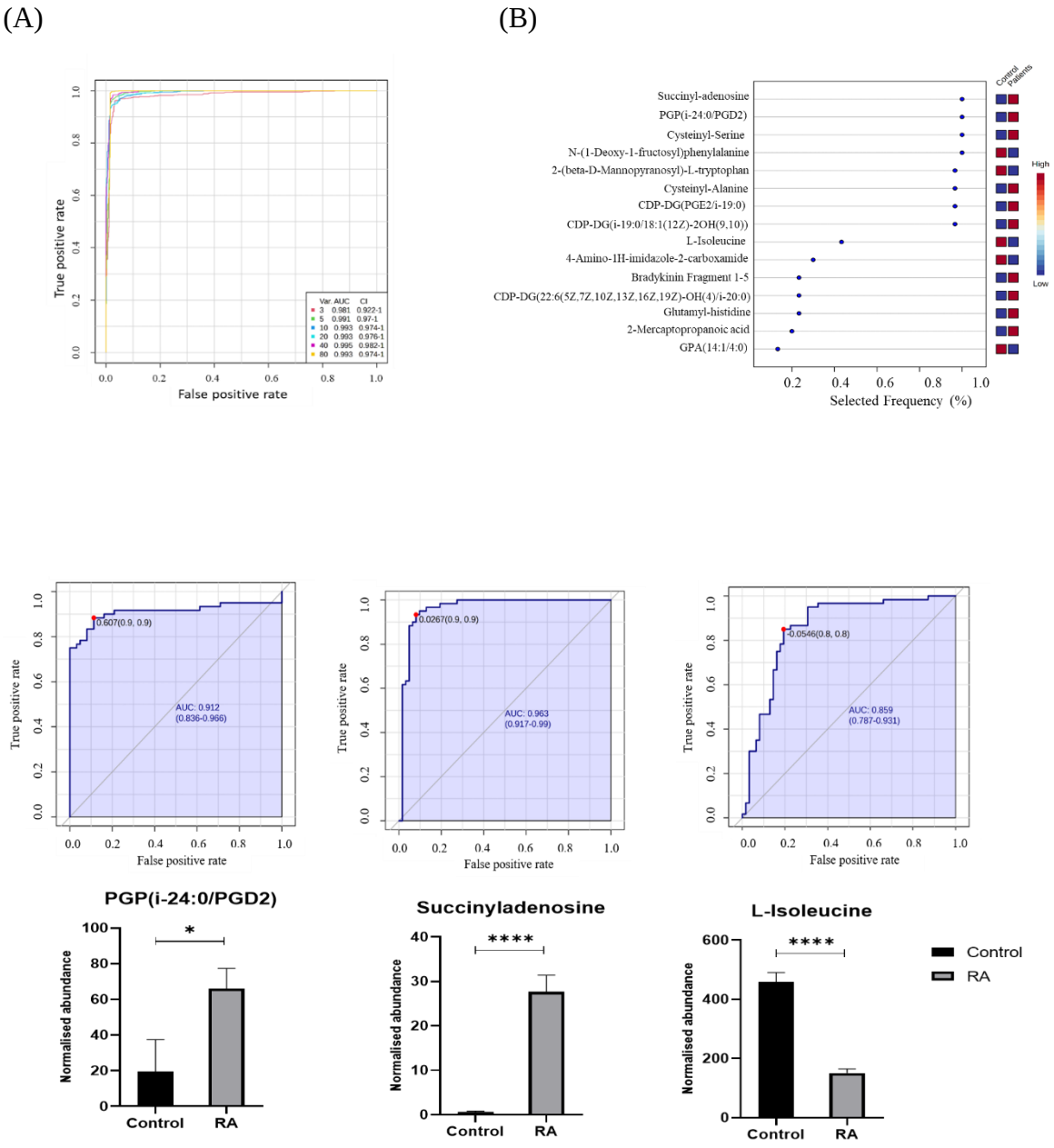


Figure 3-4 Biomarker evaluation in patients with RA and controls. (A). The Receiver Operating Characteristics (ROC) curve was generated by the OPLS-DA model, with Area Under the Curve (AUC) values calculated from the combination of 3, 5, 10, 20, 40, and 80 metabolites. (B). Frequency plot for the top 15 metabolites identified with the highest selected frequency. (C, D) Three metabolites in patients with RA had the largest AUC (FDR $p \leq 0.05$ and fold change ≥ 1.5), and their respective bar diagrams. * indicates significance <0.05 and **** indicates significance <0.0001

Pathway topology analysis using MetaboAnalyst revealed several significantly altered metabolic pathways in RA patients compared to controls (Figure 3- 5A). The most enriched pathways included phenylalanine, tyrosine, and tryptophan biosynthesis, pyrimidine metabolism, and tyrosine metabolism, which are all relevant to the chronic inflammatory and immune activation state seen in RA. Additionally, the IPA generated a molecular interaction network between the metabolites and identified humoral immune response, inflammatory response, hematological system development, and function as the network pathways affected with the highest score between the RA and control groups (score of 19 and 8 focus molecules) (Figure 3- 5B). The pathway suggests that RA-related metabolic changes are tightly integrated with immune and inflammatory networks, highlighting IL6, IL2, IL1, MAPK, and kininogen as central hubs, connecting metabolomics to inflammation, vascular response, and immune regulation in RA. The top canonical pathways included G alpha (q) signaling events ($1.39E^{-05}$, 1.8 %), Class A/1 (Rhodopsin-like receptors) that were significantly enriched and predicted to be inhibited in RA patients compared to controls (z-score < 0, —log (B-H p-value) > 1.3) (Figure 3- 5 C).

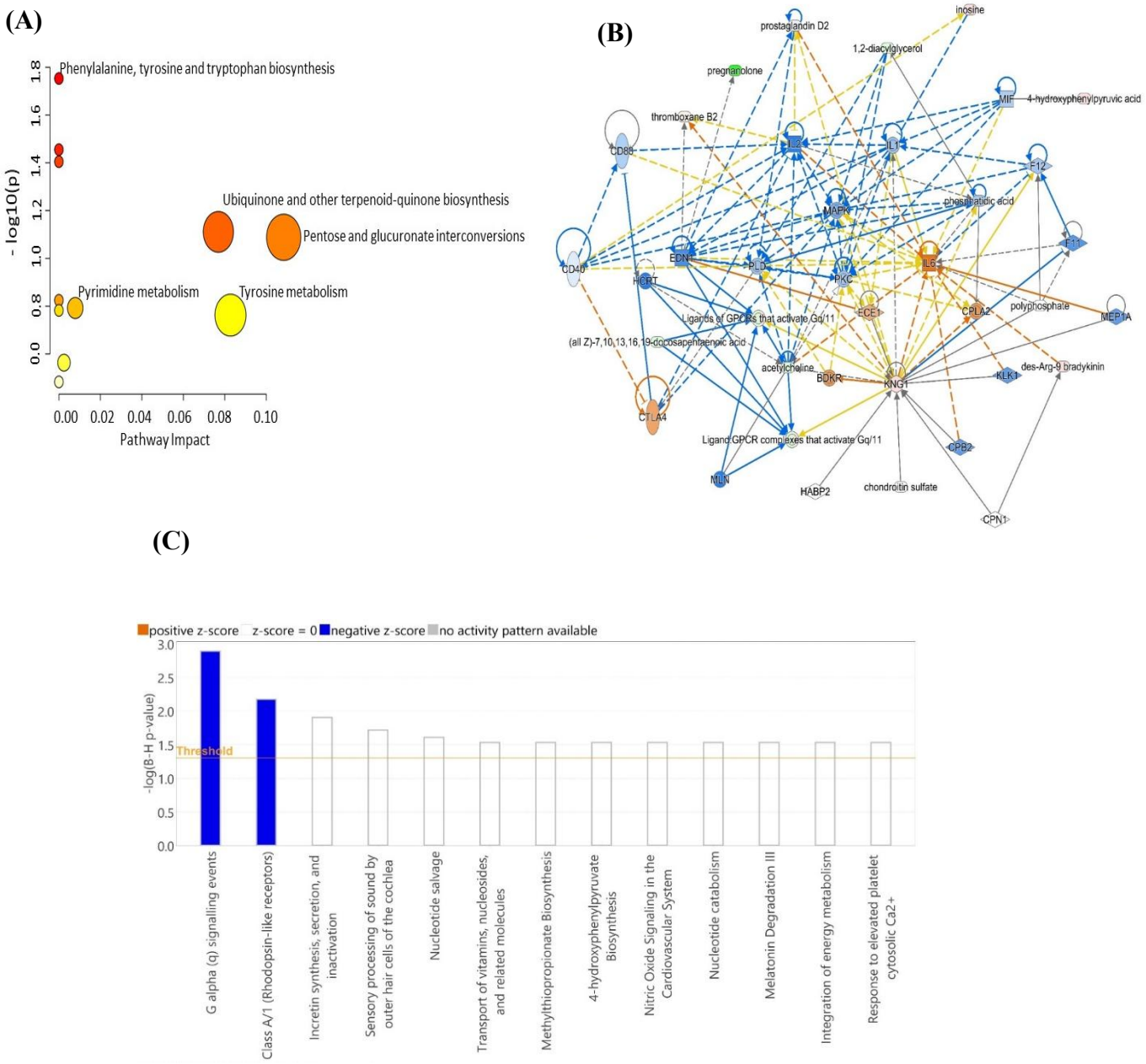


Figure 3- 5 Schematic representation of the highest-scoring network pathways depicting the involvement of the differentially regulated metabolites (A) The impact of the metabolites on the metabolic pathways (B) The interactions between the metabolites with biological context with dysregulation of PKc, interleukins (1,2 and 6), kininogen as the central nodes and (C) The top canonical pathways ranked by the p-values obtained by the IPA. The interaction networks were generated through IPA (QIAGEN Inc., Hilden, Germany).

3.4 Discussion

Rheumatoid arthritis (RA) is recognized not only as a joint-centered autoimmune disease but also as a systemic disorder characterized by profound metabolic reprogramming. These underlying changes in the metabolite profile can be captured by studying the plasma metabolome. Our untargeted metabolomics analysis revealed statistically significant dysregulation in levels of 77 (42 upregulated and 35 downregulated) circulating metabolites between patients with established RA and controls. The major metabolites belonged to the lipid class and included fatty acids and lipid-like molecules, nucleosides and nucleotides, amino acids, and others. These findings point to an inflammatory phenotype seen in RA and involve chronic immune activation, oxidative stress, vascular dysfunction, and tissue remodeling, all of which are central to RA pathogenesis. In this regard, elucidating the pathophysiological mechanisms and molecular signatures that drive either progression or remission in RA disease activity would be important (Aletaha et Smolen, 2018).

3.4.1 Dysregulation of plasma lipids and lipid-related metabolites with RA

The plasma lipidome in RA showed changes across multiple classes, including glycerophospholipids, ceramides, fatty acids, and acylcarnitines. Lipids are known not only as structural components but also as bioactive signaling molecules, and their systemic imbalance contributes to both local joint damage and systemic comorbidities in RA (Park *et al.*, 2021). It is well known that inflammation underlies the pathophysiology of RA and involves changes in innate and adaptive immune systems and their cells. Multiple cell types, including T cells, B cells, dendritic cells, neutrophils, macrophages, and fibroblast-like synoviocytes, actively contribute to disease activity and progression. Previous studies indicate that lipid metabolism is significantly altered in RA by regulating the activity and function of these cells (Lei *et al.*, 2023). In our analysis, we found notable dysregulation in lipid metabolites, with 19 metabolites decreased and 20 increased. The most substantial changes occurred in glycerophosphoglycerols (PGP), phosphatidic acid (PA), monogalactosyl diacylglycerols (MGDG), diglycerides (DG), phosphatidylethanolamine (PE), ceramides, acylcarnitines, and various fatty acids and sterol derivatives.

Phospholipids, especially glycerophospholipids, are integral to membrane architecture and function as precursors for potent lipid mediators that orchestrate immune activation, inflammation, and tissue remodeling in RA. They are also active precursors and carriers for inflammatory mediators. In our study, levels of several phosphatidylglycerol-containing

prostanoid species, PGP(i-24:0/PGD_2), and PGP(PGE_2/i-21:0), were decreased, and the level of PGP($PGF_1\alpha$/20:1(11Z)) was increased. In RA, PGD2 has been shown to suppress Th1-mediated inflammation and also contributes to eosinophilic inflammation and mast cell activation. The conjugation of PGD2 to phospholipids such as PGP may enhance its bioavailability and stability in synovial fluid. Elevated levels of PGP(24:0/PGD2) in RA patients may reflect active lipid mediator signaling and remodeling of membrane phospholipids—a common feature in chronically inflamed joints (Kim *et al.*, 2023). The dysregulation of these lipid species indicates alterations in arachidonic acid metabolism, which is known to be involved in inflammation and RA (Sano *et al.*, 2020; Wang *et al.*, 2021). Similarly, phosphatidic acid, PA(8:0/20:3(8,11,14)-2OH(5,6)) was dysregulated, highlighting their role as intermediates in both glycerophospholipid biosynthesis and intracellular signaling, which regulate immune cell activation and cytokine production (Fang *et al.*, 2001). We also observed an increase in phosphatidylethanolamine PE(PGE_2/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), suggesting coupling between inflammatory prostaglandin synthesis and omega-3 fatty acid—derived lipid mediators, potentially reflecting a mixed pro- and resolution-phase lipid signaling environment. Collectively, these lipidomic changes support the concept that in RA, glycerophospholipid remodeling not only reflects heightened immune activation but also directly contributes to the propagation and persistence of synovial inflammation.

