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MOLECULAR PROFILING OF INFLAMMATORY ARTHRITIS

A TRANSLATIONAL EFFORT UTILIZING PROTEOMICS AND BIOINFORMATICS

BY
CHRISTOPHER ABOO

DISSERTATION SUBMITTED 2023

AALBORG UNIVERSITY
DENMARK

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by

Christopher Aboo

AALBORG UNIVERSITY
DENMARK

October 2023

Dissertation submitted:	October 2023
PhD supervisor::	Associate Prof. Allan Stensballe, Aalborg University
Assistant PhD supervisor:	Associate Prof. Søren Andreas Just, OUH Odense Universitetshospital – Svendborg Sygehus
PhD committee:	Associate Professor Pablo Pennisi (chair) Aalborg University
	Associate Professor Veit Schwämmle University of Southern Denmark
	Professor Sædis Sævrasdóttir University of Iceland
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CV – CHRISTOPHER ABOO

Education

2017-2019 MSc in Translational Medicine, Aalborg University

2014-2017 BSc in Medicine with Industrial Specialisation, Aalborg University

Experience

2022-Present Research Assistant - Translational Pain Biomarkers and Precision Medicine, Department of Health Science and Technology, Aalborg University.

2019-2022 PhD Fellow - Translational Pain Biomarkers and Precision Medicine, Department of Health Science and Technology, Aalborg University and Sino-Danish Center for Education and Research, University of Chinese Academy of Sciences.

2016-2017 Research Assistant - Research Unit at Department of Surgical Gastroenterology, The North Denmark Region.

Honors & Awards

2021 Teacher of the Year (2020), Study Board of Medicine, Aalborg University. I received this award while teaching students at the bachelor's program in Medicine and Medicine with Industrial Specialization.

Dissemination and conference activity

2022 Oral presentation, Danish Proteomics Society (DAPSOC) Symposia.

2022 Oral presentation and co-host on bioinformatics workshop, 6th Annual Danish Bioinformatics Conference.

2023 Attendance, Human Proteome Organization (HUPO) World Congress.

2023 Poster Tour Presentation, European League Against Rheumatism Congress.

2019-2023 Lecturer and case supervisor (~1300 hours excluding project supervision), and co-supervisor on various student projects including four master thesis projects.

2020-2023 Lecturer, Sino-Danish Center for Education and Research.

2022 Lecturer, The Data Science Workshop Network Analyses and Data Fusion, University of Copenhagen

Papers included in this thesis:

Paper 1: Prediction and early diagnosis of immune-checkpoint inhibitor-induced inflammatory arthritis from molecular biomarkers – Where are we now?. Aboo, C., Krastrup, T. W., Tenstad, H. B., Ren, J., Just, S. A., Ladekarl, M. & Stensballe, A., 22 dec. 2022, I: Expert Review of Precision Medicine and Drug Development, 7:1, 162-168, DOI: 10.1080/23808993.2022.2156785.

Paper 2 (Submitted): Synovial Tissue Proteomics Unravels Pathological Trajectories in Rheumatoid Arthritis and Identifies Determinants of Synovial Heterogeneity. **Aboo, C.**[*], Just, S. A.[*], Nielsen, C., Schrøder, H. D., Andersen J. S., Thomsen, M. E., Déjean, S., Bennike, T. B., Lindegaard, H. & Stensballe, A. [*]Shared first authorship.
- This paper is collection of two separate manuscripts/sub-studies that were combined into one due to publication strategy.
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Impaired Abcb1a function and red meat in a translational colitis mouse model induces inflammation and alters microbiota composition. Stensballe, A., Bennike, T. B., Ravn-Haren, G., Mortensen, A., **Aboo, C.**, Knudsen, L. A., Rühlemann, M. C., Birkelund, S., Bang, C., Franke, A., Vogel, U., Hansen, A. K. & Andersen, V., 31 Jul. 2023, I: Frontiers in Medicine. 10, 1200317.

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Global proteomics profiling of serum and synovial fluid identifies biomarkers associated with improved PainDetect scores after intraarticular gold for management of painful knee osteoarthritis. Rasmussen, S., Petersen, K. K-S., **Aboo, C.**, Andersen, J. S., Skjoldemose, E., Jørgensen, N. K., Arendt-Nielsen, L. & Stensballe, A., Sep. 2022. Presented at IASP 2022 World Congress on Pain - Toronto, Canada.

Global proteomics profiling of serum and synovial fluid identifies biomarkers of outcome after intraarticular gold for management of painful knee osteoarthritis. Rasmussen, S., Petersen, K. K-S., **Aboo, C.**, Andersen, J. S., Skjoldemose, E.,

Jørgensen, N. K., Arendt-Nielsen, L. & Stensballe, A., Sep. 2022. Presented at IASP 2022 World Congress on Pain - Toronto, Canada.

Gold micro-particles for knee osteoarthritis. Rasmussen, S., Petersen, K. K., Kaae Kristiansen, M., Andersen, J. S., **Aboo, C.**, Thomsen, M. E., Skjoldemose, E., Jørgensen, N. K., Stensballe, A. & Arendt-Nielsen, L., 2022. Presented at EFIC2022 European Pain Federation - Dublin, Irland.

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Proteomic Insights into C3dg as Biomarker of Systemic Lupus Erythematosus. Trolborg, AM., Poulsen, TBG. Andersen, JS., Thomsen, ME., **Aboo, C.** & Stensballe, A. Presented at the 1st International Electronic Conference on Biomedicine, March 2021; Available online: https://ecb2021.sciforum.net.

ENGLISH SUMMARY

This PhD thesis explores the molecular landscape of rheumatoid arthritis (RA), with a focus on its translational implications for immune-checkpoint inhibitor induced inflammatory arthritis (ICI-IIA). The relationship between adverse events of cancer treatment and RA might seem counterintuitive at first thought. ICIs work by removing the "brakes" of the immune system and produces an anti-tumour effect. However, such an overactive immune system can mistakenly attack healthy tissue, resulting in ICI-IIA. RA is likewise considered to be the result of an overactive immune system that primarily attacks the synovial joints. Thus, ICI-IIA and RA could possibly share underlying immunological mechanisms, and studying this could provide valuable insights into both conditions. For instance, by employing proteomics to study elucidate the mechanisms of RA, this knowledge can be translated into an ICI-IIA context, and vice versa, by employing proteomics to elucidate the mechanisms of ICI-IIA, this knowledge can be translated into an RA context.

The thesis starts with an introduction to ICI-IIA and RA, followed by an introduction to the employed methods. These methods include proteomics, the large-scale study of proteins, and statistical tools that facilitate the interpretation of the complex proteomics data. The thesis then highlights the findings of four sub-studies, contained in two papers and one unpublished study, where these methods are central.

Paper 1 is a non-systematic review that sought to review studies aiming to identify blood biomarkers for early diagnosis and prediction of ICI-IIA (1). A lack of substantial research in this field and an apparent lack of reliable diagnostic criteria for ICI-IIA became evident. This, in turn, led to unreliable clinical endpoints within these studies. Recognizing these limitations, we proposed a set of recommendations to facilitate and guide future research on biomarker discovery for ICI-IIA. Specifically, we discussed the use of different diagnostic approaches to define robust clinical endpoints and discussed the potential of employing omics technologies for biomarker discovery in ICI-IIA.

Paper 2 lays the foundation for the anticipated future research on ICI-IIA, and secondly, it aims to answer fundamental questions about RA in two sub-studies. The study employed proteomics to study RA, yielding a comprehensive understanding of the pathological mechanisms occurring locally and systematically at different disease stages. Specifically, it answers some questions that are fundamental to our understanding of inflammatory arthritis in RA such as: *What biological pathways are dysregulated in early untreated RA and what happens following treatment initiation? What biological pathways are dysregulated in longstanding RA and what happens following treatment intensification? What are the molecular differences between early RA and longstanding RA? What are the cellular and molecular determinants of*

synovial heterogeneity? And can determinants of synovial heterogeneity predict treatment outcomes?

The thesis concludes with an unpublished study that aimed to answer how proteins covary with different measures of disease activity following treatment initiation or intensification. This investigation identified five plasma proteins that have the potential to serve as a biomarker signature of disease activity in RA.

Cumulatively, this thesis expands our understanding of the fundamental pathological mechanisms in RA, informs future translational research strategies for both ICI-IIA and RA, and paves the way for more personalized and effective treatments for these disabling conditions.

DANSK RESUME

Denne Ph.d.-afhandling udforsker det molekylære landskab af reumatoid artrit (RA) med fokus på dets translationelle implikationer for immun checkpoint inhibitor (ICI)-induceret inflammatorisk artrit (ICI-IIA). Sammenhængen mellem bivirkninger af kræftbehandling og RA kan virke lidt ulogisk ved første tanke. ICI'er virker ved at "fjerne bremserne" i immunforsvaret hvilket frembringer en anti-tumor effekt. Imidlertid kan sådan et overaktivt immunsystem også angribe raskt væv, hvilket resulterer i ICI-IIA. RA betragtes ligeledes som værende et resultat af et overaktivt immunsystem, der angriber de synoviale led. Således kan ICI-IIA og RA muligvis dele underliggende immunologiske mekanismer, og undersøgelser af dette kunne give værdifulde indblik i begge tilstande. Ved at bruge proteomik til at studere RA, kan denne viden for eksempel oversættes til en ICI-IIA-kontekst, og omvendt, ved at bruge proteomik til at belyse ICI-IIA-mekanismer, kan denne viden muligvis oversættes til en RA kontekst.

Ph.d.-afhandlingen begynder med en introduktion til ICI-IIA og RA, efterfulgt af en introduktion de anvendte metoder. Disse metoder inkluderer proteomik, som er den storskalaede undersøgelse af proteiner, og statistiske metoder der faciliterer fortolkningen af disse komplekse proteomik data. Ph.d.-afhandlingen fremhæver derefter fundene fra fire delstudier, fordelt over to artikler og et upubliceret studie, hvor disse metoder er centrale.

Artikel 1 er en ikke-systematisk gennemgang af litteraturen, der gennemgår studier som havde til formål at identificere blod-baserede biomarkører til tidlig diagnose og forudsigelse af ICI-IIA (1). Der var dog et klart mangelfuldt forskningsgrundlag på dette område og en tilsyneladende mangel på pålidelige diagnostiske kriterier for ICI-IIA. Dette medførte til upålidelige kliniske endepunkter inden for disse studier. Som en anerkendelse af disse begrænsninger foreslog vi en række anbefalinger til at faciliterer og vejlede fremtidig forskning der har til formål at identificere ICI-IIA biomarkører. Vi diskuterede specifikt brugen af forskellige diagnostiske fremgangsmåder til at definere robuste kliniske endepunkter, og drøftede dernæst potentialet af at anvende omics-teknologier til identifikation af ICI-IIA biomarkører.

Artikel 2 ligger grundlaget for den forventede fremtidig forskning indenfor ICI-IIA og søger, i to delstudier, at besvare spørgsmål der er fundamentale for vores forståelse af RA. Studiet anvendte proteomik til at undersøge RA, hvilket resulterede i en omfattende belysning af de patologiske mekanismer, der finder sted lokalt i de synoviale led og systemisk på forskellige sygdomsstadier. Specifikt besvarer studiet spørgsmål, der er fundamentale for vores forståelse af inflammatorisk artrit, såsom: Hvilke biologiske processer er dysreguleret i tidlig ubehandlet RA, og hvad sker der efter initiering af behandlingen? Hvilke biologiske processer er dysreguleret i langvarig RA, og hvad sker der efter intensivering af behandlingen? Hvad er de

molekylære forskelle mellem tidlig RA og langvarig RA? Hvilke cellulære og molekylære forskelle ligger til grund for synovial heterogenitet? Og kan disse forskelle forudsige behandlingsresultatet?