PAs are central intermediates in glycerophospholipid biosynthesis and serve as precursors for DG and CDP-diacylglycerols (CDP-DGs). Their elevation in plasma suggests enhanced membrane turnover and lipid signaling activity in circulating immune cells, which may contribute to the chronic inflammatory state characteristic of RA. Notably, CDP-DG species exhibited mixed expression patterns. In contrast, CDP-DG species, such as CDP-DG(22:6-OH/i-20:0) and CDP-DG(20:5-3OH/i-12:0), were significantly downregulated, suggesting impaired systemic availability or utilization of anti-inflammatory ω-3 polyunsaturated fatty acids (Duchez *et al.*, 2019). These shifts point to a pro-inflammatory plasma lipid profile favoring eicosanoid synthesis while limiting resolution mediators. In contrast, phosphatidylinositols and platelet-activating factor (PAF) were decreased, indicative of reduced anti-inflammatory lipid signaling. These shifts collectively represent an eicosanoid-dominant, pro-inflammatory lipid remodeling in the plasma compartment of RA.

In addition to these, we identified multiple carnitine species that exhibited differential expression, with significant upregulation of octadecenoylcarnitine, and L-acetylcarnitine and a

downregulation of 13-(3,4-dimethyl-5-pentylfuran-2-yl)tridecanoylcarnitine, revealing distinct perturbations in fatty acid transport and mitochondrial metabolism in RA. Alterations in carnitine metabolism in RA reflect a broader disruption in systemic energy homeostasis and immune cell metabolism. Carnitines are essential for the mitochondrial β-oxidation of long-chain fatty acids, serving as shuttles that transport acyl groups across the mitochondrial membrane. They play a central role in mitochondrial fatty acid oxidation, energy homeostasis, and immunometabolic regulation, and their dysregulation in the plasma of RA patients indicates the presence of a systemic pathophysiology (Xiang *et al.*, 2025). The striking elevation of long-chain acylcarnitines, particularly octadecenoylcarnitine, likely reflects an imbalance between lipid influx and mitochondrial processing capacity. Long-chain acylcarnitines have been noted to increase Th17-associated cytokine production in T cells due to compromised β oxidation, which in turn promotes inflammation (Nicholas *et al.*, 2019). Accumulation of long-chain acylcarnitines may indicate incomplete fatty acid oxidation, a metabolic phenotype associated with inflamed immune cells and mitochondrial stress. This metabolic inflexibility is consistent with reports of impaired oxidative phosphorylation and elevated glycolytic dependency in RA T cells and macrophages (Weyand *et al.*, 2017). An increase in the levels of circulating long-chain acylcarnitines has also been found in other chronic inflammatory diseases, including chronic heart failure patients (Ahmad *et al.*, 2016). Mitochondrial inefficiency and impaired fatty acid oxidation may contribute to systemic energy deficits. At the same time, elevations in L-acetylcarnitine, a medium-chain acylcarnitine, could signal a shift towards glycolysis and acetyl-CoA overflow (Izzo *et al.*, 2023), consistent with the metabolic reprogramming seen in activated immune cells (Andonian *et al.*, 2022). In animal models, L-acetyl carnitine was found to inhibit the expression of inflammatory factors and antioxidants to suppress the development of atherosclerosis (Wang, S. *et al.*, 2020). Our findings are in line with et al, who identified increase in seven acylcarnitine metabolites with lower disease activity (Hur *et al.*, 2021)

The significant changes in lipid metabolites in our study underscore inflammation-driven metabolic reprogramming, oxidative stress, and immune dysregulation linked to RA. These findings demonstrate the profound reorganization of lipid metabolism in RA, aligning with previous multi-omics studies that connect lipid disturbances to immune dysregulation, synovial inflammation, and joint destruction.

3.4.2 Dysregulation of Amino acids and peptides related metabolites with RA

Amino acids serve essential roles in immune cell metabolism, inflammation, and tissue remodeling. In RA patients, we observed significant perturbations in crucial amino acids, bioactive peptides, post-translationally modified residues, and oxidative derivatives, pointing toward interconnected biochemical pathways that sustain inflammation and joint damage. Changes in the levels of amino acids have been linked to abnormal immune response in RA. Previous metabolomic studies suggest a direct link between amino acid metabolism and T cell and macrophage responses by promoting and modulating inflammation, which could be involved in RA pathogenesis (Ren *et al.*, 2017). Notably, in our study, branched-chain amino acids (BCAAs), such as L-isoleucine, were markedly decreased. BCAAs are critical energy substrates for immune cells, and their depletion suggests increased consumption by inflammatory leukocytes, especially activated macrophages and lymphocytes, which rely on these amino acids to support anabolic pathways necessary for cell proliferation and inflammatory mediator synthesis (Dimou *et al.*, 2022). Statistically significant increases in N2-acetyl, N6-methyllysine, a double-modified product of lysine acetylation and methylation, were observed between patients with RA and controls. Lysine is an essential amino acid that has been implicated in RA pathogenesis. Post-translational modifications of lysine are abundant in histones and transcriptional regulators and are key determinants of gene expression. Elevated circulating levels likely arise from increased turnover of nuclear proteins in activated immune and synovial cells. This is in concert with the fact that RA is characterized by persistent epigenetic reprogramming, particularly histone acetylation and methylation that locks fibroblast-like synoviocytes into a pro-inflammatory, tissue-invasive phenotype (Yang *et al.*, 2022).

We additionally identified elevated levels of 2-(β-D-mannopyranosyl)-L-tryptophan (C-mannosyl-tryptophan), a C-glycosylated post-translational modification of tryptophan. C-mannosylation in the endoplasmic reticulum is known to play critical roles in the folding, sorting, and/or secretion of substrate proteins. It is produced in part by degradation of C-mannosylated proteins through the autophagic pathway in cells and is considered a product of protein C-mannosylation turnover (Minakata *et al.*, 2021). Previous studies have shown that it is a circulating biomarker for kidney function, peripheral artery disease, and ovarian cancer. An interesting finding is that the C-mannosyl-tryptophan modification is important for, be required for the intracellular transport of IL-21R (Siupka *et al.*, 2015). IL-21 is a key cytokine involved in the activation and proliferation of B cells and T cells, including Th17 cells and follicular helper T cells, both of which are implicated in RA pathogenesis. IL-21 can promote

inflammation by increasing the production of other pro-inflammatory cytokines and by impairing the function of regulatory T cells, and contributes to the inflammation and tissue damage characteristic of the disease (Shbeer et Ahmed Robadi, 2024). Studies have shown that IL-21 levels correlate with disease activity and radiographic damage in early RA (Dinesh et Rasool, 2018; Rasmussen *et al.*, 2010). Levels of cystathionine sulfoxide and S-(3-oxo-3-carboxy-n-propyl) cysteine, which are both derivatives of Cystathionine, an intermediate compound in the transsulfuration pathway, were decreased in patients with RA compared to controls. These are sulfur-containing amino acids formed through the oxidation of cystathionine by ROS. These compounds are intermediates in the methionine–cysteine–glutathione pathway, a central axis for redox balance (Mateen *et al.*, 2016; Pajares *et al.*, 2015). The decrease in their levels points to increased evidence of oxidative stress in patients with RA.

Beyond metabolic and oxidative stress pathways, our findings also implicate the neuroimmune interface in RA. We observed decreased levels of O-acetylcholine, a key neurotransmitter in the cholinergic anti-inflammatory pathway. Acetylcholine inhibits NF-κB activation and reduces pro-inflammatory cytokine release from immune cells (Tracey, 2002). Its suppression may remove an important control on systemic inflammation. On the other hand, we also observed an elevation in the levels of dynorphin B (10–13), a breakdown metabolite from the dynorphin metabolic pathway. An increase in its levels points to activation of the endogenous opioid system that may provide short-term analgesia in chronic pain syndromes and RA (Millan, 2002). Dynorphin acts on opioid receptors to produce analgesia, but it also has non-opioid effects, including activating bradykinin receptors. Bradykinin receptors (B1 and B2) are involved in inflammatory pain and hyperalgesia. Interestingly, our metabolomic analysis also identified increased levels of bradykinin and bradykinin fragments in our data set, in line with previous studies (Vanarsa *et al.*, 2020).

Plasma levels of the dipeptides cysteinyl-serine, cysteinyl-alanine, phenylalanylvaline, and tyrosyl-arginine were increased while Arginylarginine levels were lowered in patients with RA compared to controls. RA is known to increase levels of multiple cysteine/serine proteases, which lead to heightened extracellular proteolysis, in turn generating low-molecular-weight circulating peptides. This aligns with RA peptidomics studies showing an expanded low-molecular-weight peptide/dipeptide signature in serum/plasma compared with controls. Tyrosylarginine is a dipeptide which was identified as an opiod with analgesic properties. A

decrease in its levels in RA patients suggests the heightened pain response in these patients (Ueda, 2021). A decrease in arginylarginine may reflect increased consumption of L-arginine by inducible nitric-oxide synthase in activated myeloid and stromal cells, in line with previous studies that documented robust NO/iNOS signaling in RA.

3.4.3 Dysregulation of Nucleotides, nucleosides, and related metabolites with RA

We found several nucleotide- and nucleoside-related metabolites altered in the plasma of patients with RA. Persistent activation of immune cells due to hypoxia and chronic inflammation in RA leads to accelerated DNA and RNA turnover in metabolising cells, influencing circulating nucleotides in plasma. (Kishikawa *et al.*, 2021). In our study, we identified intermediates of purine/pyrimidine metabolism inosine, cytidine, and AICAR, and modified nucleosides succinyladenosine, N6-Methyladenosine, and 1-methylguanine. Levels of succinyladenosine and 1-methylguanine were elevated, while the others were down. Succinyladenosine, a modified adenosine bound to succinate, sits on the adenylosuccinate lyase arm of de novo purine biosynthesis and accumulates when flux through this pathway is high. Adenosine and succinate are known to act as signaling molecules and modulate immune responses, with elevated levels linked to inflammatory conditions. In the setting of chronic immune activation, a rise in circulating succinyladenosine is therefore consistent with heightened purine synthesis/turnover in proliferating immune and stromal cells (Huang *et al.*, 2024; Magni et Ceruti, 2020). Inosine, an adenosine-pathway metabolite with anti-inflammatory actions via adenosine receptors, was also reduced; lower inosine levels fit enhanced catabolism during immune activation and the loss of an adenosinergic anti-inflammatory tone. RA and other inflammatory models document inosine's capacity to limit cytokine output through A2A/A3 signaling (Kim et Jo, 2022).

Conversely, depletion of certain nucleosides, such as inosine and cytidine, may reflect their rapid utilization during immune-cell proliferation or altered enzymatic processing, such as increased cytidine deaminase activity. Several intermediates with immunoregulatory potential were lower. AICAR, an intermediate of de novo purine synthesis, was decreased, compatible with brisk flux toward IMP and a relative loss of AMPK control that otherwise restrains inflammatory programs (Daignan-Fornier et Pinson, 2012). We also saw evidence of RNA modification/turnover changes. N^6-methyladenosine (m^6A), the dominant internal mRNA modification in plasma, was decreased. It is known to modulate stability/translation of inflammatory transcripts; emerging rheumatology work links m^6A machinery to RA

pathogenesis, so altered circulating m⁶A nucleosides are compatible with shifted RNA turnover during immune activation (Wu *et al.*, 2021).

In contrast, 1-methylguanine was elevated. Modified purine bases/nucleosides (including 1-methylguanine/1-methylguanosine) are released during RNA turnover and are not efficiently salvaged; increases in biofluids are widely used as systemic markers of heightened RNA metabolism. The levels of the pyrimidine nucleoside, cytidine, were also observed to be reduced in patients with RA compared to controls. Beyond serving RNA synthesis, cytidine is consumed by activated cells and is regulated by cytidine deaminase. Clinical RA cohorts have previously shown elevated serum CDA activity, providing a plausible enzymatic route to lower circulating cytidine during active immune remodeling. (Fatima *et al.*, 2025; Gatarek *et al.*, 2020) Taken together, an increase in succinyladenosine and 1-methylguanine coupled with a decrease in AICAR, inosine, m⁶A, and cytidine supports accelerated de novo purine synthesis and increased RNA turnover in RA. The relationship between m6A enzymes, immune cells, and RA suggests that m6A modification offers evidence for the pathogenesis of RA.

Patients with established disease or in remission are known to have continuing underlying inflammation, and a persistently increased ESR level indicates this. An elevated erythrocyte sedimentation rate (ESR), and/or positive rheumatoid factor (RF) test support a diagnosis of rheumatoid arthritis (RA). ESR ≥ 28 mm/h and/or abnormal CRP are frequent inclusion criteria for RA clinical trials1. American College of Rheumatology (ACR) remission criteria for RA include ESR < 20 mm/h for men and < 30 mm/h for women (Wolfe et Michaud, 1994). In our study, all the patients had a high ESR level (Table 3- 1) compared to controls. Among the patients, more than half had an ESR level exceeding 35 mm/Hr. When we stratified patients by ESR, dysregulation in three plasma metabolites, cysteinyl-serine, tyrosyl-arginine, and N2-acetyl, N6-methyllysine levels was observed when the metabolites in RA vs control were overlapped with those in high-ESR vs low-ESR metabolites. We observed that levels of cysteinyl-serine were increased both in patients with RA compared to controls and in patients with high-ESR, suggesting a consistent association with both disease state and systemic inflammation. On the other hand, levels of tyrosyl-arginine and N2-acetyl, N6-methyllysine showed an opposite regulation, being increased in in patients with RA compared to controls and were decreased in patients with high ESR levels. The dysregulation in the dipeptides cysteinyl-serine and tyrosyl-arginine point to alterations in systemic proteolysis consistent with the up-regulation of cysteine and serine proteases in RA that increases chronic inflammation,

which in turn is measured by ESR (Solau-Gervais *et al.*, 2007). N2-acetyl, N6-methyllysine a doubly modified lysine is part of the lysine metabolic pathway, which has been reported to be associated with early RA patients who achieved remission. The increase in its levels in low-ESR patients, points to an ongoing systemic inflammation increases transcriptional activity and histone acetylation/methylation dynamics, activating an epigenetic activation/turnover signature in RA (Lu-Culligan *et al.*, 2023). Similar to our results, previous studies also demonstrated an association between N2-Acetyl, N6-methyllysine with lower RA disease activity (Pinals *et al.*, 1981; Teitsma *et al.*, 2018). These metabolites together read as a composite of mitochondrial stress, proteolysis, and epigenetic reprogramming that converge on IL-6 and the hepatic acute-phase axis that influences chronic inflammation and ESR in patients with RA.