Ph.d.-afhandlingen afsluttes med et upubliceret studie, der søger at besvare, hvordan proteiner kovarierer med forskellige mål for sygdomsaktivitet efter initiering eller intensivering af behandling. Studiet identificerede frem plasma proteiner, der har potentialet til at blive brugt som en biomarkør-signatur for sygdomsaktivitet i RA.

Samlet set bidrager denne afhandling til vores forståelse af de fundamentale patologiske mekanismer i RA, informerer fremtidige translationelle forskningsstrategier indenfor både ICI-IIA og RA, og baner, på sigt, vejen for mere effektive behandlinger, der er skræddersyede til hver enkelt patient, som er påvirket af disse invaliderede tilstande

PREFACE AND ACKNOWLEDGEMENTS

The journey towards completing this PhD has been full of surprises and learningful experiences. The project started in December 2019 with the initial aim to study pathological mechanisms underlying chronic chemotherapy-induced peripheral neuropathy. However, it was difficult to establish a cohort and obtain biological samples. This challenge, together with the long follow-up periods - that it takes to develop chronic chemotherapy-induced peripheral neuropathy - made the initial PhD project timewise unfeasible to complete. Thus, 13 months into my PhD study I had to rethink the whole PhD project together with my main supervisor. Following several discussions, the focus was shifted towards immune checkpoint inhibitor-induced inflammatory arthritis (ICI-IIA), a type of rheumatic immune-related adverse event, that occurs in some cancer patients following treatment with immune checkpoint inhibitors. While studying ICI-IIA, it became apparent that that this condition is very poorly understood. However, since a suitable ICI-IIA cohort was not available to study its proteomic complexities, it seemed logical to lay a foundation and understand the proteomic landscape of rheumatoid arthritis (RA) first. The association between adverse events of cancer treatment and RA might seem abstract at first thought. However, RA is also characterized by inflammatory arthritis, and patients with ICI-IIA often present with RA-like symptoms. Applying the insights I gained from my master's in Translational Medicine, I sought to apply the core principles this discipline. My aim was to gain insights into RA that also can also help us understand ICI-IIA. Likewise, understanding ICI-IIA might offer valuable insights into RA in the future. For instance, if inflammatory arthritis in ICI-IIA turns out to be molecularly similar to that in RA, then cancer patients without a prior history of inflammatory arthritis could help us understand why RA develops. Thus, my PhD project has evolved into an exploratory project, aiming to understand the pathological mechanisms in RA and ICI-IIA and contributing to the growing body of knowledge on these fields. I hope that this will ultimately pave the way for better treatments of these two complex conditions and make a difference for the affected patients.

First of all, I would like to thank Professor and MD Ursula Gerda Inge Falkmer, Professor and MD Marianne Tang Severinsen, Professor Karen Dybkær, Associate Professor and MD Laurids Østergaard Poulsen, PhD Hanne Due Rasmussen, Professor and MD Niels Ejskjær and MD Eva Futtrup Maksten from Aalborg University Hospital, and Associate Professor Carsten Dahl Mørch from Aalborg University for your efforts in establishing a cohort of cancer patients to study chemotherapy-induced peripheral neuropathy. The journey took a different path, but the experience and knowledge gained from collaborating with you have been very enriching. Your dedication and hard work are sincerely appreciated!

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I would like to thank Sébastien Déjean from the Institute of Mathematics of Toulouse for hosting me during my research exchange in France. Your mentorship has been truly invaluable. It has indeed offered me a glimpse into the mind of statisticians, and truly improved my scientific work. Also, thank you for introducing me to French culture and French food, and for welcoming me into your home and introducing me to your family. You made feel very welcome! I would also like to thank Mitja Briscik who became a good friend but also enriched my understanding of statistics. You have truly enriched my time in Toulouse, and I am looking forward to welcoming you to Denmark.

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ABBREVIATIONS

2-DE	Two-dimensional Gel Electrophoresis
ACPA	Anti-citrullinated Protein Antibodies
AS	Ankylosing Spondylitis
bDMARDs	Biologic Disease-modifying Antirheumatic Drugs
CRP	C-reactive Protein
csDMARDs	Conventional Disease-modifying Antirheumatic Drugs
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
DAS28	Disease Activity Score in 28 joints
DAS28CRP	Disease Activity Score in 28 joints + C-reactive Protein
DDA	Data Dependent Acquisition
DIA	Data Independent Acquisition
DIABLO	Data Integration Analysis for Biomarker Discovery using Latent Components
DMARDs	Disease-modifying Antirheumatic Drugs
ECM	Extracellular Matrix
ERA	Early Rheumatoid Arthritis
EULAR	European League Against Rheumatism
FDR	False Discovery Rate
HC	Healthy Controls
HLA	Human Leukocyte Antigen
ICI-IIA	Immune-checkpoint Inhibitor-Induced Inflammatory Arthritis
ICIs	Immune-checkpoint Inhibitors
IL6Ri	IL-6 Receptor Inhibitors
irAEs	Immune-related Adverse Events
LC	Liquid Chromatography
LC-MS	Liquid Chromatography - Mass Spectrometry
LFQ	Label-free Quantification
LMM	Linear Mixed Model
LRA	Longstanding Rheumatoid Arthritis
MALDI	Matrix-assisted Laser Desorption/Ionization
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry

MS1	Mass-to-charge Ratio of Precursor Ions
MS2	Mass-to-charge Ratio of Fragment Ions
MTX	Methotrexate
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
OA	Osteoarthritis
OMERACT	Outcome Measures in Rheumatology
PASEF	Parallel Accumulation - Serial Fragmentation
PCA	Principal Component Analysis
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death Ligand 1
PLS	Partial Least Squares
PLS-DA	Partial Least Squares Discriminant Analysis
RA	Rheumatoid Arthritis
RAMRIS	Rheumatoid Arthritis Magnetic Resonance Imaging Scoring System
RF	Rheumatoid Factor
TIMS	Trapped Ion Mobility Spectrometry
TMT	Tandem Mass Tag
TNFi	TNF Inhibitors
UGSB	Ultrasound-Guided Synovial Biopsy

CHAPTER 1. INTRODUCTION

1.1. IMMUNE-CHECKPOINT INHIBITOR-INDUCED INFLAMMATORY ARTHRITIS

Immune-checkpoint inhibitors (ICIs) have improved the field of cancer treatment and become an integral part of standard therapy for various malignancies, especially for patients with advanced stage cancer (2–5). These drugs work by targeting and blocking programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) that are key immune regulatory checkpoints (6). By targeting such immune checkpoints, ICIs blocks the inhibitory pathways that normally limit T cell activation, and thereby produces an anti-tumour immune response (6). Unfortunately, this potentiation of immune responses may also lead to the development of various autoimmune and autoinflammatory conditions known as immune-related adverse events (irAEs) (1,7,8). Most of these irAEs are transient, but some of the rheumatic irAEs may become chronic (9). One such rheumatic irAE is immune-checkpoint inhibitor-induced inflammatory arthritis (ICI-IIA), a newly recognized condition with relatively unknown aetiology and pathophysiology, that poses a great challenge to oncologist and rheumatologists (9–13).

1.1.1. PREVALENCE AND RISK FACTORS

The prevalence of ICI-IIA varies greatly among studies, ranging from 1% to 7-8% of patients undergoing ICI therapy, with half of these cases presenting as rheumatoid arthritis (RA)-like inflammatory arthritis (1,9,11,14–17). However, as stated in Aboo et al. (2022) these number may underestimate the true incidence, because of under-recognition and under-reporting of ICI-IIA due to the non-specific presentation, immature diagnostic criteria and overlapping symptoms with arthralgia and/or myalgia (1,9,18). The reported incidence of ICI-IIA tends to be higher in patients receiving PD-1 inhibitors as mono- or combination therapy (18), and the clinical presentation may also differ depending on type of therapy (19). In a study focusing on patients who developed ICI-IIA, Braaten et al. (2020) found that age, gender, family history of autoimmune disease and C-reactive protein (CRP) levels, did not increase the risk of developing persisting ICI-IIA (13). However, the duration of ICI treatment and combination therapy did increase the risk of persistence (13). Genetics may also contribute to the risk of developing ICI-IIA (20,21). Specifically, a study by Cappelli et al. (2019) suggested that the frequency of a specific human leukocyte antigen (HLA) DRB1 allele was higher in patients who developed ICI-IIA compared to healthy controls (21). However, more genetic studies are indeed needed to confirm this association.

1.1.2. PATHOLOGY

The pathogenesis of ICI-IIA remain poorly understood, but possible mechanisms have been proposed as to why (rheumatic) irAEs might occur (Figure 1) (9,22,23). These mechanisms include an increase of proinflammatory cytokines leading to heightened systemic inflammation, an increase in levels of pre-existing autoantibodies that unmasks pre-symptomatic autoimmune disease, and the binding of CTLA-4 inhibitors (Anti-CTLA-4 antibodies) to CTLA-4 expressed in healthy tissue that causes complement activation (9,22,23). Additionally, off-targets effects of T cell immunity caused by epitope spreading or cross reactivity between tumour antigens and synovial antigens may also be involved (9,22,23). A notable example of this is observed in melanoma patients treated with ICIs (24). Some of these patients may develop vitiligo, likely because the melanoma cells and healthy melanocytes express similar antigens (24). Generally, there is a lack of histological studies that have investigated the local synovial molecular and cellular mechanisms underlying ICI-IIA, with most of existing studies being case reports (25,26). These case reports will be presented in the discussion and are not elaborated further on here.

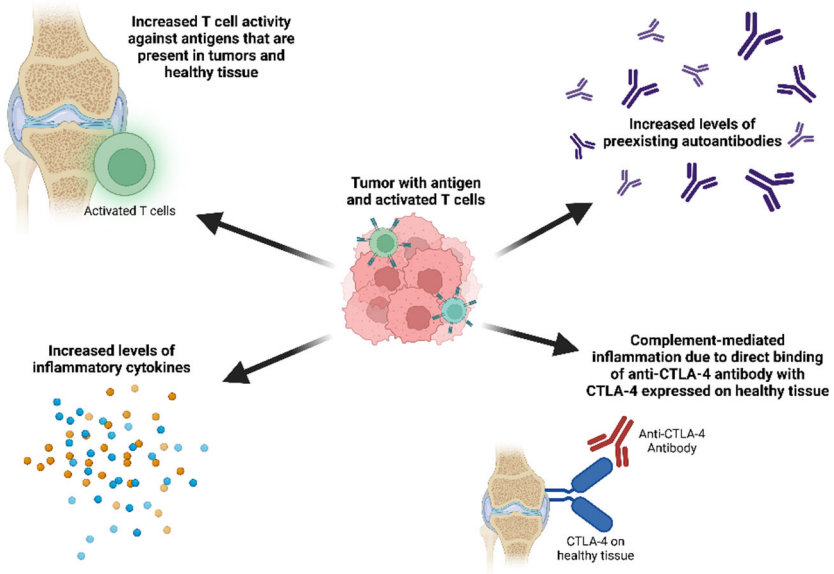

Figure 1. Proposed mechanisms as to why rheumatoid immune-related adverse events occur. Reproduced with permission from Postow et al. (2018) (8), Copyright Massachusetts Medical Society. Created with Biorender.com.

1.1.3. TREATMENT

The management of ICI-IIA is a multidisciplinary entity that should involve both oncologists and rheumatologists (1,12,17,27). Treatment strategies are generally based on the severity of ICI-IIA and may initially include Nonsteroidal anti-inflammatory drugs (NSAIDs) or analgesics for mild ICI-IIA (12,17,27). If NSAIDs are insufficient, or in cases of moderate to severe ICI-IIA, oral or intraarticular glucocorticoids may be considered (12,17,27). In addition, disease-modifying antirheumatic drugs (DMARDs) may also be considered in cases of refractory ICI-IIA that cannot be managed with NSAIDs and glucocorticoids, or when we want to minimize the long-term adverse effects of glucocorticoids (12,17,27). Examples of conventional DMARDs (csDMARDs) that can be used to manage ICI-IIA include Methotrexate (MTX), hydroxychloroquine and sulfasalazine (12,17,27). In cases where csDMARDs are ineffective or not well-tolerated, some biologic DMARDs (bDMARDs) may also be considered (17,26,27). Examples of these include IL-6 receptor inhibitors (IL6Ri) such as tocilizumab, and TNF inhibitors (TNFi) such as infliximab (17,26–29).

A very important aspect of treating ICI-IIA involves navigating through challenges arising from potential interactions between immunomodulatory therapies and ICI therapies (12,17,27,28). This is because immunosuppressive agents might disrupt the immune activation induced by ICI therapy, potentially reducing the anti-tumour efficacy of ICIs (12,17,27). The extent to which these interactions might affect treatment outcomes remains a subject of ongoing investigation (12,17,27). Back when Aboo et al. (2022) was published, there were no comprehensive comparative studies on the safety and effectiveness of DMARDs in ICI-IIA (1). However, Bass et al. (2023) recently explored the trade-off between rapid arthritis control and cancer progression risk in the treatment of ICI-IIA (30). The (retrospective) study included 147 patients treated with TNFi, IL6Ri, or MTX (30). Results underscored that TNFi and IL6Ri facilitated faster ICI-IIA control, but at the expense of faster cancer progression (30). Conversely, MTX demonstrated slower ICI-IIA control, however, with lesser interference in cancer progression (30)**.** However, there is indeed an unmet demand for larger prospective randomized studies on how different treatments may affect survival in ICI-IIA patients. Until more evidence is established to support clinical decision making, achieving a balance between managing ICI-IIA effectively and maintaining the therapeutic efficacy of ICI therapy presents a very complex clinical challenge (12,17,27). It is crucial to assess the potential risks and benefits of using immunosuppressive medications on an individual basis, considering factors like tumour type, cancer stage, ICI therapy and ICI-IIA severity, a task that requires collaboration between oncologists, rheumatologists and patients (12,17,27).

1.2. RHEUMATOID ARTHRITIS

"RA is a chronic systemic autoimmune disease that predominantly affects the synovial joints" – Cited from Debreova et al. (2022) (31–36) (Aboo et al., unpublished – Paper 2). The disease is marked by ongoing joint inflammation, which causes ongoing degradation of cartilage and bone of synovial joints, ultimately leading to significant functional impairment and decreased well-being (31,32).

1.2.1. PREVALENCE AND RISK FACTORS

RA affects approximately 0.5% of the world's population, with a slightly higher prevalence of approximately 1% in highly developed countries (32,37,38). RA is also more prevalent among women, with the female-to-male ratio being approximately 2:1 (32,38,39). Lifestyle and environmental factors, such as heavy cigarette smoking, excessive weight, infections and dust exposure are thought to play a role in the onset of RA (32,38,40–44). Likewise, genetics are also thought to play a role in RA susceptibility (45). Some of the strongest associated genes to RA are primarily immune-related genes such as HLA-DRB1 (46), STAT4 (47), TRAF1/C5 (48), AIRE (49), CTLA4 (50), CD40 (51), and genes involved in citrullination such as PTPN22 (52). The precise trigger of RA is difficult to pinpoint, but it is very likely to be a combination of both genetic and environmental factors (53). Tang et al. (2023), for instance, reported that exposure to occupational inhalable agents was associated with increased risk of anti-citrullinated protein antibodies (ACPA)-positive RA (54). That risk became even higher as the number and duration of exposure to these agents increased (54). However, the risk increased dramatically in patients who were genetically predisposed, smoked, and were exposed to inhalable agents simultaneously (54).

1.2.2. PATHOLOGY

The development of RA and the subsequent progression is thought to involve a complex series of interactions between immune cells, synovial fibroblasts, cytokines, and other molecular components (Figure 2) (31–33,55). There is a general belief that the process begins with citrullination of proteins, a critical process in RA pathogenesis that refers to a post-translational modification that transforms arginine residues in proteins into citrulline (31–33,55). This modification causes proteins to become self-antigens that are perceived as foreign by the immune system, leading to the production of autoantibodies (31–33,55,56). The crucial step from generation of self-antigens to production of autoantibodies involves immune cell activation of T cells, B cells, dendritic cells, and plasma cells (31–33,55). Antigen loading and migration takes place in lymph nodes, where dendritic cells present antigens to T cells via major histocompatibility complex molecules (31–33,55,57). T cell activation then occurs through the T cell receptor, with a subsequent release of cytokines that activates B cells and causes autoantibody production (31–33,55). The autoantibodies can then

bind to the self-antigens and generate immune complexes that contribute to inflammation and joint damage (31–33,55). The immune complexes can also result in complement activation that further amplify the inflammatory response and recruit additional immune cells (31–33,55). Synovial fibroblasts and macrophages have also been long implicated in RA pathogenesis (58). They can release matrix metalloproteases that are extracellular matrix (ECM) degrading enzymes and secrete pro-inflammatory cytokines, including TNFα, IL-1 and IL-6, that can intensify joint inflammation, and stimulate osteoclast differentiation and activation, resulting in subsequent bone erosion and joint destruction (31–33,55,58–62).

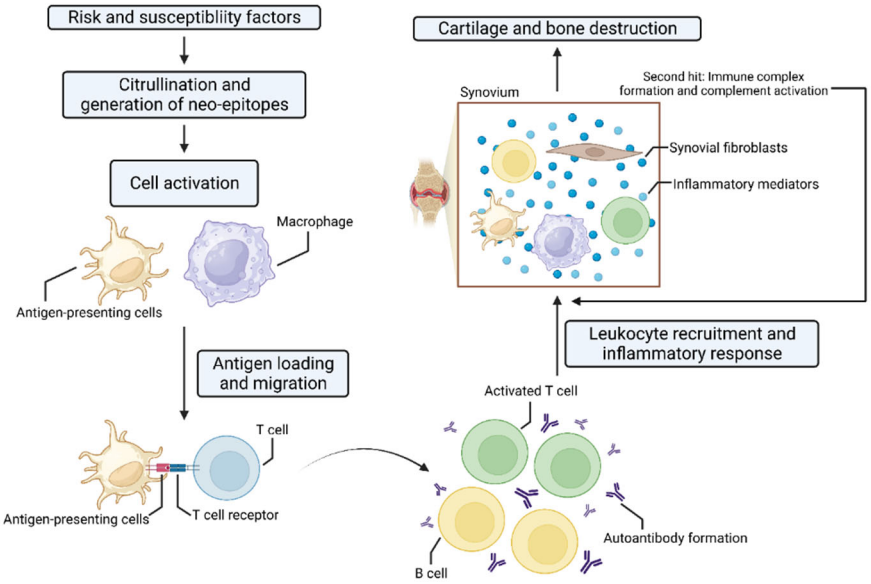

Figure 2. Onset and progression mechanisms of rheumatoid arthritis (32). Citrullination of proteins may initiate the onset of RA by creating altered proteins that provoke an immune response (32). Subsequent persistent inflammation and immune activation lead to disease progression and joint damage (32). Reproduced with permission from Smolen et al. (2018) (32), Springer Nature. Created with Biorender.com.

The synovial lining, which encapsulates the joint space, is characterized by immune-infiltration and pannus formation, a dense layer of synovial tissue primarily containing synovial fibroblasts and macrophages, that infiltrates and erodes both cartilage and bone (31,32,60,63). Additionally, angiogenesis (formation of new blood vessels) also occurs in the synovium and promote the delivery of nutrients and immune cells to the affected joints (31,32,59,64–66).

In conclusion, the current understanding of RA pathology encompasses an extremely complex interplay of various cellular and molecular mechanisms, and this section have barely scratched the surface. However, further advancement in our understanding of these pathophysiological processes is of vital importance to development more effective treatment strategies for RA patients, such as personalized medicine approaches, which could significantly improve the management of this debilitating disease.

1.2.3. TREATMENT OF RA

As of today, RA not a curable disease, so primary goal of the treatment is to achieve remission with the aim of alleviating pain and preventing joint damage (67). csDMARDs, such as MTX is often used as first-line therapy, either alone or in combination with other csDMARDs including leflunomide, sulfasalazine (68). bDMARDs can also be used in patients that do not respond adequately to csDMARDs, or if patients do not tolerate the csDMARDs (68). These bDMARDs include TNFi (Adalimumab and Etanercept), IL6Ri (Tocilizumab and Sarilumab), IL-17 inhibitors (Secukinumab and Bimekizumab), IL-1 inhibitors (Anakinra), CTLA-4 fusion protein (Abatacept), and Anti-CD20 monoclonal antibody (Rituximab) (69,70). In addition, targeted synthetic DMARDs can also be used as an alternative to the bDMARDs (70). These primarily include Janus Kinase inhibitors such as Upadacitinib (70).

The treatment of RA follows the treat-to-target approach that *"encompasses several distinct elements: choosing a target and a method for measuring it; assessing the target at a pre-specified time point; a commitment to change the therapy if the target is not achieved; and shared decision-making."* - Cited from Ronald van Vollenhoven (2019) (71). Thus, treatment strategies using various combinations of the abovementioned DMARDs are cumulatively revised based on 1) disease severity and 2) treatment outcomes including treatment efficacy and adverse events, with the aim of reaching the "target" that is remission or low disease activity (71,72).

1.2.4. MONITORING DISEASE ACTIVITY IN RA

Monitoring disease activity is essential for evaluating treatment response and adjusting the (treat-to-target) therapeutic strategies (73,74). The following sections will present various methods to assess disease activity, including blood biomarkers, composite scores, ultrasound, and magnetic resonance imaging (MRI).

1.2.4.1 Blood biomarkers

CRP and erythrocyte sedimentation rate (ESR) are commonly used blood biomarkers that reflect degree of inflammation in RA (75–77). Elevated levels of CRP and ESR are associated with higher disease activity and consequently more joint damage (76,78). *"CRP is an acute-phase protein that is synthesized by the liver in response*

to inflammation" – Cited from Liu et al. (2023) (79–81). ESR on other hand, *"measures the settlement rate of the red blood cells in a test tube"* – Cited from Passos et al. (2022) (82–85). A high degree of inflammation, as observed in RA (26,32), causes red blood cells to aggregate more, which makes them sediment faster and thereby increasing the ESR (82).

Rheumatoid factor (RF) and ACPAs on the other hand, are not measures of disease activity but diagnostic biomarkers (86–88). *"RF is an autoantibody that targets the Fc portion of immunoglobulin G*" antibodies – Cited from Yap et al. (2018) (87,89,90), and ACPAs are also autoantibodies that target citrullinated proteins present in the synovial joints (91–93). Patients who test positive for these autoantibodies (i.e., seropositive RA), generally experience a more severe disease and increased radiographic progression compared to those patients that are seronegative (94–96). Thus, although RF and ACPA are not measures of disease activity, they are still be put into use in the clinical decision making because they have a prognostic value (68).