ROC analysis identified a panel of ten metabolites that showed the highest discriminatory power between RA and controls with an AUC of 0.993. These metabolites included succinyladenosine, PGP(24:0/PGD2), cysteinyl-serine, N-(1-Deoxy-1-fructosyl) phenylalanine, 2-(beta-D-Mannopyranosyl)-L-tryptophan, cysteinyl-alanine, CDP-DG(PGE2/i-19:0), CDP-DG(i-19:0/18:1(12Z)-2OH(9,10)), L-Isoleucine and 4-Amino-1H-imidazole-2-carboxamide (Figure 3- 4A). Network Pathway analysis of the differentially regulated metabolites using the Ingenuity Pathway Analysis (IPA, QIAGEN), humoral immune response, inflammatory response, hematological system development and function, with a score of 19, and involved 7 metabolites. The actions of these metabolites centered around regulating MAPK, PKC, IL6, IL1, and IL2, which are key inflammatory and signaling molecules, suggesting involvement in immune signaling, vascular function, and cytokine regulation (Ding *et al.*, 2023). The network pathway also showed the involvement of IL-21, a cytokine involved in stimulating follicular T helper cells, which was identified in our previous proteomic analysis (Masood *et al.*, 2025). Canonical pathway analysis of the significantly altered metabolites revealed several biologically relevant processes associated with rheumatoid arthritis. Notably, G alpha (q) signaling events and Class A/1 (Rhodopsin-like receptors) pathways were significantly enriched and predicted to be inhibited in RA patients compared to controls (z-score < 0, —log(B-H p-value) > 1.3). These pathways are primarily involved in G-protein—coupled receptor signaling, which plays a critical role in immune cell activation, cytokine release, and inflammation. The observed inhibition suggests a possible disruption or downregulation of GPCR-mediated signaling in the RA metabolic landscape.

3.5 Conclusion

Our plasma metabolomics analysis reveals that rheumatoid arthritis is characterized by coordinated changes across lipid, amino acid, nucleotide, and glycan metabolism, reflecting the biochemical imprint of persistent inflammation and immune metabolic dysregulation. Alterations in glycerophospholipids, acylcarnitines, dipeptides, nucleosides and their intermediates align with the fact that implies that autoimmune disorders like RA trigger multi-metabolite changes rather than singular alterations. These metabolite signatures not only deepen understanding of RA pathophysiology but also offer potential biomarkers for disease activity and treatment response, supporting the clinical value of metabolomics in precision patient stratification.

CHAPTER 4 — (BOOK CHAPTER)

METABOLOMICS OF RARE ENDOCRINE, GENETIC DISEASE: A FOCUS ON THE PITUITARY GLAND

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Abstract

Endocrine diseases and disorders are influenced by individual genetic and environmental factors that directly influence metabolite levels revealing unique metabolomic disease profiles. In the last few years, metabolomic-derived biomarkers and quantitative traits have helped identify the underlying mechanisms for many rare endocrine diseases and demonstrated a high potential for use in precision medicine. Using the metabolomic platform has also provided disease-specific biomarkers for diagnosis and monitoring treatment beyond the use of conventional biochemical immunoassays. Although the development of metabolomic profiling in pituitary disorders is at an early stage, recent advances have shown it to be a promising approach for identifying specific disease biomarkers in cases of growth hormone disorders (acromegaly, short stature) and Cushing's syndrome. Implementing high-performance metabolomic analysis techniques in pituitary disease will be a helpful clinical tool for significantly improving diagnosis and, potentially, the therapeutic approach by identifying highly specific disease biomarkers and novel molecular pathogenic mechanisms.

Keywords Rare endocrine disease, Rare genetic disease, Metabolomics, Biomarkers, Pituitary adenomas, Growth hormone, Acromegaly, Short stature, Pituitary dwarfism, Cushing's syndrome

In Context

The broader scientific and translational potential of metabolomics was explored through Chapter 4. This Chapter broadens the scientific and translational context of the work and to demonstrate the wider applicability of metabolomics to complex human disease and relating genetic and genomic variation to downstream changes. The metabolomics approach is not confined to one disease area, but rather represents a versatile and broadly informative analytical approach for investigating disorders characterized by biological complexity, phenotypic heterogeneity, and incomplete mechanistic understanding. In this respect, rheumatoid arthritis and rare endocrine and genetic disorders, although clinically distinct, share important conceptual challenges. Both involve heterogeneous clinical manifestations, variable disease trajectories, and limitations in current diagnostic or monitoring tools, thereby creating a need for improved molecular approaches that can provide deeper biological insight and more precise disease characterization. The chapter is intended to complement serve as a broader illustration of how metabolomics can be applied across different pathological settings to address these challenges. It highlights how metabolic profiling can uncover disease-associated biochemical disturbances that are not readily captured by conventional clinical or laboratory measures alone. This strengthens the broader significance of the approach to disease characterization, biomarker discovery, and translational molecular medicine.

4.1 Introduction

Endocrinology is a branch of medicine that deals with studying the endocrine system. The endocrine system comprises a system of specialized ductless glands responsible for synthesizing, storing, and releasing their secretions, that is, hormones, directly into circulation. Classically, hormone action occurs at different peripheral target tissues having receptors for the specific hormones. In addition to the classical endocrine signaling, hormones also act on neighboring cells (paracrine effect), on the cell secreting the hormone itself (autocrine effect), or locally within the cell without actually getting released from it (intracranial effect).

Hormonal action can be broadly described as important in controlling and coordinating whole-body metabolism and maintaining body homeostasis. Hormones are responsible for maintaining energy balance, regulating metabolic pathways, reproduction, growth and development, and response to injury, stress, and environmental factors. The hormonal synthesis, production, and secretion are tightly regulated processes, broadly, through complex regulatory feedback control loops or axes between different endocrine glands. The feedback

mechanisms control the circulating concentrations of the hormones within a tight physiological range necessary for optimal hormone action at the cellular level. The most well known among these are those under hypothalamic-pituitary control, such as the hypothalamo-pituitary-thyroid axis and hypothalamo-pituitary-adrenal axis that control and regulate thyroid and adrenal hormone action via the regulatory and organ-stimulating hormones. Endocrine dysfunction arises as a result of either subnormal (e.g., hypothyroid) or excess production (e.g., hyperthyroid) of hormones or due to peripheral resistance to hormone action (e.g., insulin resistance (IR)), which leads to different disease states.

Clinical evaluation of endocrine dysfunction is generally based on measuring single effector hormones using immunoassays in the clinical laboratory. Although these methods are well established and widely used in different hospital laboratories, single hormone measurements, as commonly perceived, do not completely represent disease or hormonal defects (Wallaschofski, 2012). The complexity in endocrine disease diagnosis arises as virtually all complex processes are regulated at any point with one hormone whose functions are closely intertwined. Therefore, measurement of single hormone levels may not be sufficient in identifying disease. The immunoassays are further limited in their detection capabilities due to their narrow specificity, sensitivity, and a high coefficient of variation between the different immunoassays for the same hormone (Barth, 2008; Hillebrand *et al.*, 2021). With the advent of deeper genome sequencing, an increasing number of endocrine diseases are grouped as a single disease with a heterogeneous presentation but are now identified as distinct disorders based on their genetic makeup. In the present chapter, we will look at the metabolomics of diseases of the pituitary gland.

4.2 Endocrine Diseases and Genetics

Although the paradigm of studying hormonal action and related diseases focused on the concept of one anatomical gland and its associated hormone, this view is changing with many interrelated complex interactions being identified between the different hormones. Hormones do not act in isolation; a single hormone affects multiple organs and functions, and several hormones control each function. Identification of various genetic polymorphisms conveying an increased risk of developing endocrine disease has been identified through genome-wide association (GWAS) studies. These studies have greatly improved knowledge and genetic testing, becoming a significantly important component for the thorough clinical diagnostic

workflow in endocrine diagnostics and the routinely performed biochemical laboratory analysis (De Sousa *et al.*, 2018; Eggermann *et al.*, 2020).

Recent studies using GWAS have identified many genetic mutations (such as single nucleotide polymorphisms, allelic loss or gene amplification, and epigenetic changes, usually by promoter methylation chromosomal defects) and their variants that are known to lead to endocrine disorders and pathogenic or benign neoplastic endocrine disease conditions (Eggermann *et al.*, 2020; Goodarzi, 2016; Hirschhorn *et al.*, 2002; Song *et al.*, 2016). This has been facilitated through clinical genetic studies in patients carrying mutations with well-characterized presentations and manifestations. These genetic mutations have been identified in genes involved in regulating the cell cycle, growth factors, signaling pathways, and hormone receptors. The genetic mutations may either result in gain of function or loss of function of the specific genes and their downstream protein products. These defects in proteins translate into biochemical enzymatic defects resulting in alterations in the hormonal levels due to disorders of hormone synthesis or receptor uptake, to name a few, resulting in endocrine disease (Eriksson *et al.*, 2021; Fang *et al.*, 2016; Hwangbo et Park, 2018; Persani *et al.*, 2018; Rahmioglu *et al.*, 2017).

Additionally, advances in cellular biology and transcriptomic studies have shown the significant role that hormones play in regulating gene expression. Mutations in the germline result in familial diseases, while somatic mutations may present with expression only within specific tissues. Evaluation of rare endocrine genes besides the characterization of the hormonal defects requires a comprehensive genetic workup (De Sousa *et al.*, 2018; Eggermann *et al.*, 2020).

Genetic diagnostic testing in rare endocrine conditions has become important for achieving an early molecular diagnosis and identifying presymptomatic individuals. Although significant, genetic evaluation is not without disadvantages as it is highly specialized and not easily available routinely. Recent advances in OMIC technologies, specifically in metabolomics, have shown that changes in the metabolite pattern reflect the associated disease conditions (Jacob *et al.*, 2019). The disease is often seen to exceed the influence of clinical factors. The variations in many metabolites have been heritable and linked to distinct loci identified by GWAS (Rhee *et al.*, 2013). Genetic variants, particularly of the enzyme coding genes, are associated with changes in the proteins regulating the homeostasis of key lipids, carbohydrates, or amino acid metabolisms and metabolites.

Over the last decade, the understanding of physiological and pathological processes underpinning endocrine and endocrine-related disease has significantly expanded, aided by advances in mass spectrometry (MS) approaches and novel molecular biological and computational tools. The metabolic compound dysregulation is associated with changes in physiology which are consequences of a unique expression of the genes. A small variation in protein expression can significantly affect the metabolic pathway activity with alterations in concentrations of metabolites related to the relevant proteins. Thus, compared to both the proteome and transcriptome, metabolomic profile is far more sensitive. The development of the high-throughput omic platforms has helped identify the specific metabolites altered with endocrine diseases and more so in the cases of rare genetic, endocrine diseases. Determining metabolomic features as causes of endocrine disorders provides a better holistic opportunity to understand the related pathophysiology and develop approaches for personalized therapy (Macel *et al.*, 2010).

4.3 Metabolomics of Endocrine Disease

Endocrine diseases deeply impact the human metabolome as hormonal defects affect the functions of enzymes, causing alterations in the normal metabolic activity of tissues or glands, leading to disease. Metabolomics has contributed significantly to endocrinology by providing means to understand better the inner workings of individual cells. It also helped to understand the interactions between different organ systems and providing insight into physiological and pathological processes. The main application of metabolomics in endocrine disease has been clinical research dealing with disease metabolomic profiling and biomarker discovery for diagnostic purposes. Plasma profiling of the disease and control states has allowed the characterization of a comprehensive set of metabolites in biological samples. Fluctuations in concentration of these small molecules, in response to different stimuli, serve as markers for monitoring of disease. Two principal analytical platforms have been most used to analyze metabolites in body fluids, primarily derivatives of blood (serum, plasma), tissues, and urine. These include nuclear magnetic resonance (NMR) spectroscopy and liquid/gas chromatography coupled with mass spectrometry (LC-MS). The analysis of lipid metabolites was enhanced by using ultra-performance liquid chromatography quadrupole time-of-flight (UPLC Q-TOF) MS and GC-MS. TMS major complementary approaches have been applied. One involves the targeted analysis of metabolites, which measures the concentrations of known disease-associated metabolites. The second is an untargeted hypothesis-driven approach that

provides a global profile of a large set of metabolites associated with the disease semiquantitatively.

Alterations in metabolite levels or a specific group of metabolites reflect the intricate relationships between genes and their products, as well as the activity of related enzymes within metabolic pathways either physiologically or in a disease state. In combination with genetics, untargeted metabolomics has provided additional information to determine the association between genetic variants and disease. Combining the clinical (phenotype) with the different molecular datasets (OMIC technologies) in a bioinformatic-based clinical decision-making system also has the advantage of advancing personalized medicine. These advances are in understanding the pathophysiology, diagnosis, stratification, management, and assessment of treatment efficacy in endocrinology. Metabolomics adds to this milieu by including metabolic information, furthering the accuracy of diagnosis in individuals and discovering novel metabolites as reported in the human metabolome database with the disease. For each metabolic gene, prediction models can then be developed for predicting changes in concentrations of metabolites in different biological fluids (serum, plasma, urine, saliva, etc.) or tissue specimens with various genetic mutations (Table 4- 1).

Table 4-1. The Table shows the different endocrine glands with the associated disease, gene locus, and the major metabolites altered with the disease.

Endocrine gland	Hormones	Disease	Genes	Metabolomics platform	Major significant Metabolites	References
Pituitary	GH Defects	GH Acromegaly	*AIP, GNAS, GPR101, USP8, USP48, BRAF. MEN1, PRKAR1A, GPR101, SDHx, CDKN1B, MAX*	GC-MS (untargeted), LC-MS (targeted), ^{1}H-NMR (untargeted)	Serine, 5-aminovaleric acid, glyceric acid, L-dithiothreitol, dihydrocoumarin, N-acetyl-L-glutamic acid, gluconic acid, and monoolein, dimethylamine (↑). BCAA, lysine, D-erythronolactone, taurine, carbamoyl-aspartic acid, mucic acid, lactate (↓)	24-26
		Short Stature	*PIT-1, HESX13, SOX3, FGFR3, PAPP-A2*	NMR (untargeted)	Citrate, mannitol, alanine, lactate, and DMA (↑). Creatine, creatinine, TMAO, and acetoacetate (↓).	32
				GC-MS (untargeted)	Threonic acid, cysteine, cystine, and palmitoleic acid (↓). Glutamic acid, aspartic acid, hypoxanthine-like, uridine, and glyceric acid (↑)	31

Endocrine gland	Hormones	Disease	Genes	Metabolomics platform	Major significant Metabolites	References
				NMR (untargeted)	Citrate, phenylalanine, creatinine, and tyrosine (↑) Glucose, serine, betaine, inositol, lysine, glycerol, and glutamine (↓)	33
	ACTH Increase	Cushing's disease	*USP8*	LC-MS/MS (targeted),	11-deoxycortisol, cortisol, cortisone, corticosterone, 11-deoxycorticosterone,androstenedione,18-oxocortisol, DHEA, DHEA-SO4,(↑) and aldosterone (↓)	38,40

4.3.1 Metabolomics of Hypothalamus and Pituitary Gland Dysfunction

The hypothalamus along with pituitary gland together constitutes a functional unit responsible for secreting regulatory hormones that exert control over the functions of nearly all the endocrine glands. The pituitary is the master regulatory gland of the body, acting through several hypothalamic-pituitary-target organ regulatory axes. Any defects arising within these regulatory systems, synthesis, storage, or release of the hormones impact the human body's overall physiological functions, leading to disorders and diseases.

Primary dysfunction of the pituitary gland is uncommon. The majority of the cases of hypo-, hyper-, or panhypopituitarism arise secondary to the presence of either neoplastic or benign tumors such as adenomas. Pituitary adenomas (PA) are benign neoplasms that are highly heterogeneous with varying subtypes and accounts for nearly 15% of primary tumors arising in the brain. They are either secretory based on the increase or decrease in the hormones secreted by the affected cell types or nonsecretory. Inherited genetic mutations, although rare, have been identified by GWAS and are known to lead to the development of benign PA, with several genetic genes encoding for guanine nucleotide-binding protein G(s) subunit alpha (*GNAS*) and G protein-coupled receptor 101 (*GPR101*), aryl hydrocarbon receptor-interacting protein (*AIP*), and deubiquitinase genes: *USP8, USP48,* and *BRAF*. PA can also occur in familial syndromes, including *MEN1, SDHx* (mutated in the PA, pheochromocytomas, and paragangliomas), von Hippel-Lindau, DICER1, *PRKAR1A* (mutated in Carney complex), succinate dehydrogenase-related familial PA, *GPR101* (involved in X-linked acrogigantism), neurofibromatosis type 1 (NF1), and Lynch syndromes (Gadelha *et al.*, 2017). The phenotype of the secretory PA depends on the affected cell types and the hormones secreted; growth hormone (GH)-secreting somatotroph adenomas result in acromegaly, corticotroph cell adenomas-secreting corticotropin hormone result in Cushing's disease, lactotroph cell adenomas-secreting prolactin result in hyperprolactinemia, and thyrotropin-secreting adenomas result in hyperthyroidism. The nonsecreting adenomas, on the other hand, manifest as incidental pituitary sellar masses and lead to hypogonadism. The clinical diagnosis of pituitary disease, irrespective of the cell type affected, is challenging. This is because of the wide range of presenting symptoms, nonspecific changes in single-hormone measurements, the need for adopting stimulation or suppression testing, and the requirement for imaging studies. Metabolomic approaches using GC/LC-MS have profiled pituitary-related diseases and

identified biomarkers differentiating between patients with PA and healthy controls. Plasma secretory plasma metabolomic profiling revealed significant alterations in the metabolism of amino acid, specifically in alanine, glutamate (metabolites of the glutamate oxidationcycle), and aspartate (metabolite of the urea cycle involved in gluconeogenesis), and increase in homocysteine levels. Increased homocysteine levels in patients with PA are considered to be the cause for increased cytotoxicity and oxidative stress (Bicikova *et al.*, 2006).

In addition to the findings in plasma, pituitary tissue metabolomic profiling was also done on tissue sections removed postoperatively from patients with gonadotropin- and PRL-secreting PA. The metabolomic profile showed differential regulation of phosphoethanolamine, glutamate, glutamine, N-acetyl aspartate, aspartate, and myoinositol, which are known metabolites involved in the regulation of the central nervous system. Levels of these metabolites distinctly differentiated between the two adenomas with PRL-secreting PA showing a decrease in levels of phosphoethanolamine, N-acetyl aspartate, and myoinositol while aspartate, glutamate, and glutamine levels were increased. Women with PRL-secreting PA showed higher estrogen metabolites and 17-ketosteroids in the urine. The increase in these metabolites was most likely due to reduced enzymatic actions of 3-beta-hydroxysteroid dehydrogenase and 5α-reductase enzymes among these patients. These patients also showed characteristic increase in the ratios of levels between 5-beta and 5-alpha-hydrogensteroids and delta 5 and delta 4 steroid ratios which served as novel markers for disease detection (Lee *et al.*, 1998; Pinzariu *et al.*, 2018).

4.3.2 Growth Hormone Secretion Defects

The primary cause of GH defects is pituitary adenomas that secrete aberrant GH. They can appear as single (isolated) tumors or as component of other systemic conditions as multiple endocrine neoplasia type 1 or type 4, Carney complex, McCune-Albright syndrome, or in association with pheochromocytoma and paraganglioma. GH acts via the somatotropic axis consisting of GH, GH receptors, insulin-like growth factors (IGF) 1 and 2, their associated carrier proteins, receptors, and releasing factors, which regulate growth and body composition. GH secretion defects can manifest clinically as an increase in its circulating levels leading to acromegaly or a decrease that leads to short stature.

4.3.2.1 Growth Hormone Excess: Acromegaly

An excess of GH or its chronic hypersecretion results in acromegaly or gigantism, a rare endocrine disease that is debilitating and leads to multiple comorbidities with reduction in life expectancy. The uncontrolled production of GH stimulates the liver to increasingly synthesize and secrete insulin-like growth factor-1 (IGF-1) which in turn influences metabolic changes in various organ systems and stimulates somatic (internal organs, tissues, bones, and muscles) overgrowth and development of comorbidities, including cardiovascular and malignant diseases. In skeletal muscles, GH exerts an anabolic impact that increases amino acid intake and protein synthesis and decreases protein oxidation. This action shifts the normal metabolism from mainly utilizing glucose and protein substrates to oxidation of lipid oxidation, altering the linear body growth and organ growth during childhood. The resulting clinical phenotype of these patients presents with growth acceleration, enlarged feet and hands, increased appetite, and broadening and coarsening of the facial features.

The primary cause of pituitary gigantism in nearly 50% of individuals is an underlying genetic variant or mutation (Iacovazzo *et al.*, 2016; Rostomyan *et al.*, 2015). In 95% of cases, acromegaly occurs sporadically because of adenomas of somatotroph cells that are characterized by excessive secretion of GH and IGF-1. Fifty percent of these tumors are attributable to familial germline mutations in aryl hydrocarbon receptor-interacting protein (AIP) and probable G-protein-coupled receptor 101 (GPR101) genes. On the other hand, cases with pituitary hyperplasia are a rarity and are usually encountered as part of syndromes such as the McCune-Albright syndrome, Carney complex disease, or X-linked acrogigantism. Besides the mutations seen in AIP, germline mutations have also been documented in other genes such as GPR101, PRKAR1A, CDKN1B, GNAS, MAX, SDHx, and MEN1. Somatic mutations have been shown to be related to mutations in GNAS gene and account for 40% of tumors (Boguslawska et Korbonits, 2021).

Presently the diagnosis of active acromegaly is made by determining elevations in GH levels, after a standard oral glucose tolerance testing, and in levels of IGF-1 when compared to age and gender matched controls. Confirmation of the diagnosis is through radiological MRI evaluation that validates the findings of an adenoma. Biochemical evaluations of GH and IGF-1 levels are sometimes inconsistent, and an increase in GH levels is not paralleled with that of IGF-1. The heterogeneous clinical presentation of the disease lacks early warning signs, and the inconsistencies in the measurements of GH and IGF-1 levels lead to delay or misdiagnosis.

Molecular genetic testing using chromosomal microarray analysis or single gene duplication studies has helped identify cases with germline or somatic mutations (Iacovazzo *et al.*, 2016).

In addition to genetic testing, metabolomic analysis has aided the diagnosis of acromegaly by creating a metabolomic fingerprint of the disease and identifying characteristic metabolites that differentiate patients with active disease from controls. Elevations were noted in gluconeogenic substrates (glycerol, lactate, propionate, and glucogenic amino acids such as valine and isoleucine); serine, 5-aminovaleric acid, dihydrocoumarin, mono-olein, N-acetyl-L-glutamic acid, glyceric acid, L-dithiothreitol, and gluconic acid were observed in the metabolomic profile in active acromegaly compared with normal controls. In contrast, serum levels of D-erythronolactone, taurine, carbamoyl-aspartic acid, and mucic acid were decreased. Among the different metabolites, glyceric acid and taurine had the highest sensitivities to discriminate between patients with acromegaly from normal controls (Fellinger *et al.*, 2020; Yu *et al.*, 2020).

Patients with active disease having increased GH but normal IGF-1 levels showed significantly lower levels of essential branched-chain amino acids (BCAAs) (valine, isoleucine), lysine, and lactate, whereas levels of the metabolite dimethylamine were higher. The inverse correlation between valine and isoleucine was seen with higher GH levels and not with IGF-1 indicating that lower BCAA levels, which represent the main metabolic fingerprint of acromegaly, were influenced by GH rather than IGF-1 as the primary mediator (Biagetti *et al.*, 2019). Metabolomic analysis in patients with acromegaly also highlighted significant alterations in major metabolic pathways in these groups of patients. The pathways affected as a result of active disease included glycerolipid, glyoxylate, taurine, hypotaurine, dicarboxylate, and pyrimidine pentose phosphate pathway. Dysregulation of these pathways was proposed to be the underlying reason that supported hyperplasia of tissues observed in different organs, due to excess GH secretion (Pinzariu *et al.*, 2018). An increase in gluconeogenic metabolites along with decreased BCAA levels detected was suggested to be a result of an accelerated consumption of BCAAs. Assessing levels of BCAAs, besides identifying the active disease, were also beneficial in monitoring the response to therapy.

Metabolomic profiling in patients with acromegaly, with associated cardiovascular dysfunction, identified distinct changes in amino acids and in plasma lipids. Specific perturbations were noted in the glycerophospholipid, sphingolipid, and metabolites involved in the linoleic acid metabolic pathways. Levels of phosphatidyl ethanolamine (PE) (22:6/16:0) positively correlated with changes in left ventricular mass, while lysophosphatidyl choline

(LysoPC) (16:0) was positively correlated with alterations in fractional shortening and left ventricle ejection fraction (Wang, M. *et al.*, 2020). Patients with active acromegaly also present with increased insulin resistance (IR) accompanied with lowered hepatocellular lipids and cholesterol. This is in stark contrast to other IR-associated metabolic diseases which are commonly seen associated with fatty liver disease. The lower hepatic cholesterol levels reflect the decreased liver fat content, a lower unsaturated-to-saturated lipid ratio, and decreased levels of different carnitine species, namely, plasma butyryl carnitine hexanoyl carnitine (Fellinger *et al.*, 2020). Among the various FFA species, levels of cholesteryl esters (CEs, 18:3) were lower. In contrast, within phosphocholine (PC) lipids, increased levels of LPC (18:0), as well as decreased PC (36:5), ether PC (38:6), PC (40:7), and PC (42:5), were noted, and in the sphingomyelin class, SM (36:0) was significantly lower. The hepatic lipid content decreased from increased hepatic ATP synthesis due to excess GH.

4.3.2.2 Growth Hormone Deficiency

Insufficient production of GH leads to GH deficiency or pituitary dwarfism, a rare endocrine disease condition. The causes of GHD can be inherited, congenital, or acquired. The usual manifestation of the disease is during childhood, with children clinically presenting with abnormally short stature but normal body proportions. Lately, the importance of GH has also been observed in adult patients, especially concerning its effect on lipid profile, body composition, bone mass, and cognition (De San-Martin *et al.*, 2021). Adult growth hormone deficiency is a well-defined clinical condition that is characterized by abnormal body composition, poor quality of life, dyslipidemia, and increased risk of cardiovascular disease and death (Molitch *et al.*, 2011). Genetic analysis by whole genome sequencing has identified multiple genes that affect the normal development of the pituitary, hypothalamic signaling or influence the actions of GH and IGF-1 in target tissues by altering binding to its receptors. Isolated GH deficiency can arise due to mutations or defects in the growth hormone (GH1) gene itself or the GH-releasing hormone receptor which lead to either classical GH deficiency or produce physiologically inactive GH. More rarely, growth hormone deficiency can result from mutations in HESX13, SOX3, FGFR3 gene, and pregnancy-associated plasma protein (PAPP)-A2 (Argente *et al.*, 2019).

The diagnosis of GHD is based on laboratory evaluation, through radiological imaging examinations and the insulin hypoglycemia test, which is the gold standard test in adults. The biochemical evaluation is done through measuring levels of GH, IGF-1, and IGFBP-3 (insulin-

like growth factor binding protein 3) as indirect markers of growth hormone action. Although these markers are used routinely, they lack sensitivity, and among them, IGF-1 is a least sensitive marker and a poor diagnostic indicator of GHD.