1.2.4.2 Disease Activity Score in 28 joints

The Disease Activity Score in 28 joints (DAS28) is a measure that uses clinical parameters to assess disease activity (97). Specifically, it combines the number of tender joints and number of swollen joints out of 28 joints and the patient's global healthy (97,98). DAS28 is often used in combination with plasma CRP levels (DAS28CRP), but it can also be used in combination with the ESR (98,99). *"A DAS28CRP score of less than 2.6 indicates remission, a score of 2.6 to 3.2 indicates low disease activity, a score of 3.2 to 5.1 indicates moderate disease activity, and a score greater than 5.1 indicates high disease activity"* (100–103)[*]. The relatively simple nature of DAS28CRP has made it a valuable, widely implemented, and preferred measure of disease activity in both clinical and research settings (104). However, DAS28CRP is not a perfect measure of disease activity and should, if possible, be used in combination with other clinical assessments. Orr et al. (2018), for instance, found that 71% of patients in DAS28CRP-defined remission had evidence of synovial inflammation, and many patients with no so signs of synovial inflammation had a high DAS28CRP score (105). Furthermore, DAS28CRP does not consider other important aspects such as extra-articular manifestations, that are common in RA patients (106).

1.2.4.3 Ultrasound imaging

Ultrasound is a non-invasive imaging technique that is often utilized to detect and assess synovitis (i.e., synovial inflammation) (107). It does so by measuring the thickness of the synovial lining and degree of effusion in greyscale mode and

[*] This sentence reflects a standardized definition/terminology/nomenclature in the field.

measuring active inflammation by visualising the synovial vascularity in power Doppler mode (107,108). One of the widely recognized standardized scoring system for ultrasound assessment in RA is the European League Against Rheumatism (EULAR) Outcome Measures in Rheumatology (OMERACT) ultrasound score (107). This scoring system evaluates the severity of synovitis in assessed joints by combining the scores of synovial hypertrophy/effusions (from 0 to 3) and the scores of power Doppler activity (from 0 to 3) into a semi-quantitative score of synovitis that likewise ranges from 0 to 3 with 0 indicating a normal joint and 3 indicating severe synovitis (107). Overall, the EULAR-OMERACT ultrasound score is sensitive and responsive to change, which has made it a valuable tool for detecting subclinical synovitis (109) and assessing disease activity/severity (and consequently, for monitoring treatment response) (108,110) . Nonetheless, ultrasound scores may be prone to intraobserver and interobserver variability (107,111,112). However, automated ultrasound solutions, like ARTHUR by ROPCA (Odense, Denmark), have been developed to address these limitations by standardizing the acquisition of ultrasound images (111,113,114). This solution uses artificial intelligence to scan the hand joints and capture ultrasound images automatically, thereby providing a faster and more standardized measure of synovitis (111,113,114). Such automated ultrasound solutions have significant clinical implications as they can inform clinical decision making more accurately (by reducing the variability associated with manual assessments) and more frequently (by saving time and making ultrasound more accessible in the routine clinical assessments) (111,113,114)

1.2.4.4 Magnetic resonance imaging

MRI is an advanced imaging technology that offers exceptional visualization of joint structures such as bone, cartilage, and connective tissue (115,116). MRI can detect early abnormalities that are not visible using other radiological techniques, making it particularly useful when other radiological techniques are inconclusive (115,117). The EULAR-OMERACT Rheumatoid Arthritis Magnetic Resonance Imaging scoring system (RAMRIS) is a validated used scoring system to assess synovitis, bone erosions and bone marrow oedema based on MRI (115,118–122). More specifically, RAMRIS scores 1) the degree of synovitis in three wrist regions and the metacarpophalangeal joints from 0 to 3 with 0 being normal and 3 indicating severe synovitis, 2) the degree of bone erosions in the wrist joints and metacarpophalangeal joints from 0 to 10, with 0 indicating 0% erosion and 10 indicating 90-100% bone eroded, and 3) the degree of bone marrow oedema/osteitis in the same joints with 0 indicating no oedema, and 3 indicating oedema in 67-100% of the bone (115,118,121,122). MRI is indeed powerful tool for assessing synovial joints in RA (116,117), and is even commonly used as a standard for validating the accuracy of ultrasound measures (123). However, it does have many major disadvantages. For instance, it is much more expensive and time-consuming than other imaging modalities, and it requires an experienced radiologist to interpret the results, which cumulatively makes it much less available in clinical settings (116,117,123,124).

Furthermore, seen from a patient perspective, MRI may be more unpleasant compared to ultrasound and X-ray, and may require contrast agents (116,117,123).

1.3. SYNOVIAL TISSUE BIOPSIES

Liquid biopsies such as plasma and synovial fluid are minimally invasive and easy to collect in outpatient settings and have both been extensively used to study RA (125,126). However, while RA is indeed a systemic disease, it primarily affects synovial joints (32,125,126). For the same reason, synovial tissue biopsies are highly relevant in RA research, and perhaps also in clinical settings where they might be used aid diagnosis, predict treatment responses, and disease progression (125–132).

1.3.1. ULTRASOUND-GUIDED SYNOVIAL TISSUE BIOPSIES

As briefly described in Paper 2, the collection and availability of synovial tissue biopsies have been historically challenging, and the generalizability of proteomics studies has been limited to late-stage disease (Aboo et al., unpublished – Paper 2). This is because the tissue biopsies were often collected post-mortem, following traumas, or during joint replacement surgery with the concurrent presence of osteoarthritis (OA) (Aboo et al., unpublished – Paper 2) (133–140). Although being a fairly old technique (141,142), only recent validation studies of the ultrasound-guided synovial biopsy (UGSB) technique have renewed the relevance of synovial tissue biopsies by addressing many of these challenges (143,144). Consequently, UGSB has become a promising tool for advancing our understanding of the pathological mechanisms in RA, but also for the management (125,126,145). As the name implies, UGSB uses ultrasound guidance to collect the synovial tissue, offering some major advantages compared to gold standard, arthroscopic guidance (142–144). For instance, it enables the collected of synovial tissue biopsies from smaller joints, which are more relevant than larger joints in an RA context (126,142,143). Furthermore, the procedure is minimally invasive, well-tolerated, and can be performed in outpatient settings (143,146,147). Thus, UGSB has now facilitated the collection of synovial tissue from RA patients at various disease stages (such as prior to treatment initiation in newly diagnosed patients) and from smaller joints (130–132,148,149).

1.3.2. ADVANCES IN RA FACILITATED BY UGSB-DRIVEN RESEARCH.

Dennis et al. (2014) identified four distinctive synovial phenotypes within the synovial tissue of RA patients: lymphoid, myeloid, low inflammatory, and fibroid (150). The study also found that patients with a prominent baseline myeloid gene signature were more responsive to TNFi therapy (150). Although this research was based on synovial tissue that was collected during arthroplasty or synovectomy from patients with more than three years disease duration, it laid the foundation for a new concept of synovial phenotypes (which later became known as pathotypes) (130,132,150). This concept of synovial pathotypes gained more attention shortly

after validation of the UGSB technique (143), which revolutionized the way researchers could access tissue samples from RA patients at different disease stages, thus expanding the extent of possible studies such as Humby et al. (2019) and Lewis et al. (2019) (130,132). Building on these advancements, Humby et al. (2019), identified the presence of distinct synovial pathotypes in early RA patients that were treatment naïve (132). Specifically, by employing histology in combination with transcriptomics, the study identified three distinct synovial pathotypes: a pauci-immune fibroid, a diffuse-myeloid and a lympho-myeloid pathotype (132). They also found positive associations between the expression of myeloid and lymphoid pathotype-associated gene signatures and 1) the disease activity, 2) levels of acute phase reactants and 3) and the response to csDMARDs therapy (132). Additionally, they found that a higher expression of lymphoid-associated gene signatures was associated with seropositivity and a higher risk of bone erosion at 1-year follow-up, and vice versa that patients with a pauci-immune fibroid phenotype had less risk of bone erosion at 1-year follow-up (132). Similarly, Lewis et al. (2019) identified distinct transcriptional signatures for each of the RA pathotypes (130). They found positive associations between myeloid-associated gene signatures and clinical response to initial drug treatment (~88% of patients received MTX alone or in combination with hydroxychloroquine and/or sulfasalazine) (130). Additionally, high expression of plasma cell gene signatures, as observed in the lymphoid pathotype, was associated with more bone erosion at 1 year follow-up (130). Humby et al. (2021) then demonstrated, in the R4RA clinical trial (a UGSB-driven, multicentre randomized trial), that transcriptomics-based stratification of synovial tissue could predict clinical responses better than the conventional histopathological classification (127). Specifically, they observed that patients who did not respond well to or tolerate csDMARDs or at least one bDMARDs (excluding tocilizumab and rituximab), had a superior treatment response to Tocilizumab (an IL6Ri) compared to Rituximab (an anti-CD20 monoclonal antibody), when they had a low or absent B cell gene expression signature (127). Utilizing repository single-cell transcriptomics data, Micheroli et al. (2022) then studied the association between subsets of synovial fibroblasts and synovial pathotypes in early untreated RA patients (149). They identified four distinct subsets of synovial fibroblasts, and observed that these four subsets were differentially present in the synovial pathotypes (149). Additionally, they found that different synovial fibroblast subsets correlated with measures of disease activity depending on the pathotype (149).

1.4. PROTEOMICS - EVOLUTION AND APPLICATION IN BIOLOGICAL SCIENCES

1.4.1. WHAT ARE PROTEINS?

Proteins can be seen as the engine that runs the complex machinery of life, and the functional machinery itself (151). These complex macromolecules are made of chains of amino acids, that are determined by the genes in our DNA (152,153). These chains of amino acids follow a defined sequence that determines their spatial conformation, which in turn provides each protein with a unique set of functions (154), and changes in this amino acid sequence can have varying degrees of impact on the protein's function (155). Similarly, changing a letter in a word can result in a word conveying its original meaning (gray → grey) or a completely different meaning (gray → pray). The functions of proteins are numerous and very important (151,155,156). In fact, they are essentially involved in every aspect of biological activity (157). They make up the core components of cellular structures, shape the cells, and provide mechanical support (156,157). But their functions go far beyond structural roles: They also carry out and coordinate the numerous biological processes that sustain life, serving as the enzymes that catalyse chemical reactions and the signalling molecules that regulate cellular responses (156,157). Therefore, it is no exaggeration to state that understanding proteins and their interactions is crucial for understanding the fundamental mechanisms of diseases (156,158–160).