Untargeted metabolomic profiling using GC-MS identified numerous metabolites and potential biomarkers for diagnosing GHD and was also used for monitoring recombinant human growth hormone (rhGH) replacement therapy in these patients. Levels of 13 metabolites including threonic acid, cystine, cysteine, palmitoleic acid, glutamic acid, glyceric acid, aspartic acid, uridine, and hypoxanthine-like were reported to be significantly dysregulated in adult patients with GHD. On the other hand, successful treatment with rhGH showed a reversal in glutamic acid, glyceric acid, hexadecanoic acid, and palmitoleic acid to normal control levels (Hoybye *et al.*, 2014). The effectiveness of growth hormone treatment was similarly assessed in a patient with a rare pituitary-specific positive transcription factor (PIT-1) genetic defect that caused short stature. PIT-1 is responsible for the normal development of the anterior pituitary gland. Urine metabolomic profile from this patient using NMR-based metabonomics showed that higher levels of creatine, creatinine, lactate, and trimethylamine-N-oxide and lower levels of urinary citrate, dimethylamine (DMA), and alanine observed before GH treatment were reversed after treatment and returned to normal values (Abd Rahman *et al.*, 2013). It was also possible to determine the metabolic differences between children with short stature (SS) and healthy controls, using NMR-based blood metabolomic analysis, to identify metabolites which can serve as potential biomarkers for the diagnosis of SS. Serum levels of creatinine, citrate, phenylalanine, and tyrosine were increased, and levels of inositol, lysine, glycerol, glucose, betaine, serine, and glutamine were lower in comparison to normal controls. These dysregulated metabolites were associated with disturbances in metabolic pathways related to carbohydrate and amino acid metabolism (Xu *et al.*, 2019). Metabolomic profiling was also used to monitor therapy and assess the metabolic effects of IGF-1 treatment in PAPPA2-deficient patients. The metabolomic analysis highlighted profound changes in lipid and protein metabolism after replacement. An increase in BCAA, hydroxyproline, glutamic acid, glutamine, and asparagine was noted along with a decrease in levels of glycerol and FFA, palmitate, oleate, arachidonate, linoleate, palmitoleate, and stearate (Mastrangelo *et al.*, 2018). Metabolomics in GHD extends beyond identifying metabolites as biomarkers for disease diagnosis as it also helped to assess the risk of patients for developing cardiovascular disease. The identified metabolites have been linked in adults with untreated GHD to a number of CV risk factors, including morphological changes in the heart, metabolic alterations, and visceral

obesity (Biagetti *et al.*, 2019). More recently, epigenetics and metabolomics have been increasingly developed to detect distinctive fingerprints, which could predict an increased risk of CVD in patients with GHD.

Because of its anabolic effects on protein metabolism and muscle growth, athletes have used GH to improve their performance. Another aspect of metabolomics has been its extension toward analyzing biological fluids, urine, and plasma samples for evidence of doping. In the treated groups, application of direct discriminant analysis distinguished the treatments and was also used to classify them accordingly (Narduzzi *et al.*, 2020).

4.3.2.3 Hypercortisolism (Cushing's Syndrome and Cushing's Disease)

Hypercortisolism is a term that encompasses an increase in cortisol production and is a feature of both Cushing's syndrome (CS) and Cushing's disease. It can be classified into three types: ACTH-dependent (due to pathology of the pituitary gland), ectopic ACTH secretion, and ACTH-independent (due to adrenal pathology). The most common pituitary pathology is adenoma of the pituitary gland affecting the corticotrophic cells resulting in glucocorticoid (cortisol) excess production from the adrenal glands. The uncontrolled ACTH secretion from a pituitary adrenocorticotrophic adenoma is a rare pathology and is termed as endogenous Cushing's disease.

Clinically cortisol excess manifests by a well-characterized group of signs and symptoms that include centripetal obesity, hypertension, muscle weakness, moon face, fatigue, diabetes mellitus, cognitive difficulties skin changes, osteoporotic fractures, headaches, and changes in mood. In the female patients, menstrual irregularities and hirsutism (female) are also additionally seen (Nieman *et al.*, 2008). Diagnosis of Cushing's syndrome (CS) (and Cushing's disease) is a tedious multistep process requiring verification of hypercortisolism as the first step, followed by identifying the cause of hyperfunctioning of adrenocortical axis. This is further hampered by the poor diagnostic utility of the glucocorticoid metabolic pathway indices such as plasma cortisol levels, urinary-free cortisol, and other clinically measurable bio-chemical parameters, such as 11-deoxycortisol. Assessing the cause of hypercortisolism is challenging as each factor in the differential diagnosis of the disease needs to be ruled out.

Metabolomic evaluation of patients suspected of CS, with clinical features of hypercortisolism but indeterminate pituitary imaging, identified many affected metabolic pathways. These mainly involved metabolic pathways related to fatty acids, amino sugars, carbohydrates

(glycolysis/gluconeogenesis), purines, glycated nucleotides, amino acids (glutamate, alanine, aspartate, and lysine), vitamin B metabolism, aminoacyl-tRNA biosynthesis, and starch and sucrose metabolism (Oklu *et al.*, 2014). Patients with ACTH-secreting pituitary adenomas showed distinct changes in plasma metabolite levels identified using LC-MS/MS compared to the control group. These included 2-hydroxybutyric acid, 3-hydroxyphenyl acetic acid, hypoxanthine, L-aspartic acid, aminoadipic acid, 4-pyridoxic acid, quinolinic acid, deoxycholic acid, xanthine, 3-methyladipate, and sucrose and glucose 6-phosphate. Besides the plasma and urine metabolomics, tissue metabolomic analysis from ACTH-secreting pituitary adenoma showed increased short-chain fatty acids (hexanoic, capric, heptanoic, octanoic, and nonanoic fatty acids)(Arlt *et al.*, 2017). At the same time, glucose-6-phosphate was decreased compared to the control group (Pinzariu *et al.*, 2018). Short- and medium-chain acylcarnitines, branched-chain and aromatic amino acids, and polyamine levels were also lower in patients with hypercortisolism. Independently, the severity of hypercortisolism is linked to changes in intermediate metabolism, which are consistent with skewed protein synthesis and catabolism and incomplete β-oxidation, indicating the presence of metabolic inflexibility exists (Di Dalmazi *et al.*, 2017; Pinzariu *et al.*, 2018).

Differentiating between the different subtypes of hypercortisolism is also clinically challenging. Using metabolomic approaches through serum and urine steroid profiling has helped identify sensitive markers in patients with CS. Steroid metabolite profiling has shown promise for accurately subtyping patients with CS. The profiling revealed that patients with ACTH-dependent CS (caused by ACTH-secreting pituitary adenomas or ectopic malignancies) have elevated androgens and related metabolites, whereas patients with CS having glucocorticoid hypersecretion from the adrenals which is autonomous or independent from ACTH have lower levels (Arlt *et al.*, 2017; Eisenhofer *et al.*, 2018). Targeted metabolomic analysis using a combination panel of ten CS with a high discriminatory capacity between the three subtypes of CS. The various steroids in the panel (11-deoxycortisol, cortisol, cortisone, corticosterone, 11-deoxycorticosterone, androstenedione, 18-oxocortisol, DHEA, DHEA-SO4, and aldosterone) demonstrated significantly distinct profile patterns among patients with ACTH-independent and ACTH-dependent forms of CS. From the metabolites mentioned earlier, increases in cortisol levels, its precursor 11-deoxycortisol and derivative 21-deoxycortisol, cortisone, corticosterone, 11-deoxycorticosterone, and decrease in plasma aldosterone and 18-oxocortisol differentiated between patients with and without CS. Higher levels of 11-deoxycorticosterone and 11-deoxycortisol were higher in patients with pituitary

and adrenal CS, while patients with ectopic CS disease showed lower plasma levels of 18-oxocortisol and aldosterone. The plasma levels of adrenal androgens, androstenedione, DHEA, and DHEAS were higher in pituitary and ectopic CS compared to adrenal CS. On the other hand, ectopic CS had highest increase in glucocorticoids.

The urine metabolomic profile was also evaluated in patients with CS and showed a higher urinary excretion of DHEA and DHEAS metabolites in pituitary disease compared to adrenal CS (Oklu *et al.*, 2014; Pinzariu *et al.*, 2018). The ratio of cortisol to metabolites of cortisone was higher, indicating the suppression of activity of the cortisol-inactivating enzyme HSD11B2. The unrestricted action of cortisol on the mineralocorticoid receptor explains the clinical symptoms of hypertension and hypokalemia commonly seen in these patients.

Cushing's syndrome leads to various metabolic dysfunctions, including cardiovascular disease due to a proatherogenic shift in the circulating lipids. Metabolomic analysis identified increased urinary ceramides, glycerophospholipids, and sphingolipid metabolites, independently associated with higher urinary-free cortisol. In the study by Vega-Beyhart, patients with CS showed significant alterations in 93 metabolites showing an increase in sulfur containing amino acids, ceramides, triacylglycerols, cholesteryl esters, and glycerophospholipids. On the other hand, a decrease was seen in essential and nonessential AA, polyunsaturated fatty acids, conjugated bile acids, and second messenger glycerolipid concentrations. Twenty-four-hour urinary-free cortisol was associated with alterations in the concentration of many lipids and amino acid metabolites. The identified metabolites comprising of ten amino acids and ten lipid metabolites demonstrated an AUC-ROC of 95% and served as a metabolic signature for the classification of CS. The identified amino acids, acylcarnitines, ceramides, and glycerophospholipid markers were suggested as potential biomarkers of cardiovascular risk in patients with CS (Vega-Beyhart *et al.*, 2021).

4.4 Conclusion

Metabolomic profiling of endocrine diseases with its high-power discrimination is a powerful tool for the clinical investigation and diagnosis of rare pituitary endocrine diseases. The realm of metabolomics, in managing pituitary diseases, will in the future extend beyond identifying metabolites as biomarkers for disease diagnosis and towards personalized medicine. This approach will aim to deliver targeted individualized therapy that optimizes the effectiveness of treatments and avoid unwanted adverse effect reactions or procedures. Establishing disease-

specific metabolite panels on a chip could further revolutionize the diagnostic industry. Achieving this milestone would require further studies with larger populations, meta-analysis, and a uniform standardization of analytical and statistical steps to yield reproducible results.

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVES

In this chapter I summarize the contributions of the thesis, highlighting the main findings from each chapter, and discussing their biological and translational significance. Rheumatoid arthritis is a chronic, systemic autoimmune disease that affects around one percent of the global population and remains a major cause of long-term disability. It is characterized by persistent inflammation of the synovial joints, leading to pain, stiffness, swelling, and eventual destruction of cartilage and bone. In addition to joint involvement, rheumatoid arthritis has systemic effects, influencing cardiovascular health, bone metabolism, and overall immune function. Early diagnosis is often difficult; treatment responses vary greatly between patients and some of them continue to present with pain even when in remission. Despite the availability of advanced treatments, many patients experience delays in diagnosis, unpredictable responses to therapy, and progressive joint damage. Conventional biomarkers such as rheumatoid factor and anti-citrullinated protein antibodies, while useful, do not fully capture the complexity of the disease or its heterogeneity among patients. There is therefore a pressing need for new biomarkers and diagnostic strategies that reflect the molecular basis of RA more accurately to guide more precise clinical decisions.

The hypothesis underlying this work was that an untargeted proteomic and metabolomic profiling of plasma from patients with rheumatoid arthritis could reveal novel dysregulated pathways and identify candidate biomarkers that are not captured by conventional diagnostic assays. The aims of the project followed directly from this hypothesis. The first aim was to conduct high resolution mass spectrometry to carry out untargeted proteomic profiling of plasma samples from patients with RA compared to healthy controls, followed by validation of significant proteins using multiple reaction monitoring mass spectrometry. The second aim was to perform high resolution mass spectrometry untargeted metabolomic profiling using LC/MS in the same cohort, thereby identifying small-molecule changes that accompany protein-level alterations. By using the results from the untargeted proteomic and metabolomic results, the goal was to generate a more complete understanding of disease biology.

The aims were two fold. First, to perform discovery proteomic profiling of plasma samples from rheumatoid arthritis patients and controls using 2D-DIGE coupled with mass spectrometry, followed by validation of significant candidates using multiple reaction

monitoring (MRM). Second, to conduct untargeted metabolomic profiling on the same cohort, thereby capturing small-molecule changes that complement the protein-level alterations, and to integrate these findings through bioinformatics to provide a more comprehensive view of disease biology.

This thesis achieved several important outcomes. Proteomic profiling revealed a panel of differentially abundant proteins with clear relevance to rheumatoid arthritis. Among the most notable were mitochondrial dicarboxylate carrier, which was increased and points to mitochondrial dysfunction and oxidative stress, and hemopexin, another protein elevated in patients, which links inflammation with iron and heme metabolism. Several proteins were decreased, including osteocalcin, apolipoproteins A-I and A-IV, haptoglobin, metallothionein-2, and CCDC124. These findings highlight diverse biological processes: osteocalcin points to altered bone metabolism, apolipoproteins to impaired lipid transport and immune regulation, haptoglobin and metallothionein-2 to reduced antioxidant buffering, and CCDC124 to dysregulation of RNA-binding and translation. Together, they reveal systemic disturbances that extend far beyond the joints, reinforcing the view of rheumatoid arthritis as a complex systemic disease. Importantly, some of these proteins, such as osteocalcin and CCDC124, showed excellent discriminatory power between patients and controls, with high area-under-the-curve values, marking them as potential biomarkers of diagnostic value.