However, *"proteomics is not an island, entire of itself."* – Cited from Zhang et al. (2019), which means: proteins are a part of a larger whole (161). Therefore, it is essential to understand the nuanced relationship between genes, transcripts, proteins, and other biological layers to understand the full picture (161–163). The genetic code carries important information about susceptibility (164), and due to its fairly static nature, it can be an exceptionally early predictor of diseases including RA (45–52). Furthermore, it can inform downstream analyses by generating new hypotheses (165,166). The first step towards functional manifestation of this genetic information occurs when genes are transcribed into transcripts (152,153,167,168). These transcripts very are dynamic in nature as their expression changes in response to cellular needs and both endogenous and exogenous stimuli (168,169). Yet, mRNA expression and protein abundance do not have a straightforward linear relationship, and one cannot uncritically employ transcript levels as a surrogate for proteins abundance (170–172). This is the result of very complex post-transcriptional and post-translational mechanisms that control protein synthesis and stability of proteins, ultimately affecting the final abundance of proteins, but also their function and functionality (173–177). Proteins may also undergo degradation, accumulation, and transportation/relocation, which can also affect their abundance and function (178–180). Thus, proteins are extremely dynamic molecules that evolve and adapt constantly in response to various factors (154,181). This dynamic nature of proteins makes them a very powerful tool for assessing the immediate (or "the actual") state

of biological systems and (dys)functions, while simultaneously capturing the effects of lifestyle choices, environmental exposures, genetics, epigenetic modifications, post-transcriptional, and post-translational regulation (172,182–185). Not to overlook the metabolites, that are even more powerful than proteins when it comes to assessing the actual phenotype (186,187). A fascinating example of this, that is commonly used by researchers in the proteomics field, can be found in the spoken metaphor involving the evolution of butterflies (Figure 3): *"The genetic sequence is largely the same regardless of whether the organism exists as a larva or an adult butterfly. The transcripts undergo extensive changes over time. Thus, the transcripts, although not precisely reflecting the immediate state of the organism, can shed light on the regulatory mechanisms and give hints on the potential state of the organism. However, one cannot be sure if the transcripts have been translated into actual proteins, or if the proteins have undergone subsequent changes, and this is important, because proteins are eventually the functional machinery and structural constituents through which genes and transcripts are expressed. Thus, assessment of the proteins (and metabolites) is essential to capture the immediate state of an organism, although one needs to inspect the organism visually to confirm the phenotype (i.e., the "truth"), which is a larva or an adult butterfly."* In conclusion, the genetic sequence and transcripts can answer fundamental biological questions such as *How did the larva become a butterfly? And why is it orange?* and the proteins and metabolites can provide a reflection of the immediate biological state and answer question such as *Is the organism a larva or a butterfly? And is the butterfly orange or brown?*

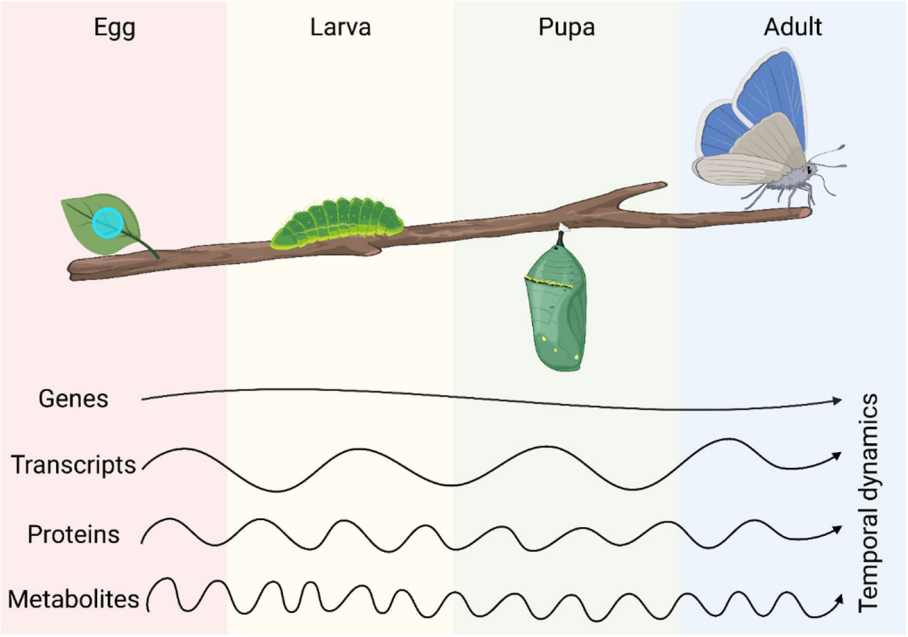

Figure 3. Temporal dynamics of genes, transcripts, proteins, and metabolites during the evolution of butterflies. The genetic code remains (fairly) constant, but the transcripts, proteins and metabolites are increasingly more temporal dynamic. Created with Biorender.com.

1.4.2. LIQUID CHROMATOGRAPHY MASS-SPECTROMETRY BASED PROTEOMICS.

The field of proteomics, that *"is the large-scale study of proteins"* – Cited from Wikipedia (188), has made it possible to measure (almost) the entire set of proteins (i.e., proteome) within an organ, or even in single cells (189–193). One of the key technologies in proteomics is Liquid Chromatography (LC)-Mass Spectrometry (MS) (LC-MS)-based proteomics, that constitutes an advanced and robust method for protein identification and quantification (190,194). The working principle of LC-MS-based proteomics is to separate complex protein/peptide mixtures via liquid chromatography, ionize the proteins/peptides, and subsequently identify and quantify the proteins/peptides through MS (190,194,195). Based on this principle, it is possible to identify and quantify proteins in complex mixtures through two different approaches known as top-down proteomics or bottom-up proteomics (190,196). In top-down proteomics, intact proteins are analysed directly on the LC-MS without prior enzymatical digestion (197–199). This approach can be used to map the full amino acid sequence of proteins and subsequently study proteoforms, a term that

"designate all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications" – Cited from Smith et al. (2013) (197–199). In bottom-up proteomics, the proteins are enzymatically digested into smaller peptides and then analysed on the LC-MS (190,194,195). This approach can be used to identify and quantify many thousands of proteins simultaneously in very complex mixtures, enabling a comprehensive protein profiling of biological samples (189,190,194,195,200,201). In Paper 2, we employed a variant of bottom-up proteomics known as shotgun/discovery/untargeted proteomics with label-free quantification (LFQ), which is covered in the following sections (Figure 4) (202).

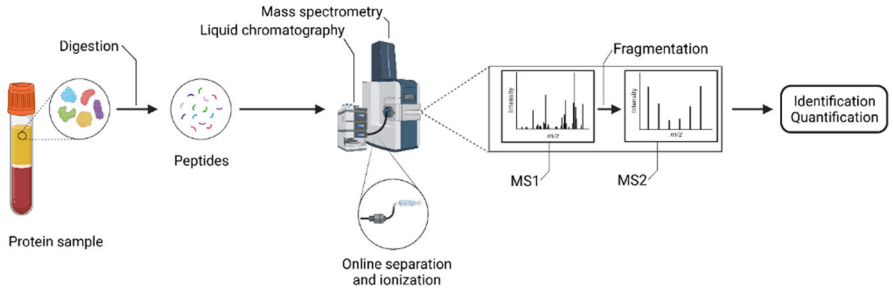

Figure 4. General workflow of bottom-up mass spectrometry-based proteomics. Proteins are digested and analysed using liquid chromatography mass spectrometry followed by identification and quantification of the proteins using software (190,194).

1.4.2.1 Sample preparation

Sample preparation is important to ensure a reproducible and reliable identification and quantification in LC-MS-based bottom-up proteomics (203–205). The general principle of the sample preparation involves *"protein extraction, solubilization, denaturation, enzymatic digestion into peptides followed by their purification"* – Cited from Supasri et al. (2021), to remove remaining cellular debris, lipids, and contaminants such as detergents (204–208). One of the earliest sample preparation methods was in-gel digestion, that involves one- and two-dimensional gel electrophoresis (2-DE) to separate the proteins before digestion (209–211). This method is effective at solubilizing and digesting proteins (212). However, it is very time consuming, and requires a lot of hands-on work (205). In-solution digestion provides a solution to overcome these limitations by reducing time, costs, and hand-on workload substantially, and thereby increasing the scalability of proteomics sample preparation (205,212). In addition, it is particularly useful for very low sample quantities, as seen in the field of single-cell proteomics today (213). However, In-solution digestion has a major limitation, that is poor solubilization and incomplete digestion of proteins, resulting in fewer identified proteins and poor quantitative

reproducibility (212,214). The filter-based sample preparation techniques (as used in Paper 2) were then developed to overcome these limitations by combining the advantages of in-gel digestion (efficient solubilization and digestion of proteins), and the advantages of in-solution digestion (higher throughput and scalability) (215–218). The principle of these filter-based techniques is to utilize strong detergents to solubilize proteins, which yields a more efficient digestion (215,216,218). This is possible because the filter can retain the proteins and peptides while washing out the LC-MS-incompatible detergents prior to analysis (214,219,220). This ability to retain proteins/peptides during the sample preparation also minimizes sample loss (205,215,217). More recently, sample preparation techniques based on magnetic beads have emerged (221–223). These methods utilize magnetic beads to retain the proteins, which enables the use of strong detergents to solubilize proteins (and improve digestion efficiency), and subsequently washing out the detergents (221–223). As such, the magnetic beads-based sample preparation techniques offer largely the same advantages as the filter-based sample preparation techniques (217,221–223). In addition, it does not require lengthy centrifugation steps, and the manual transferring associated hereto (217,221–224). This results in a very high throughput and the ability to be fully automized, which is essential for the scalability and reproducibility, and the subsequent advancement of LC-MS-based proteomics to clinical settings (224,225).

1.4.2.2 Data acquisition modes

The clean peptides are then separated by LC, ionized by the ionization source, and subsequently introduced to the MS (190,194,226). The MS then measures the mass-to-charge ratio (m/z) of the precursor ions (MS1), that are intact peptides (190,201,227). The precursor ions are then fragmented, typically using collision-induced dissociation, that accelerates the precursor ions and collides them with a gas, subsequently generating multiple fragment ions from each precursor ion (190,201,227). The MS then measures the m/z of these fragment ions (MS2), adding another dimension of information that can be used for identification and quantification of the proteins (190,201,227). Depending on what data acquisition mode is used to operate the MS, the process for selecting precursor ions for fragmentation can vary (190,201,227). The two primary data acquisition modes in untargeted LC-MS-based proteomics, are called Data Dependent Acquisition (DDA) and Data Independent Acquisition (DIA) (190,201,227). DDA preferentially selects the most abundant/intense precursor ions for fragmentation (190,201,227). This is a very simple approach, but it has some major limitations. For instance, less abundant peptides may not be fragmented as often if there are other very high abundant peptides in the sample (190,201,227). This variable selection of ions results in a low sensitivity for low abundant peptides, and large amounts of missing data across different runs (190,201,227–229). DIA, on the other hand, does not select the most abundant/intense precursor ions for fragmentation (190,228–230). Instead, it sequentially fragments all ions within a specified m/z mass range (190,228–230). This more unbiased approach

offers a more comprehensive proteome coverage and reduces amount of missing data (190,228–230). It does not depend on random fragmentation of precursor ions, and theoretically fragments the entire sample, including low abundant ions (190,228–230). Consequently, DIA has improved reproducibility across different runs and makes the comparison of different samples more reliable (229,231–233).

Recent advances in MS, that is the incorporation of Trapped Ion Mobility Spectrometry (TIMS) into Quadrupole Time-of-Flight mass spectrometers, have further leveraged the capabilities of DIA (234–239). TIMS can separate ions based on their collisional cross section, a measure that is associated to their size and shape, but also accumulate and retain ions for a specific duration before releasing them for further analysis (235,236,239). The Parallel Accumulation-Serial Fragmentation (PASEF) method is based on this concept (235,236,239). Specifically, it accumulates ions based on their collisional cross section and releases them sequentially in synchronization with the positioning of the quadrupole (235,236,239). This has given rise to DIA-PASEF, a data acquisition mode that integrates the capabilities of DIA with the rapid sequencing and additional separation dimension of PASEF (that is collisional cross section), and thereby enhances the complexity of DIA data and results in a more comprehensive and efficient method for analysing complex protein mixtures (236,239).

1.4.2.3 Data analysis

After the raw LC-MS data has been acquired, the data is preprocessed in several steps prior to protein identification and quantification (226,240–242). The preprocessing steps include filtering out background noise, peak detection, deisotoping, and charge state deconvolution (226,240–242). Retention time alignment between runs is also performed to ensure that the peaks from identical peptides have the same retention time in all samples (226,240–244). This is because retention time of peptides (i.e., the time at which a specific peptide elute from the LC) may shift over the course of an experiment (226,240–244).