The metabolomic profiling provided a complementary perspective. It revealed significant disturbances in amino acid metabolism, purine turnover, and peptide signaling. Bradykinin and its fragments were elevated, in keeping with their known role in vascular permeability, pain generation, and recruitment of immune cells. Dynorphin was increased, suggesting an underappreciated neuro-immune crosstalk where pain modulation intersects with inflammation. Purine metabolism was perturbed, with higher levels of inosine, N6-methyladenosine, and 4-amino-1H-imidazole-2-carboxamide, molecules that reflect intense nucleotide turnover and immune activation. These metabolites also highlight potential epigenetic regulation of immune function, particularly through RNA modifications. Amino acid derivatives such as cysteinyl-serine, tyrosyl-arginine, and N2-acetyl-N6-methyllysine were linked with disease activity measures such as ESR, indicating their relevance for disease monitoring. Taken together, the metabolomic findings show that rheumatoid arthritis involves not just immune cell activation, but also widespread metabolic reprogramming that supports chronic inflammation.

Table 5-1 Summary of the principal findings, biological significance, and translational relevance of this thesis

Protein / Analyte	Molecular Function / Protein Class	Biological Role & Pathway Significance	Direction in RA
CHAPTER 2 — PROTEOMICS (2D-DIGE / MALDI-TOF-MS): Key Findings in Established Rheumatoid Arthritis (RA)			
CCDC124 (Coiled-Coil Domain-Containing Protein 124)	Putative mRNA-binding protein	Regulates cytokinesis, and RNA metabolism. Decrease in RA may reflect aberrant cell-cycle/apoptotic regulation in immune cells.	↓
Osteocalcin (OC / Bone GLA Protein)	Non-collagenous bone matrix protein; metabolic hormone	Marker of bone formation/remodeling; modulates whole-body glucose metabolism, insulin secretion, and cognition. Decreased levels may have distinct roles in RA.	↓
Apolipoprotein A-I (ApoA-I)	Major HDL apolipoprotein	Regulates lipid metabolism and reverse cholesterol transport. Acts as a negative acute-phase protein, supports regulatory T cells.	↓
Hemopexin	Acute-phase protein; heme-binding glycoprotein	Downregulates TNF and IL-6 secretion from macrophages; negative regulator of Th17 differentiation. Multiple proteoforms identified and carbamylated form is an early-RA autoantigen.	↑
Haptoglobin (HPT)	Hemoglobin-binding acute-phase protein	Scavenges free hemoglobin; protects from oxidative stress; modulates cytokine production and binding to CD8 T cells and B cells. HPT deficiency facilitates autoimmune inflammation. Genetic proteoforms (Hp2-2) independently linked to CVD risk in RA. Multiple glycosylated proteoforms detected.	↓
Mitochondrial Dicarboxylate Carrier (DIC / SLC25A10)	Solute carrier family 25 (SLC25)	Transports dicarboxylates in TCA cycle; regulates cellular energy production and mitochondrial glutathione transport. Influences immune cell activation in RA.	↑
Apolipoprotein A-IV (ApoA-IV)	Multifunctional apolipoprotein	Anti-inflammatory: reduces pro-inflammatory cytokine secretion and immune cell infiltration in mouse models. Involved in lipid metabolism, antioxidant defense, coagulation, and glucose homeostasis.	↓
Muskelin	CTLH E3 ubiquitin ligase complex component	Mediates ubiquitination and proteasomal degradation; and lymphocyte migration. Decrease may impair normal proteasomal clearance of autoreactive proteins, contributing to RA immune dysregulation.	↓
Proteins Validated by Multiple Reaction Monitoring - OC, CCDC124, ApoA-I, HPT, Hemopexin			

Bioinformatics, Pathway & Biomarker Analysis - Top IPA Network (Score = 37): Central nodes include STAT1, TNF, IL-4, Cell-to-cell signaling & interaction, Hematological system development & function, Immune cell trafficking			
CHAPTER 3 — METABOLOMICS (Untargeted LC-HRMS): Key Findings in Established Rheumatoid Arthritis (RA)			
Metabolite	**Metabolite Class**	**Biological Role & Pathway Significance**	
Succinyladenosine	Modified purine nucleoside	Marks heightened de novo purine biosynthesis. Elevated adenosine and succinate signaling are linked to chronic immune activation and inflammation.	↑
N2-acetyl, N6-methyllysine	Doubly modified lysine (post-translational)	Product of histone acetylation and methylation turnover. Probably reflects epigenetic reprogramming in RA. Elevated circulating levels arise from nuclear protein turnover in activated immune cells.	↑
Cysteinyl-Serine	Dipeptide (cysteine–serine)	Generated by cysteine/serine proteases elevated in RA; reflects heightened extracellular proteolysis. Consistent with an expanded low-molecular-weight peptide signature in plasma of RA patients.	↑
Dynorphin B (10–13)	Endogenous opioid neuropeptide fragment	Activates opioid receptors for short-term analgesia in chronic pain (RA); also activates bradykinin receptors (B1/B2) involved in inflammatory pain and hyperalgesia.	↑
Bradykinin & Bradykinin Fragments	Kinin peptides	Mediators of inflammatory pain, vasodilation, and vascular permeability. Elevated levels reflect activation of the kallikrein–kinin system in RA.	↑
Inosine	Purine nucleoside (adenosine pathway)	Anti-inflammatory; limits cytokine output via adenosine A2A/A3 receptor signaling. Decreased levels reflect enhanced catabolism during immune activation and loss of adenosinergic anti-inflammatory tone.	↓
N6-Methyladenosine (m^6A)	Modified mRNA nucleoside	Dominant internal mRNA modification regulating transcript stability and translation of inflammatory mRNAs. m^6A machinery is directly linked to RA pathogenesis in emerging literature.	↓
AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide)	Purine synthesis intermediate	Activates AMPK, which restrains inflammatory programs. Decrease indicates brisk flux toward IMP (purine synthesis) and relative loss of AMPK-mediated anti-inflammatory control.	↓
O-Acetylcholine	Cholinergic neurotransmitter	Inhibits NF-κB activation and suppresses pro-inflammatory cytokine release from immune cells. Suppression may remove an important brake on systemic inflammation in RA.	↓

Tyrosyl-Arginine	Dipeptide (opioid-like analgesic)	Endogenous analgesic dipeptide with opioid-like properties. Decreased levels in RA patients suggest diminished endogenous pain suppression as seen in patients with RA.	↓
Arginylarginine	Dipeptide	Decrease may reflect increased L-arginine consumption consistent with documented NO/iNOS signaling in RA.	↓
ESR Subgroup Analysis: High ESR (>35 mm/hr) vs Low ESR - 3 Metabolites Overlapping RA vs Control AND High vs Low ESR: Cysteinyl-Serine ↑ in both comparisons, Tyrosyl-Arginine ↑ in RA vs control, but ↓ in high ESR, N2-acetyl, N6-methyllysine ↑ in RA vs control, but ↓ in high ESR			
Bioinformatics, Pathway Analysis - IPA Network (Score = 19, 8 focus molecules): Central nodes (IL-6, IL-2, IL-1, MAPK, Kininogen), Humoral immune response, Inflammatory response, Hematological system development & function,			

An important strength of this thesis is the analysis of the proteomic and metabolomic data in the same cohort of patients. When considered together, these two omics layers' highlight key converging pathways. For example, mitochondrial dysfunction revealed at the protein level aligns with increased oxidative stress metabolites. Dysregulated lipid and apolipoprotein pathways link with changes in amino acid metabolism and inflammatory mediators. The PAI-1–bradykinin axis exemplifies the connection between proteomic and metabolomic findings, bringing together coagulation, fibrinolysis, vascular biology, and pain signaling. This integrative view reinforces the notion that rheumatoid arthritis is a systemic immunometabolic disease. It is not limited to synovial inflammation, but involves coordinated disruptions across immunity, metabolism, vascular biology, and tissue remodeling.

In addition to these molecular discoveries, this thesis has also set the stage for the next phase of research: the use of aptamers as diagnostic tools. While the work here was focused on identifying biomarkers through proteomics and metabolomics, the aptamer framework will be designed as continuation of this work, with CD80 selected as an initial target. Once identified and validated, the biomarkers from the proteomics and metabolomics platforms will serve as the basis for developing aptamer-based affinity capture and diagnostic systems, offering a new technological pathway that would be both scientifically powerful and clinically practical. The groundwork is now in place to extend the findings of this thesis into aptamer-based assays that can measure these newly identified proteins and metabolites with high sensitivity and specificity. This is not unfinished work but rather a natural continuation, where the discoveries of this thesis directly inform the development of a new generation of diagnostic technologies.

The advantages of aptamers are considerable. Unlike antibodies, they can be synthesized reproducibly, modified chemically, and remain stable under conditions where antibodies may degrade. Their small size and structural versatility allow them to bind to epitopes that antibodies cannot access. This means they can expand the range of detectable biomarkers and improve both sensitivity and specificity. In addition, aptamers can be designed to detect not only proteins but also metabolites, which makes them ideally suited for multiplex assays. This opens the possibility of creating aptamer-based platforms that simultaneously measure osteocalcin, apolipoproteins, bradykinin, inosine, and other molecules identified in this thesis. Such a multiplex approach could provide a more comprehensive and reliable diagnostic picture than any single marker alone. By embedding aptamers into nanomaterial-based sensors or electrochemical platforms, these assays could also be made portable and cost-effective, suitable for point-of-care use. This would bring biomarker testing closer to the clinic and even to the patient bedside, significantly improving early diagnosis and disease monitoring in rheumatoid arthritis.

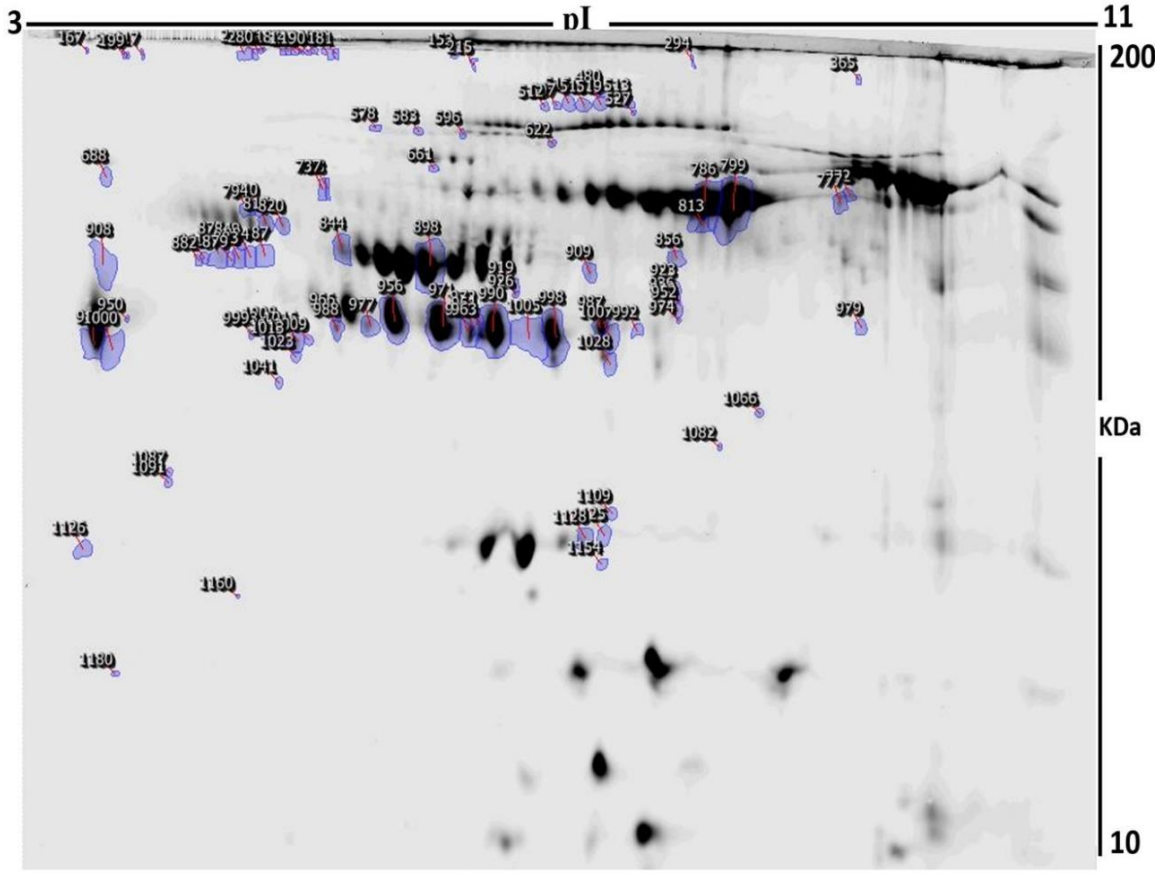
Looking ahead, several steps remain important. Candidate biomarkers from this thesis need validation in larger, independent, and longitudinal cohorts, especially in early and seronegative cases. Integration with transcriptomics, lipidomics, and immunopeptidomics will enrich the molecular picture and help define subtypes of disease. Aptamer development should now move forward, first by optimizing affinity capture methods for selected targets, and then by developing multiplex biosensor platforms. The long-term vision is to translate the biomarker discoveries of this thesis into robust diagnostic assays that change clinical practice.

In conclusion, this thesis has achieved significant advances by identifying novel proteins and metabolites associated with rheumatoid arthritis, demonstrating the power of integrated proteomic and metabolomic analysis, and establishing the conceptual foundation for aptamer-based diagnostics. Far from being an endpoint, this work provides both discoveries and directions. It shows that rheumatoid arthritis is best understood as a systemic immunometabolic disease, and it provides concrete molecular candidates that can be built into the next generation of diagnostic technologies. The continuation of this research through aptamer-based platforms promises to improve diagnostic capability, making tests more sensitive, more specific, and more accessible. This thesis therefore represents both a contribution in itself and a foundation for future progress, bridging discovery and translation, and moving closer to earlier diagnosis, better monitoring, and ultimately improved care for patients with rheumatoid arthritis.