The preprocessing steps are followed by peptide identification (194,195,226,240–242). This typically involves matching the peptides up against a database that contains the amino acid sequences of thousands of proteins (195,201,240,242). The principle of this process is to computationally “digest” the database proteins using the same enzyme that was used to digest the samples during sample preparation (195,201,240,242). This creates a theoretical list of peptides for each protein in the database, and a list of their theoretical MS2 spectra that is generated by considering their most likely fragmentation patterns (195,201,240,242). These theoretical peptides and their MS2 spectra should resemble the peptides in the sample (195,201,240,242). This is because certain proteases will digest proteins at specific sites and not randomly (195,201,240,242). For instance, trypsin will specifically digest the proteins at the carboxyl side of lysine or arginine, unless these are followed by a proline (245,246).

The computationally generated theoretical spectra are then compared with the acquired experimental MS2 spectra, and potential matches are scored based on how well m/z peak values and peak intensities aligns (247). Potential matches are then statically validated by calculating the False Discovery Rate (FDR), where the cut-off is typically set at 1% FDR (201,226,247,248). This is done by generating false peptide sequences (i.e., a decoy database) from the same database that used to generate the theoretical peptides and their MS2 spectra (i.e., the target database) (201,226,247,248). The proportion of decoy hits above a set cut-off, compared to the total number of hits, provides an estimate of the FDR (201,226,247,248).

Once the peptides within the experimental sample have been identified, protein inference is carried out (201,240). The principle is to map the identified peptides back to the protein they originated from (201,240). Sometimes the peptides can map to multiple proteins because the sequences of these proteins are very similar, for instance if there are several proteoforms (201,240). In such case, proteins are grouped together in protein groups which simplifies the data (201,240). The more peptides that map to a protein, the more confident the protein identification (201,240). Once the proteins in the samples have been identified, quantification is carried out (201,240). In LFQ, this can be done using spectral counting and ion intensity-based quantification (226,249–252). Spectral counting involves counting the number of MS2 spectra to estimate relative protein abundancies, with the assumption that more abundant proteins results in more MS2 spectra (226,249–251). The intensity-based, on the other hand, measures the MS1 peak intensity (height of the peak, or by integrating the peak area) of each peptide, and then summarizes (all or some of) the peptide ion intensities to estimate relative protein abundancies (226,249–251). Finally, normalization is carried out to account for technical variability across different LC-MS runs, using, for instance, the well-established MaxLFQ algorithm (253).

The outlined process of using database searching cannot be directly applied to DIA data, because each MS2 spectra in DIA data originates from several precursor ions (254–257). Traditionally, this has necessitated the use of spectral libraries that are generated in a preceding DDA experiment where project samples are pooled and fractionated, and then analysed (254–257). *"The experimental MS2 spectra are then compared with those in the spectral library to identify the proteins in the sample"*[*], but the process of generating spectral libraries is labour intensive, costly and may be done repeatedly for each project (254–257). However, (spectral) library-free searching tools have recently been developed to bypass the need for spectral libraries, subsequently enabling peptide/protein identification directly from DIA data using database searching (255,258–261). The principle of directDIA™ in Spectronaut® (Biognosys, Schlieren, CH) that was used in Paper 2 enables this by deconstructing the MS2 spectra (that originates from several precursor ions) into a pseudo MS2 spectra that resembles normal MS2 spectra (260,261). This is done computationally

[*] This sentence reflects a commonly used definition/terminology/nomenclature in the field.

by determining which fragment ions came from the same precursor ion based on the chromatographic coelution of precursor and fragment ions (260,261). Following this step, the DIA data can be analysed using database searching, comparable to the method used in traditional DDA workflows (260,261). This have significantly streamlined the process of analysing complex mixtures of proteins in DIA mode, and subsequently generate comprehensive datasets that contain the identity and relative quantity of thousands of proteins (255,260,261).

1.5. STATISTICS IN PROTEOMICS AND MULTI-OMICS DATA INTEGRATION

The field of proteomics has made large-scale identification and quantification of proteins more accessible than ever, providing novel opportunities for discovering biomarkers and investigating disease mechanisms. However, navigating the highly complex and highly dimensional proteomics data to extract relevant information that can answer the research question being addressed, requires appropriate statistics and statistical finesse. Such statistical methods are described in the following sections, that were inspired my research stay at Toulouse Mathematics Institute, during which I was mentored by Prof. Sébastien Déjean and all the mathematicians and statisticians I interacted with daily.

1.5.1. UNIVARIATE METHODS

Univariate statistics are methods that examine one variable separately (262), which can provide a fundamental understanding of each protein's individual behaviour (263). They do not consider the relationships between proteins but serve as the key initial steps in the data analysis, laying the foundation for more complex downstream multivariate analyses (263).

1.5.1.1 Linear mixed models

The complexities of study designs, such as repeated measures experiments with multi-group comparisons as seen in Paper 2, require statistical methods that can take into consideration several factors to understand how these affect the complex behaviours of proteins (264). One such method is linear mixed model (LMM), a statistical method that addresses these complexities by incorporating both fixed and random effects (264–267). Fixed effects are known experimental factors, such as experimental groups and type of interventions, that are intentionally controlled because the aim is to study how these factors affect the outcome (264). Random effects, on the other hand, are factors that are not of primary interest to the research question (264–267). Instead, they are included in the statistical model to account for the variability they introduce (264–267). A hypothetical example could be a multi-site clinical trial where the primary research aim is to study if two different treatments affect CRP differently over time. In this example, the fixed effects would be the treatment (MTX vs

Etanercept) and time (baseline versus follow-up). If we then assume that the response to the two treatments varies depending on the clinical trial sites, then "sites" can be included as a random effect. The LMM will then account for the between-subjects variability that "sites" introduce and ensure less biased estimates of the fixed effects.

LMMs are also very useful in longitudinal studies where data are repeatedly collected from the same subjects over time (264–267). This adds another layer of complexity because the repeated measurements will be dependent of each other (264–267). Building on the previous example, if CRP was collected at baseline and follow-up from the same patients (i.e., repeated measures), then baseline and follow-up CRP abundance would likely be related to each other. For instance, a patient with high CRP at baseline may have a high score at follow-up, or perhaps a larger decrease in CRP over time. The LMM can account for such within-patient correlation by including "patient" as a random effect. By doing so, the LMM can simultaneously account for between-patients variability such as patients having different baseline CRP scores and different response to treatments.

Overall, the LMMs are very versatile statistical tools that can model the effects of several factors on an outcome (264–267). This is particularly relevant when it is necessary to account for biological variability and repeated measures or handle unbalanced designs and missing data (264). The ability to address these challenges makes LLM a powerful tool for proteomics statistical analysis, where it can facilitate the identification of proteins whose abundance change in response to different conditions and various factors (268–270).

1.5.1.2 Post-hoc analysis and visualization

Once a LMM has been performed, and a significant effect (for instance, of group) has been identified, the LMM suggests there is a difference between group (267,271). However, it does not tell which groups are significantly different (271). To identify these differences, pairwise comparisons must be performed (271). T-tests are widely used for this purpose in a proteomics context (272). It essentially determines whether the mean abundance of a given protein is significantly different between two groups (272). Results can then be presented with data visualizations such as bar plots, box plots or violin plots to show the distribution of data (273). However, creating individual plots for each protein in Paper 2 would be impractical due to the high dimensionality of proteomics data. Volcano plots can overcome this challenge by summarizing the results of multiple t-tests (274–276). They achieve this by plotting the negative log10 p-values (indicating statistical significance) against the log2 fold change (indicating the difference between two groups) (274–276).

However, while volcano plots can visualize thousands of t-test results, performing thousands of pairwise comparisons will increase the risk of Type I errors (false positives) substantially (277). This is especially true when dealing with high-

dimensional proteomics data where thousands of pairwise comparisons are often made simultaneously (277,278). Therefore, it is critical to adjust the p-values for multiple comparisons and control the number of Type I errors (277,278). The Benjamini-Hochberg procedure is useful for this purpose in proteomics statistical analysis (277–280). This method, unlike the conservative Bonferroni method, minimizes the risk of Type I error without increasing the risk of Type II errors to the same extend (i.e., false negatives) (281,282). This is achieved by controlling the FDR, that is the expected proportion of wrongly identified significant results (280,283). The Benjamini-Hochberg procedure works by ranking p-values from the multiple tests from smallest to largest (280,281). *"A critical value is then calculated by dividing the rank of each p-value with the number of tests and multiplying this with the chosen FDR"* (277,281,282) [*]. This critical value is then compared with each p-value, and p-values that fall below the critical value are considered to be significant (277,281,282). Controlling the FDR has proven to be an efficient way to limit the number of false discoveries and ensure more reliable results when analysing high-dimensional proteomics data (278,279,283). However, adjusting the p-values threshold, regardless of the method, always comes at a risk of increasing Type II errors (false negatives) (278,279). This may not be appropriate in some cases where true proteomic differences are so small that they are regarded as being non-significant following FDR correction (278,279). Thus, and this is my personal view, when applying FDR controlling procedures, it is essential to employ critical evaluation and make informed decision-making by considering findings in a biological context-specific manner, rather than accepting the statistical output without further examination. A hypothetical example could be a plasma proteomics study where 10 acute phase proteins are significantly upregulated in an inflammatory condition, but this significance disappears following FDR correction. However, from a biological point of view, it seems very plausible that 10 acute phase proteins would be upregulated as the results of an underlying inflammatory condition, and especially if these proteins are known to interact with each other. Thus, instead of ignoring the initial findings due to a stringent FDR correction, one might still consider these results to be significant based on their relevancy and the biological plausibility. Likewise, significant findings should not always be regarded as relevant just because they passed stringent statistical conditions. For example, if a study has investigated the proteomic differences between RA and OA, and it turns out a gender-specific protein is significantly upregulated in the RA group, this difference could indeed be the result of RA, but it may also be related to other factors than the disease itself. Such factors could be an overrepresentation of females in the RA group (if the groups were unmatched) or perhaps an incorrectly identified protein (if the groups were matched). Thus, and this is also my personal view, while statistical tools like FDR correction are important to control the risk of Type I errors, they should not be a standalone substitute for

[*] This sentence reflects a commonly used definition/terminology/nomenclature in the field.

scientific intuition. Interpretation should always be a balance between statistical stringency and common sense that is informed by context-specific biological insights.

1.5.2. MULTIVARIATE METHODS

Unlike univariate statistics, that examine one variable separately (262), multivariate methods consider multiple variables simultaneously, and has the potential to unravel complex relationship between variables that univariate statistics might overlook (263,284–286). They provide a holistic view into biological systems by identifying and summarizing the complexities and dynamics within large datasets (263,284–286). Multivariate methods are consequently particularly useful when analysing high-dimensional omics data (including proteomics), where they can facilitate interpretation by reducing dimensionality, identifying true signals among noise, and visualize large amounts of data (263,284–286). Multivariate methods can also be used combine proteomics data with other omics layers and/or clinical data (263,284–286). This field, known as data integration or multi-omics, can be used to understand the relationships between multiple datasets, and subsequently unravel coupled molecular and cellular mechanisms underlying diseases and clinical presentations (263,284–287).

1.5.2.1 Principal component analysis

Principal Component Analysis (PCA) is a dimension reduction method that reduces high dimensional data by retaining only the most relevant information (288–292). It is particularly useful for analysing high-dimensional data like proteomics, where it can facilitate visualization and interpretation of thousands of proteins simultaneously (263). Proteomics data are often characterized by many (redundant) strongly covarying variables (293,294). PCA works by identifying these covarying proteins and combines them *"into a new set of variables called principal components, which are linear combinations of the original variables"* - Cited from Symoniuk et al. (2023) (288–292,295). A principal component is constructed for each variable in the dataset, with each succeeding component capturing less of the remaining variability (288–292). *"For instance, the first principal component captures the highest proportion of variability in the data, and the second principal component captures the highest proportion of the remaining variability"* (288–292)[*]. Dimension reduction is then achieved by focussing on the principal components that captures most of the variability in the data, and ignoring the succeeding principal components that contain less information (288–292). Most often, in a biological context, only the first two-three principal components are retained and used for visualization because the aim is to explore the major sources of variability in the data (296). Subsequent visualization is achieved using scoreplots and loading plots (263,284). The scoreplots project each observation (such as patient) based on their scores on component one and component

[*] This sentence reflects a commonly used definition/terminology/nomenclature in the field.

two (284). This provides an overview of the data and trends herein such as clusters of patients and outliers (263,284). The PCA is an unsupervised model, and therefore it does not take any response variable (i.e., group membership of observations) into consideration (263,284). However, if such groupings exist and they give rise to large variability in the data, they could still be detected by the PCA (263,284). For example, if the major sources of variability in a proteomics dataset arises from proteomic differences between RA and OA patients, then the patients will cluster naturally according to their disease group on the first component. The loadings on component one (i.e., the contribution of each protein on the first component), can then be visualized and examined using loading plots to identify what proteins are responsible for discrimination of RA and OA samples (263,284). The PCA can also detect potential bias (such differences in sample collection) or confounders (such as disease duration or medication use) if these give rise to large variability in the proteomics dataset, and thereby highlight the need for addressing these limitations before proceeding with downstream analysis (263). Thus, PCA is indeed a powerful tool for exploring high-dimensional data and trends herein and might reveal patterns in the data associated with various factors/conditions (263).

1.5.2.2 Partial least squares analysis

Partial Least Squares (PLS) (297–299) analysis is another useful unsupervised multivariate method for proteomics data analysis (263,284,300). PLS reduces dimensionality of the data by constructing a new set of variables/linear combinations (components) (286,298,299,301,302). However, unlike PCA where the linear combinations (principal components) maximize the variability of data, the PLS constructs linear combinations that maximizes covariance between two datasets of continuous variables that were collected from the same subjects (predictor variables and response variables) (286,301,302). By doing so, PLS summarizes the variability of each dataset while simultaneously capturing their shared variance (286,301,302). This can be used to study the relationship between two datasets (263,286,300–302). For instance, proteomics data can be integrated with clinical data to identify predictors of treatment response or measures of disease activity (as in Paper 3), or proteomics data can be integrated with other types of omics data to identify coupled responses/covarying variables across different biological layers (263,284,300).

1.5.2.3 Partial least squares for discriminant analysis

Partial Least Squares Discriminant Analysis (PLS-DA) is an adaption of PLS that can be used for classification of categorical variables based on high-dimensional data (284,302–304). The principle of PLS-DA is largely the same as that of PLS (284,302–304). However, instead of maximizing the covariance between two datasets of continuous variables, the PLS-DA reduces the dimensions of a continuous dataset (predictor variables) by creating linear combinations that maximize its covariance with a dummy block matrix of the categorical response variable (284,302–304). Thus,

PLS-DA can be used to identify (linear combinations of) variables from a high-dimensional dataset that characterize or differentiate between different categorical groups (284,302–304). This is particularly relevant in a proteomics context, where it can facilitate identification of proteins that can discriminate between conditions such as responders versus non-responders, or diseased versus healthy. However, the PLS-DA, like any other supervised models, shall be used with caution because applying them to any high-dimensional omics dataset result in noisy and misleading results (305).

1.5.2.4 Multi-block partial least squares discriminant analysis - Data Integration Analysis for Biomarker discovery using Latent cOmponents

Multiblock PLS-DA, widely known as Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO), is an extension of the PLS-DA, that allows prediction of categorical classes based on multiple datasets (blocks) of continuous predictor variables (284,306). This stands in contrast to the basic PLS-DA model, that can only handle one dataset at a time, and the basic PLS model, that cannot handle more than two datasets at a time or perform prediction of categorical classes (284,306). DIABLO works by creating linear combinations of each block that 1) maximizes its covariance with the other blocks, and 2) maximize its covariance with a dummy block matrix of the categorical response variable (284,306). Thus, it can be used to identify related variables across several high-dimensional datasets that simultaneously characterize and differentiate between different categorical groups (284,306). This has a range of applications, that are particularly relevant when analysing high-dimensional omics data (307–311). For example, DIABLO can be used to identify “minimalistic” biomarker signatures that contain a few proteins (284,306). These small biomarker signatures could pave the way for more personalized treatments by facilitating development of multiplex arrays, that are more feasible to implement in clinical settings (312,313). DIABLO can also be used in a systems biology context to identify large biomarker signatures containing hundreds of proteins along with other variables (284,306). This broader systems biology approach can provide a comprehensive and holistic insight into the molecular characteristics underlying various biological and clinical conditions, and can generate data for subsequent functional enrichment analyses, facilitating investigations into underlying dysregulated biological pathways and/or protein-protein interactions (263,284,306).

Figure 5. Overview of multivariate methods described in this chapter, and what they can be used for. These methods are available in the MixOmics R package (301). Principal component analysis (PCA) handles one omics dataset at a time and can, for example, answer if synovial tissue proteomes from rheumatoid arthritis (RA) patients and healthy controls cluster together without considering group affiliation of each patient. Partial least squares (PLS) can be used to integrate omics data with other omics data or clinical data and can, for example, answer if synovial tissue proteins covary with measures of disease activity, as demonstrated in Paper 3 (Unpublished results). PLS for discriminant analysis (PLS-DA) handles one omics dataset at a time together with a categorical response variable and can, for example, help identification of synovial tissue proteins that discriminate between RA and healthy controls in a supervised manner by considering group affiliation of each patient. Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) can handle multiple omics datasets together with a categorical response variable. This method can, for example, facilitate identification of covarying synovial proteins and plasma proteins that can discriminate between RA patients and healthy controls. Reference: (286,301).

1.6. FUNCTIONAL ENRICHMENT ANALYSIS – TRANSLATING GENE LISTS INTO MEANINGFUL BIOLOGY.

Once the statistical analyses have identified multiple proteins of interest, these lists of proteins must be translated into meaningful biology. Inferring biological relevance of a few proteins may be "manually" feasible by reviewing the literature. However, inferring biological relevance of hundreds or thousands of proteins is complicated and practically unfeasible without computational tools. Functional enrichment analysis is one such tool that can translate long lists of proteins into interpretable biology (314,315). One of the methods by which functional enrichment analysis achieves this is over-representation analysis (316–319). This method tests whether a functional term is overrepresented by comparing a test set of proteins (for instance differentially abundant proteins) with a reference set of proteins (316–319). By analysing the overlap between the test set of proteins and functional terms obtained from databases (i.e., gene lists associated with specific biological processes or functions), it is possible to identify functional terms that are statistically overrepresented (i.e., more represented in the test set of proteins than one can expect by random chance) (314,316–320). This can be done using Fisher's exact test, that calculates a *p*-value indicating whether the overrepresentation is statistically significant (317,319).

1.6.1. DATABASES

There are several databases that contain information about gene and protein functions, and these are prerequisites for any functional enrichment analysis. Among these databases, the Gene Ontology Resource contains information about gene functions, including associated biological processes, cellular components, and molecular functions (321–323). The Kyoto Encyclopedia of Genes and Genomes and Reactome databases, on the other hand, contain information about genes and their associated biological pathways, interactions networks, molecular reactions, etc (324–328). Additionally, there are other expert-curated resources that contain information about genes and their associated canonical pathways (329,330). These canonical pathways represent biological pathways that have been well-studied and comprehensively characterized (329,330).

1.6.2. INTEGRATING MULTIPLE DATABASES

Using one database to infer biology from a list of proteins can indeed provide valuable information. However, information from multiple databases can also be integrated to increase the depth and width of the functional enrichment analysis, thereby providing a more holistic and comprehensive view into biology (331). Metascape is one such tool that can aggregate and summarize information from several databases (331). Specifically, is can cluster functionally related terms based on their similarity, that is defined by the overlap between their gene lists (331). Once the overrepresented terms have been clustered together, each cluster will be assigned a representative cluster

label, which is typically the most significantly enriched functional term of the cluster (331). This allows for grouping of vast amounts of redundant data into interpretable information, thereby facilitating identification of major functional themes (331). Metascape can also create network representations of the functional enrichment analysis, using nodes to represent an overrepresented functional term and edges to represent overlap between their gene lists (331). These network representations can be used visualize the complex relationships between and within functional clusters and help us understand the relationships of various biological pathways (331). These integrative approaches overall enhance the utility of functional enrichment analysis, providing a more comprehensive and holistic view into biology that otherwise may be missed (331).

1.7. SYNOVIAL TISSUE PROTEOMICS IN RHEUMATOID ARTHRITIS

Proteomics analysis of synovial tissue has provided valuable insight into the molecular landscape of RA. Some of the earliest investigations were by Tilleman et al. (2005) that utilized 2-DE to separate cytosolic proteins from synovial tissue of RA and OA patients (134). These proteins were then identified using matrix-assisted laser desorption/ionization (MALDI)-MS and LC-MS (134). This novel approach at the time identified S100A8 to be differentially abundant between RA and OA (134). Subsequent studies by Chang et al. (2009), compared synovial tissue of RA, OA, and Ankylosing Spondylitis (AS) (136). They separated proteins using 2-DE to find protein spots that were more intense in RA (136). These protein spots were then analysed using MALDI-MS to identify the proteins within, and western blot and ELISA were subsequently used to quantify the proteins (136). This approach identified Ig-kappa light-chain C region, PRDX4, SOD2, TPI, and TXNDC5 to be more abundant in RA compared to OA and AS (136). Yan et al. (2012) employed a similar approach to compare the synovial tissue of RA, OA, and AS (137). However, they separated proteins using 2-DE to find protein spots that were less intense in RA compared OA and AS, not more intense (137). One spot with particularly low intensity in RA was subsequently analysed using MALDI-MS and identified to be Vitamin D Binding Protein (137). Semiquantitative quantification using western blot they confirmed that Vitamin D Binding Protein had a significantly lower abundance in RA synovial tissue compared to OS and AS (137). The throughput of proteomics studies then began to increase rapidly owing to technological advancements in the proteomics field, and investigations became increasingly comprehensive over the years. This was seen in Hayashi et al. (2015) that employed an LC-MS-based LFQ proteomics approach to compare laser-micro dissected synoviocyte lesions from RA and OA patients (139). They identified and quantified 508 proteins, 98 of which were differentially abundant between RA and OA synoviocyte lesions (139). Functional enrichment analyses were then carried out to infer biological relevancy of these 98 differentially abundant proteins (139). This identified several dysregulated pathways in RA including ribosome pathways, p53 signalling pathways, leukocyte migration

pathways, and NF-kB/MAPK (139). Ren et al. (2021) then used a LC-MS-based proteomics approach with tandem mass tag (TMT) labelling to compare synovial tissue proteome of RA and OA patients (133). Synovial tissue from 10 RA patients were divided into three pools, and synovial tissue from 12 OA patients were likewise divided into three pools (133). These six pools were then digested, labelled each with a unique TMT-label, pooled together, fractionated, and analysed using LC-MS (133). The relative protein abundance between RA and OA sample pools could then be calculated using the ratios of TMT reporter ion intensities in the MS2 spectra (133). This led to the identification 4822 proteins, 510 of which were differentially abundant in RA synovial tissue compared to OA (133). Subsequent functional enrichment analyses revealed that these proteins were associated with developmental processes, extracellular structure organization, skeletal system development, collagen catabolic process, and various developmental processes (133). More recently, Xu et al. (2023) employed a similar LC-MS-based proteomics approach with TMT labelling to compare synovial tissue proteome of RA and OA patients (332). Although their primary aim was to study the role of protein glycosylation in RA, they also reported differences in protein abundance between RA and OA (332). They managed to identify an impressive total of 7227 proteins because they fractionated the pooled TMT-labelled samples into 20 fractions, thereby decreasing the complexity of the peptide mixtures, and increasing the number of identifications (332). Of these 7227 proteins, 427 were more abundant and 241 were less abundant in RA compared to OA (332). A subsequent gene set enrichment analysis of the overabundant RA proteins, revealed several dysregulated immune-related pathways in RA (332).