APPENDIX A

SUPPLEMENTARY INFORMATION FOR "Proteomic Profiling Reveals Novel Molecular Insights into Dysregulated Proteins in Established Cases of Rheumatoid Arthritis"

Supplementary Figure 1 The original gel images, unsupervised spot details and a representative 2D-gel electrophoresis showing the spot numbers of significant DAPs (cutoffs; FC > 1.5, ANOVA FDR $p \leq 0.05$) identified on the 2D-DIGE gel between RA and control. The numbered spots were excised and taken for identification via MALDI-TOF-MS. MW—protein molecular weight; pI—isoelectric point



Supplementary Table 1 Mass spectrometry list of significant differentially abundant proteins between RA and control states identified in samples using 2D-DIGE. Protein name, accession number, Mascot score, MS% coverage, protein MW, and pI values according to the Uniprot database are listed

S No	Spot No[a]	Accession No[b]	Protein Name	MASCOT ID	Pi[c]	MW[d]	Cov%	Score
1	1016	Q96CT7	Coiled-coil domain-containing protein 124	CC124_HUMAN	9.54	25820	37	59
2	1109	P02647	Apolipoprotein A-I	APOA1_HUMAN	5.56	30759	65	146
3	1008	P02818	Osteocalcin	OSTCN_HUMAN	4.19	2935	95	59
4	977	P02818	Osteocalcin	OSTCN_HUMAN	4.19	2935	95	62
5	1013	P02795	Metallothionein-2	MT2_HUMAN	8.23	7178	36	60
6	1087	Q9P2M1	LRP2-binding protein	LR2BP_HUMAN	7.99	40154	12	57
7	952	P00738	Haptoglobin	HPT_HUMAN	6.13	45861	22	85
8	919	Q9UL63	Muskelin	MKLN1_HUMAN	5.91	85911	18	59
9	887	Q9UBX3	Mitochondrial dicarboxylate carrier	DIC_HUMAN	9.62	31718	27	62
10	963	P06727	Apolipoprotein A- IV	APOA4_HUMAN	5.28	45371	41	80
11	961	P06727	Apolipoprotein A- IV	APOA4_HUMAN	5.28	45371	42	105
12	997	P02818	Osteocalcin	OSTCN_HUMAN	4.19	2935	95	59
13	909	P02647	Apolipoprotein A-I	APOA1_HUMAN	5.56	30759	65	146

Supplementary Table 2 Using mass spectrometry, the table shows the list of differentially regulated proteins between patients with RA and controls with their respective fold changes and significance levels (ANOVA, FDRp-value < 0.05). [Analysis type: MALDI-TOF; Mascot score greater than 56, Mascot search algorithm (v2.0.04, Matrix Science Ltd., UK), database: SwissProt; taxonomy: Homo sapiens].

SL. No	Spot No[a]	Accession No	Protein Name	MASCOT ID	FDR P-value[b] (ANOVA)	Ratio[c] RA/Control	Exp[d]
1	1016	Q96CT7	Coiled-coil domain-containing protein 124	CC124	3.40E-06	1.5	DOWN
2	1109	P02647	Apolipoprotein A-I	APOA1	1.00E-04	1.5	DOWN
3	1008	P02818	Osteocalcin	OSTCN	1.00E-04	1.5	DOWN
4	977	P02818	Osteocalcin	OSTCN	1.00E-04	1.8	DOWN
5	1013	P02795	Metallothionein-2	MT2	2.00E-04	1.5	DOWN
6	1087	Q9P2M1	LRP2-binding protein	LR2BP	2.00E-04	1.92	DOWN
7	952	P00738	Haptoglobin	HPT	4.00E-04	1.63	DOWN
8	919	Q9UL63	Muskelin	MKLN1	4.00E-04	1.92	DOWN
9	887	Q9UBX3	Mitochondrial dicarboxylate carrier	DIC	6.90E-04	1.59	UP
10	963	P06727	Apolipoprotein A- IV	APOA4	7.00E-04	1.57	DOWN
11	961	P06727	Apolipoprotein A- IV	APOA4	8.00E-04	1.65	DOWN
12	997	P02818	Osteocalcin	OSTCN	8.00E-04	1.5	DOWN
13	909	P02647	Apolipoprotein A-I	APOA1	9.00E-04	1.5	DOWN
14	993	Q9Y240	C-type lectin domain family 11 member A	CLC11	1.00E-03	1.5	DOWN
15	1091	Q9H4B6	Protein salvador homolog 1	SAV1	1.00E-03	1.5	DOWN
16	983	P00738	Haptoglobin	HPT	1.00E-03	1.56	DOWN
17	988	P46939	Utrophin	UTRN	2.00E-03	1.53	DOWN
18	940	P00738	Haptoglobin	HPT	2.00E-03	1.5	DOWN

SL. No	Spot No[a]	Accession No	Protein Name	MASCOT ID	FDR P-value[b] (ANOVA)	Ratio[c] RA/Control	Exp[d]
19	926	O43715	TP53-regulated inhibitor of apoptosis 1	TRIA1	2.00E-03	1.5	DOWN
20	519	Q3ZCN5	Otogelin-like protein	OTOGL	2.00E-03	2.1	DOWN
21	972	Q96MM7	Heparan-sulfate 6-O-sulfotransferase 2	H6ST2	2.00E-03	1.6	DOWN
22	518	P04217	Alpha-1B-glycoprotein	A1BG	2.00E-03	1.5	DOWN
23	998	P00738	Haptoglobin	HPT	3.00E-03	1.57	DOWN
24	1000	A0A0C4DH73	Immunoglobulin kappa variable 1-12	KV112	4.00E-03	1.5	DOWN
25	990	P00738	Haptoglobin	HPT	5.00E-03	1.56	DOWN
26	1012	O95613	Pericentrin	PCNT	5.00E-03	1.56	DOWN
27	971	P00738	Haptoglobin	HPT	6.00E-03	1.53	DOWN
28	966	Q92522	Histone H1.10	H1X	9.00E-03	1.63	DOWN
29	879	Q9BXS6	Nucleolar and spindle-associated protein 1	NUSAP	0.01	1.65	UP
30	688	Q9H7D0	Dedicator of cytokinesis protein 5	DOCK5	0.01	1.74	DOWN
31	1023	Q92522	Histone H1.10	H1X	0.011	1.58	DOWN
32	861	P02790	Hemopexin	HEMO	0.013	1.9	UP
33	515	P01011	Alpha-1-antichymotrypsin	AACT	0.015	1.55	DOWN
34	882	Q9Y3D5	28S ribosomal protein S18c, mitochondrial	RT18C	0.015	1.58	UP
35	1154	P13647	Keratin, type II cytoskeletal 5	K2C5	0.017	1.5	DOWN
36	1066	P02647	Apolipoprotein A-I	APOA1	0.018	1.52	DOWN
37	875	Q9Y3D5	28S ribosomal protein S18c, mitochondrial	RT18C	0.018	1.5	UP
38	979	O95613	Pericentrin	PCNT	0.019	1.5	DOWN
39	507	Q02790	Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4	0.02	1.59	DOWN
40	215	A5D8V7.	Coiled-coil domain-containing protein 151	ODAD3	0.022	1.5	UP

SL. No	Spot No[a]	Accession No	Protein Name	MASCOT ID	FDR P-value[b] (ANOVA)	Ratio[c] RA/Control	Exp[d]
41	884	Q3ZCN5	Otogelin-like protein	OTOGL	0.022	1.5	UP
42	898	P01009	Alpha-1-antitrypsin	A1AT	0.023	1.69	DOWN
43	480	P02768	Albumin	ALBU	0.028	1.72	DOWN
44	786	P02787	Serotransferrin	TRFE	0.029	1.66	DOWN
45	1028	O15382	Branched-chain-amino-acid aminotransferase, mitochondrial	BCAT2	0.029	1.66	DOWN
46	820	Q15131	Cyclin-dependent kinase 10	CDK10	0.029	1.68	DOWN
47	956	P00738	Haptoglobin	HPT	0.03	1.61	DOWN
48	817	Q9HBE4	Interleukin-21	IL21	0.03	1.66	DOWN
49	1128	Q8NBN7	Retinol dehydrogenase 13	RDH13	0.034	1.5	DOWN
50	583	O95613	Pericentrin	PCNT	0.034	1.98	UP
51	992	P00738	Haptoglobin	HPT	0.035	1.6	DOWN
52	758	P60763	Ras-related C3 botulinum toxin substrate 3	RAC3	0.037	1.7	DOWN
53	974	P00738	Haptoglobin	HPT	0.038	1.5	DOWN
54	844	P01009	Alpha-1-antitrypsin	A1AT	0.041	1.5	UP
55	1005	P06727	Apolipoprotein A- IV	APOA4	0.042	1.61	DOWN
56	728	P01011	Alpha-1-antichymotrypsin	AACT	0.042	1.57	UP
57	794	P02768	Albumin	ALBU	0.044	1.69	DOWN
58	790	Q9P126	C-type lectin domain family 1 member B	CLC1B	0.045	1.74	DOWN
59	1125	P02647	Apolipoprotein A-I	APOA1	0.046	1.65	DOWN
60	661	Q9UBI4	Stomatin-like protein 1	STML1	0.047	1.75	DOWN
61	883	Q9Y240	C-type lectin domain family 11 member A	CLC11	0.047	1.51	UP
62	964	Q9Y3D5	28S ribosomal protein S18c, mitochondrial	RT18C	0.048	1.5	DOWN
63	923	Q7Z2K8	G protein-regulated inducer of neurite outgrowth 1	GRIN1	0.05	1.51	DOWN
64	512	Q92522	Histone H1.10	H1X	0.05	1.69	DOWN
65	987	P00738	Haptoglobin	HPT	0.052	1.72	DOWN
66	527	Q8N806	Putative E3 ubiquitin-protein ligase UBR7	UBR7	0.052	1.5	DOWN
67	856	Q9NWK9	Box C/D snoRNA protein 1	BCD1	0.053	1.57	DOWN

SL. No	Spot No[a]	Accession No	Protein Name	MASCOT ID	FDR P-value[b] (ANOVA)	Ratio[c] RA/Control	Exp[d]
68	849	Q6ZN19	Zinc finger protein 841	ZN841	0.055	1.5	UP
69	513	Q8IY21	Probable ATP-dependent RNA helicase DDX60	DDX60	0.058	1.57	DOWN

[a] Protein accession number for SWISSPROT Database.

[b] P-Value (ANOVA).

[c] Ratio between the groups

[d] Protein expression between the groups

Supplementary Table 3 Experimental conditions for the validation of the selected proteins signature peptides

Accession Number	Protein Name	Peptide Sequences	Length (AA)	Molecular weight (g/mol)	MRM Transition		Cone Voltage (v)	Collision Energy (v)	RT (min)
					MS1 (m/z)(z)	MS2(m/z) (ion type)			
Q96CT7	Coiled-coil domain-containing protein 124	R.AAFTAFEEAQLPR.L[171, 183]	13	1535.66	484.2490	175.1190 [y1]	20	20	5.06
P02818	Osteocalcin	EVCELNPDCDELADHIGFQEAY [72,93]	23	2666.85	837.9	182.12 [y1]	34	25	5.84
P02647	Apolipoprotein A1	EQLGPVTQEFWDNLEK[86,101]	16	1933.08	644.9828	951.4571 [y7] 1080.5 [y2]	20	20	5.05
P00738	Haptoglobin	R.TEGDGVYTLNNEK.Q [119, 131]	13	1458.58	480.5598	390.1983 [y3] 276[y2]	20	20	4.47

APPENDIX B

SUPPLEMENTARY INFORMATION FOR "Untargeted Metabolomics Reveals Immune-Metabolic Signatures in Established Cases of Rheumatoid Arthritis"

Supplementary Table 4: List of endogenous metabolites that were significantly dysregulated between RA and Control groups. One-way ANOVA (Tukey's post hoc, FDR $p < 0.05$) Table S2: List of endogenous metabolites that were significantly dysregulated between RA patients with high and low ESR levels. One-way ANOVA (Tukey's post hoc, FDR $p < 0.05$) Table S3: List of network pathways with their respective scores and number of focus metabolites identified between RA and control using IPA.

Table S1: List of endogenous metabolites that were significantly dysregulated between RA and Control groups. One-way ANOVA (Tukey's post hoc, FDR p < 0.05)

Compound name	Retention Time	Mass	p (Corr) ([patients] Vs [ctrl])	FC (abs) ([patients] Vs [ctrl])	Log FC ([patients] Vs [ctrl])	Regulation ([patients] Vs [ctrl])
3,4-Dihydroxyphenylvaleric acid	1.01	231.06	0.00	3.80	1.93	up
L-Isoleucine	3.63	173.13	0.00	2.64	-1.40	down
Succinyladenosine	12.17	425.14	0.00	11.11	3.47	up
Butyl (S)-3-hydroxybutyrate [arabinosyl-(1->6)-glucoside]	13.28	437.20	0.00	2.49	1.32	up
2-Mercaptopropanoic acid	3.46	129.00	0.00	2.79	1.48	up
Cysteinyl-Alanine	4.11	173.04	0.00	4.03	2.01	up
S-(3-Oxo-3-carboxy-n-propyl)cysteine	2.42	186.02	0.00	1.73	0.79	up

Methylthio 2—(propanoyloxy)propanoate	3.99	159.05	0.00	1.60	0.68	up
MGDG(2:0/3:0)	10.59	397.11	0.00	14.11	3.82	up
2-Hydroxy-3-methoxyestrone	4.09	323.16	0.00	3.09	1.63	up
1,9-Nonanedithiol	4.50	215.09	0.00	1.77	-0.83	down
(2R,3S)-Piscidic acid	4.69	237.04	0.00	2.19	1.13	up
PGP(i-24:0/PGD2)	7.63	528.28	0.00	3.25	1.70	up
Cytidine	1.42	288.06	0.00	2.09	-1.06	down
Glucose-6-phosphate lactate	2.39	347.04	0.00	1.93	0.95	up
9-(4-Fluoro-3-(hydroxymethyl)butyl)guanine	3.09	236.09	0.00	2.71	1.44	up
Cysteinyl-Serine	3.46	173.04	0.00	2.46	1.30	up
alpha-D-Galactopyranuronosyl-(1->4)-alpha-D-galactopyranuronosyl-(1->4)-D-galacturonic acid	12.01	547.12	0.00	2.62	1.39	up
Cystathionine sulfoxide	0.82	280.10	0.00	1.74	-0.80	down
CerP(d17:0/2:0)	17.23	205.64	0.00	1.60	0.68	up

2-Phenylethanol glucuronide	0.91	263.09	0.00	2.52	1.34	up
4-Hydroxyphenylpyruvic acid	7.08	179.04	0.00	1.96	0.97	up
Tyrosyl-Arginine	4.28	338.18	0.00	3.29	1.72	up
Mandelic acid	4.24	175.04	0.00	1.85	-0.89	down
3-(3,4-Dihydroxyphenyl)-2-methoxypropionic acid	7.29	195.07	0.00	1.91	-0.94	down
(all-E)-3,5,7-Tridecatriene-9,11-diyn-1-ol	2.83	209.09	0.00	1.51	0.59	up
CDP-DG(PGE2/i-19:0)	5.74	379.51	0.00	2.42	1.28	up
MGDG(2:0/2:0)	2.44	170.07	0.00	2.16	-1.11	down
N-(1-Deoxy-1-fructosyl)phenylalanine	3.13	310.13	0.00	2.91	-1.54	down
2-(beta-D-Mannopyranosyl)-L-tryptophan	4.00	367.15	0.00	2.83	-1.50	down
1beta-Hydroxyalantolactone	12.01	249.15	0.00	1.83	-0.87	down
O-Acetylcholine	14.33	191.09	0.00	1.62	-0.70	down
N6-Methyladenosine	0.93	280.10	0.00	3.42	-1.78	down
Bioadykinin Fragment 1-5	6.13	573.31	0.00	1.85	0.89	up

L-histidinol-phosphate	7.52	186.04	0.00	1.84	0.88	up
CDP-DG(22:6(5Z,7Z,10Z,13Z,16Z,19Z)-OH(4)/i-20:0)	5.65	547.78	0.00	2.55	1.35	up
3,3-Dimethylglutaric acid	3.22	183.06	0.01	1.93	-0.95	down
(Â±)-Glycerol 1,2-diacetate	4.21	197.04	0.01	1.64	-0.72	down
Bradykinin	5.71	530.79	0.01	2.42	1.28	up
Arginylarginine	12.22	372.25	0.01	2.20	-1.14	down
Dynorphin B (10-13)	13.20	466.26	0.01	1.54	0.62	up
CDP-DG(i-19:0/18:1(12Z)-2OH(9,10))	5.74	366.85	0.01	2.12	1.08	up
CDP-DG(20:5(7Z,9Z,11E,13E,17Z)-3OH(5,6,15)/i-12:0)	16.19	992.43	0.01	2.80	-1.48	down
Cystamine	6.63	153.05	0.01	1.71	-0.77	down
Pregnanolone	16.76	339.23	0.01	1.98	-0.99	down
4-Amino-1H-imidazole-2-carboxamide	1.71	147.03	0.01	2.14	-1.09	down
N-Acetyl-a-neuraminic acid	1.09	290.09	0.01	1.93	0.94	up

PE(PGE2/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	6.88	452.74	0.02	2.14	1.10	up
PA(8:0/20:3(8Z,11Z,14Z)-2OH(5,6))	11.59	310.18	0.02	1.87	0.91	up
Phenylalanylvaline	14.05	285.12	0.02	1.82	0.86	up
N-Acetyl-5-methoxykynuramine	11.92	257.09	0.02	1.52	0.60	up
GPA(14:1/4:0)	12.01	226.13	0.03	1.59	-0.67	down
2-Phenylethyl beta-D-glucopyranoside	7.32	249.11	0.03	1.66	-0.73	down
n2-Acetyl,n6-methyllysine	4.07	247.10	0.03	2.54	1.35	up
PGP(PGE2/i-21:0)	15.93	991.53	0.03	3.40	-1.76	down
Inosine	0.93	267.07	0.04	2.50	-1.32	down
Octadecenoylcarnitine	11.59	448.34	0.04	3.56	1.83	up
13-(3,4-Dimethyl-5-pentylfuran-2-yl)tridecanoylcarnitine	18.02	563.44	0.04	1.55	-0.63	down
PGP(PGF1alpha/20:1(11Z))	16.71	501.26	0.04	1.52	0.61	up
L-Acetylcarnitine	6.55	184.10	0.05	1.83	0.87	up

Supplementary Table 5 List of endogenous metabolites that were significantly dysregulated between RA patients with high and low ESR levels. One-way ANOVA (Tukey's post hoc, FDR $p < 0.05$)

Compound	m/z	Retention time (min)	Accepted Compound ID	Accepted Description
0.92_766.5347 m/z	766.53	0.92	HMDB0114211	PE-NMe2(20:1(11Z)/14:1(9Z))
1.11_243.0634 m/z	243.06	1.11	HMDB0000296	Uridine
10.68_526.2543m/z	526.25	10.68	GPEtn(2:0/17:2)	GPEtn(2:0/17:2)
10.83_432.3120m/z	432.31	10.83	HMDB0000328	12-Ketodeoxycholic acid
11.05_474.1843n	519.16	11.05	121186	Asp Phe Gly His
11.07_156.5495m/z	156.55	11.07	HMDB0012144	2-Amino-4-oxo-6-(1',2',3'-trihydroxypropyl)-diquinoid-7,8-dihydroxypterin
11.35_753.3610m/z	753.36	11.35	DGDG(14:1/5:0)	DGDG(14:1/5:0)
11.75_301.2894m/z	301.29	11.75	HMDB0061714	Docosadienoate (22:2n6)
11.82_495.2126m/z	495.21	11.82	GPA(18:3/2:0)	GPA(18:3/2:0)
12.00_510.2574n	509.25	12.00	HMDB0266539	PA(2:0/18:1(12Z)-2OH(9,10))
12.41_361.2019m/z	361.20	12.41	HMDB0253262	hydroxycortisol

12.76_465.3102n	488.30	12.76	HMDB0240607	Glycohyocholic acid
13.23_428.2330m/z	428.23	13.23	HMDB0266152	PA(22:6(4Z,7Z,10Z,13E,15E,19Z)-OH(17)/22:5(7Z,10Z,13Z,16Z,19Z))
13.47_656.3411n	701.31	13.47	223473	Arg Tyr Tyr Arg
13.51_246.1576m/z	246.16	13.51	HMDB0241883	(9E,12Z)-10-Nitrooctadeca-9,12-dienoylcarnitine
13.81_775.5172n	774.51	13.81	CSID24608515	25-Amino-22-hydroxy-22-oxido-16-oxo-17,21,23-trioxa-22Î»~5~-phosphapentacosan-19-yl (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19,22-tricosaheptaenoate
14.27_321.1336m/z	321.13	14.27	HMDB0240440	2-Hydroxyphenylacetic acid glucuronide
14.27_525.2575m/z	525.26	14.27	GPA(17:2/5:0)	GPA(17:2/5:0)
14.38_648.4728m/z	648.47	14.38	CerP(d14:1/24:4)	CerP(d14:1/24:4)
14.65_470.3199m/z	470.32	14.65	HMDB0005065	Oleoylcarnitine
14.65_476.3714m/z	476.37	14.65	HMDB0240746	(11Z)-Eicoseneoylcarnitine
15.01_855.5785m/z	855.58	15.01	HMDB0270888	PG(20:4(6E,8Z,11Z,14Z)+=O(5)/a-21:0)

15.03_505.2400m/z	505.24	15.03	HMDB0010351	11-beta-Hydroxyandrosterone-3-glucuronide
15.03_981.5050m/z	981.51	15.03	HMDB0275539	PGP(i-19:0/6 keto-PGF1alpha)
15.05_842.5138m/z	842.51	15.05	HMDB0281372	PS(18:0/18:1(12Z)-2OH(9,10))
15.09_483.8112m/z	483.81	15.09	HMDB0288730	PC(24:1(15Z)/PGJ2)
15.33_482.3264m/z	482.33	15.33	HMDB0011129	LysoPE(0:0/18:0)
15.37_447.2747m/z	447.27	15.37	HMDB0276071	PI(20:3(8Z,11Z,14Z)-2OH(5,6)/16:0)
15.39_295.6625m/z	295.66	15.39	HMDB0010393	LysoPC(20:3(5Z,8Z,11Z)/0:0)
15.52_978.5863n	979.59	15.52	HMDB0277774	PI(22:6(4Z,7Z,10Z,13E,15E,19Z)-OH(17)/22:2(13Z,16Z))
15.57_311.6390m/z	311.64	15.57	GPSer(17:2/4:0)	GPSer(17:2/4:0)
15.98_408.2922m/z	408.29	15.98	HMDB0116653	PG(a-13:0/i-24:0)
16.15_1106.6715m/z	1106.67	16.15	GPA(17:2/7:0)	GPA(17:2/7:0)
16.15_614.4538m/z	614.45	16.15	HMDB0008887	PE(15:0/14:0)
16.24_409.7708m/z	409.77	16.24	HMDB0286683	PC(20:5(5Z,8Z,11Z,14Z,16E)-OH(18R)/18:3(6Z,9Z,12Z))

16.27_883.6086m/z	883.61	16.27	HMDB0270990	PG(5-iso PGF2VI/a-25:0)
16.36_832.5130m/z	832.51	16.36	HMDB0289244	PC(PGD2/DiMe(9,3))
16.63_904.7085m/z	904.71	16.63	HMDB0013423	PC(O-18:0/24:0)
16.73_799.6210m/z	799.62	16.73	HMDB0056274	DG(24:1n9/0:0/22:4n6)
17.04_341.1210m/z	341.12	17.04	HMDB0002044	8-Hydroxyguanosine
17.11_495.2494m/z	495.25	17.11	GPA(20:5/3:0)	GPA(20:5/3:0)
17.14_487.2899m/z	487.29	17.14	HMDB0296911	DG(2:0/6 keto-PGF1alpha/0:0)
18.04_1092.3335m/z	1092.33	18.04	HMDB0301384	(5Z,7S,8E,10Z,13Z,15E,17S,19Z)-7,17-dihydroxydocosa-5,8,10,13,15,19-hexaenoyl-CoA
18.75_656.4206m/z	656.42	18.75	HMDB0191592	CL(10:0/i-14:0/18:2(9Z,11Z)/18:2(9Z,11Z))
18.75_724.9568m/z	724.96	18.75	HMDB0060480	Guanosine 3'-diphosphate 5'-triphosphate
19.19_407.2846m/z	407.28	19.19	HMDB0265605	PA(18:1(12Z)-2OH(9,10)/22:0)
19.23_495.3154m/z	495.32	19.23	DGDG(18:2/22:6)	DGDG(18:2/22:6)
19.94_1162.4456m/z	1162.45	19.94	HMDB0300789	21-Methyltricosanoyl-CoA

19.99_362.2763m/z	362.28	19.99	HMDB0002038	N(6)-Methyllysine
19.99_421.7418m/z	421.74	19.99	HMDB0281098	PS(15:0/PGE1)
3.46_173.0388 m/z	173.04	3.46	HMDB0028784	Cysteinyl-Serine
3.96_365.1382 m/z	365.14	3.96	CSID35014638	6-Hydroxy-6,9-dimethyl-3-methylene-2-oxo-2,3,3a,4,5,6,9a,9b-octahydroazuleno[4,5-b]furan-5-yl (2E)-2-methyl-2-butenoate
4.07_247.1029 m/z	247.10	4.07	HMDB0242186	n2-Acetyl,n6-methyllysine
4.16_247.1307 m/z	247.13	4.16	HMDB0034564	13-Oxo-9,11-tridecadienoic acid
4.28_249.0987 m/z	249.10	4.28	HMDB0028821	Glutamylhistidine
4.47_338.1837 m/z	338.18	4.47	HMDB0029099	Tyrosyl-Arginine
4.58_474.7634 m/z	474.76	4.58	HMDB0282178	PS(6 keto-PGF1alpha/20:1(11Z))
4.61_146.0605 m/z	146.06	4.61	HMDB0250803	D-Tyrosine
5.25_130.0494 m/z	130.05	5.25	HMDB0000148	L-Glutamic acid
5.28_333.0976 m/z	333.10	5.28	HMDB0062427	6-[2,3-Dihydroxy-1-(hydroxymethyl)propyl]-1,2-dihydro-7-hydroxy-9-methoxy-

				cyclopenta[c][1]benzopyran-3,4-dione
6.88_278.1195 m/z	278.12	6.88	HMDB0266559	PA(20:4(8Z,11Z,14Z,17Z)-2OH(5S,6R)/2:0)
7.49_674.3739 n	697.36	7.49	264714	Arg Glu Lys Asp Lys
7.59_362.2353 m/z	362.24	7.59	HMDB0000303	Tryptamine
8.23_408.1349 n	431.12	8.23	HMDB0248079	(2R,3R,4R,5S,6R)-2-(4-Chloro-3-(4-ethoxybenzyl)phenyl)-6—(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol
8.44_487.2681 m/z	487.27	8.44	HMDB0261956	PE(20:1(11Z)/LTE4)
9.04_445.1961 m/z	445.20	9.04	HMDB0114771	PA(8:0/8:0)
9.05_595.3510 m/z	595.35	9.05	HMDB0011529	LysoPE(24:6(6Z,9Z,12Z,15Z,18Z,21Z)/0:0)
9.35_179.0710 m/z	179.07	9.35	HMDB0039427	2-Hydroxy-3-(4-methoxyphenyl)propanoic acid

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