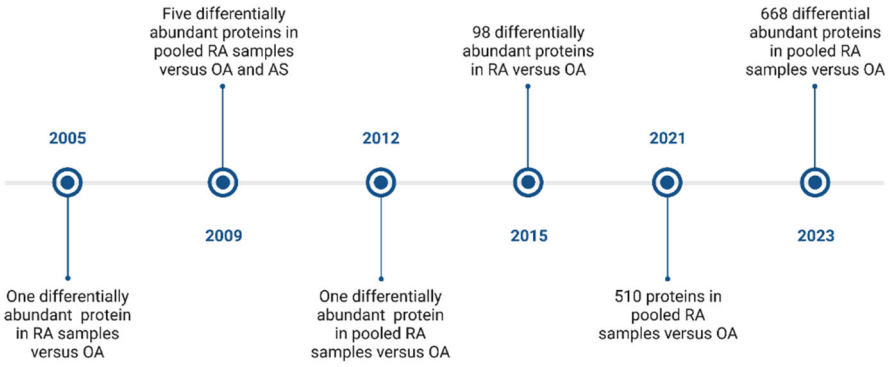

Figure 6. Evolution of synovial tissue proteomics. From identifying one differentially abundant protein in 2005 to 668 differentially abundant proteins in 2023, synovial tissue proteomics has increasingly improved our understanding of the molecular landscape of rheumatoid arthritis (RA) over time (133,134,137,139,332,333). Osteoarthritis, OA; Ankylosing Spondylitis, AS.

The evolution of proteomics and its application to synovial tissue profiling has provided increasingly comprehensive insights into the molecular landscape of RA (Figure 6). However, there is an unmet need for more comprehensive proteomics-driven investigations of RA synovial tissue, on an individual patient basis, to answer questions that are fundamental to our understanding of RA: *What biological pathways are dysregulated in early untreated RA and what happens following treatment initiation? What biological pathways are dysregulated in longstanding RA and what happens following treatment intensification? What are the molecular differences between early RA and longstanding RA? What are the cellular and molecular determinants of synovial heterogeneity? And can determinants of synovial heterogeneity predict treatment outcomes?* Facilitated by the rapid advancements in the field of proteomics, bioinformatics, and the UGSB procedure for collection of synovial tissue biopsies, this thesis aims to answer these fundamental questions. This is not only to expand our understanding of RA, but also to lay the foundation for future studies on ICI-IIA, and subsequently facilitate translational research between these two disabling conditions.

CHAPTER 2. RESULTS

2.1. PAPER 1

Prediction and early diagnosis of immune-checkpoint inhibitor-induced inflammatory arthritis from molecular biomarkers – Where are we now?
Christopher Aboo [a,b,c], Tue Wenzel Krastrup [d,e], Helene Broch Tenstad [f], Jie Ren [c], Søren Andreas Just [f], Morten Ladekarl [g] and Allan Stensballe [a]. EXPERT REVIEW OF PRECISION MEDICINE AND DRUG DEVELOPMENT 2022, VOL. 7, NO. 1, 162–168, https://doi.org/10.1080/23808993.2022.2156785, Received 2 June 2022, Accepted 6 December 2022.

[a] Department of Health Science and Technology, Aalborg University, Aalborg, Denmark; [b] Sino-Danish Center for Education and Research, University of Chinese Academy of Sciences, Beijing, China; [c]CAS Key Laboratory of Genomic and Precision Medicine, Beijing Institute of Genomics, Beijing, China; [d] Department of Biomedicine, Aarhus University, Aarhus, Denmark; [e] Department of Rheumatology, Aarhus University Hospital, Aarhus, Denmark; [f] Department of Rheumatology, Odense University Hospital, Odense, Denmark; [g] Department of Oncology and Clinical Cancer Research Center, Aalborg University Hospital, Aalborg, Denmark

This investigation was a non-systematic review that sought to review existing studies aiming to identify blood biomarkers for early diagnosis and prediction of ICI-IIA (1). We identified a lack of substantial research in this specific field (1). Moreover, we discovered an apparent lack of reliable diagnostic criteria for ICI-IIA (1). This, in turn, led to unreliable clinical endpoints within these studies (1). Recognizing these limitations, we provided a set of recommendations to facilitate and guide future research on biomarker discovery for ICI-IIA (1). Specifically, we discussed the use of different diagnostic approaches to define robust clinical endpoints and briefly discussed the potential of employing omics technologies for biomarker discovery in ICI-IIA (1).

2.2. PAPER 2

Synovial tissue proteomics unravels pathological trajectories in Rheumatoid Arthritis and identifies determinants of synovial heterogeneity. Christopher Aboo*[1,4], Søren Andreas Just*[2], Christian Nielsen[5,6], Henrik Daa Schrøder[7], Jacob Skallerup Andersen[1,4], Mikkel Thomsen[1], Sébastien Déjean[8], Tue Bjerg Bennike[9], Hanne Lindegaard[3], Allan Stensballe[1].

[1] Translational Biomarkers in Pain and Precision Medicine, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark. [2] Section of Rheumatology, Department of Medicine, Odense University Hospital, Svendborg Hospital, Svendborg, Denmark. [3] The Rheumatology Research Unit, Department of Rheumatology, Odense University Hospital and University of Southern Denmark, Odense, Denmark. [4] Sino-Danish Center for Research and Education, University of Chinese Academy of Sciences, Beijing, China. [5] Department of Clinical Immunology, Odense University Hospital, Odense, Denmark. [6] Open Patient Data Explorative Network (OPEN), Odense University Hospital, Odense, Denmark. [7] Department of Clinical Pathology, Odense University Hospital, Odense, Denmark. [8] Institut de Mathématiques de Toulouse, Université Toulouse III-Paul Sabatier, Toulouse, France. [9] Medical Microbiology and Immunology, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark. *Shared first authorship. Manuscript submitted to Annals of Rheumatic Diseases, 8th September 2023.

2.2.1. SUBSTUDY 1

By employing proteomics to map the synovial tissue and plasma proteomic landscape of RA at its various stages, this investigation sought to answer fundamental questions about RA (202) (Aboo et al., unpublished – Paper 2). We identified several proteins and biological pathways that were dysregulated in untreated early RA (ERA) compared to healthy controls (HC) (202) (Aboo et al., unpublished – Paper 2). Proteomes of ERA patients were partially normalized following treatment (primarily MTX alone or as combination therapy), whereas the proteomes of longstanding RA (LRA) patients remained static following treatment intensification (202) (Aboo et al., unpublished – Paper 2). This suggest that a therapeutic "window of opportunity" at the protein level is present in ERA but not in LRA (202) (Aboo et al., unpublished – Paper 2). Despite a partial normalization of the synovial proteome in ERA at follow-up, only a few pathways were attenuated (primarily complement cascade-related pathways) (202) (Aboo et al., unpublished – Paper 2). However, the remaining dysregulated pathways in ERA at follow-up resembled those observed in untreated ERA (202) (Aboo et al., unpublished – Paper 2). This was also observed in LRA patients where attenuation of complement cascade-related pathways was sustained at both timepoints (202) (Aboo et al., unpublished – Paper 2). However, the remaining dysregulated pathways in LRA resembled those observed in untreated ERA (202) (Aboo et al., unpublished – Paper 2). Notably, despite a sustained attenuation of complement cascade-related pathways in LRA, a more pronounced ECM-degradation was observed in LRA compared to ERA (202) (Aboo et al., unpublished – Paper 2).

This might explain the absence of the 'window of opportunity' in late-stage disease (202) (Aboo et al., unpublished – Paper 2). Given that treatment had only targeted a narrow spectrum of dysregulated pathways, we assumed that nontargeted dysregulated pathways could account for disease progression (202) (Aboo et al., unpublished – Paper 2). This was supported by our discovery of 99 synovial proteins and several dysregulated pathways that were consistently dysregulated in RA, regardless of disease state and treatment (202) (Aboo et al., unpublished – Paper 2). This observation highlights the potential of combination therapies to advance RA treatment beyond just achieving clinical remission (202) (Aboo et al., unpublished – Paper 2). Such combination therapies should not only target immune-related pathways, but also platelet/haemostasis, ECM-degradation, and oxidative stress-related pathways (202) (Aboo et al., unpublished – Paper 2).

2.2.2. SUBSTUDY 2

Utilizing the generated proteomics dataset and employing data integration, this investigation sought to identify cellular and molecular determinants of synovial heterogeneity in untreated ERA patients (202) (Aboo et al., unpublished – Paper 2). We identified a continuous molecular and cellular difference between histologically defined synovial pathotypes in untreated ERA patients (202) (Aboo et al., unpublished – Paper 2). Patients with a lympho-myeloid pathotype tended to have higher abundance of proteins involved in antibody production and lower abundance of proteins that prevent ECM-degradation (202) (Aboo et al., unpublished – Paper 2). Untreated ERA patients with a baseline lympho-myeloid pathotype also showed large improvements in CRP, DAS28CRP, MRI and ultrasound scores over time (202) (Aboo et al., unpublished – Paper 2). However, these improvements were not the result of low follow-up scores, but rather the result of high baseline scores of disease activity and severity (202) (Aboo et al., unpublished – Paper 2). By including HC in a subsequent analysis, we observed that the histologically defined synovial pathotypes might reflect molecular and cellular deviations from HC, rather than three distinct molecular conditions (202) (Aboo et al., unpublished – Paper 2). Notably, we found the pauci-immune fibroid pathotype to be molecularly and cellularly least different from HC, the diffuse-myeloid pathotype to be slightly more different from HC, and the lympho-myeloid pathotype to be most different from HC (202) (Aboo et al., unpublished – Paper 2). This is interesting because the pauci-immune fibroid pathotype - who had a non-immune-centric nature and no signs of inflammation – is known to respond poorly to csDMARDs and bDMARDs (202) (Aboo et al., unpublished – Paper 2).

2.3. PAPER 3 – UNPUBLISHED RESULTS

Protein Biomarker Signatures Covary with Measures of Disease Activity in Response to Treatment Initiation/Intensification in Rheumatoid Arthritis.

This investigation was made as another substudy of the same project as Paper 2. Utilizing the generated proteomics dataset and employing data integration, this investigation sought to study what proteins covary with different measures of disease activity, and subsequently identify a biomarker signature of disease activity in RA. The study identified several synovial tissue proteins that decrease along with measures of disease activity following treatment initiation or intensification. Notably, five proteins (ORM1, LRG1, SAA1, CRP and LBP) exhibited a direct relationship with ≥3 measures of disease activity, and the abundance of these proteins in plasma might constitute a biomarker signature for disease activity.

CHAPTER 3. DISCUSSION

The aim of this thesis was to utilize state of the art proteomics, bioinformatics, and the UGSB procedure for the collection of synovial tissue biopsies to answer questions that are fundamental to our understanding of RA. The ultimate goal was to establish the foundation for future studies on ICI-IIA and facilitate translational research that could help us understand RA trough insights into ICI-IIA, and vice versa, help us understand ICI-IIA through insights into RA. So, can insights into RA pave the way for a better understanding of ICI-IIA? And can we leverage our understanding of ICI-IIA to gain insights into RA? At the moment, we do not have all the necessary evidence to answer this conclusively. Nonetheless, early investigations, including those presented in Paper 1, Aboo et al. (2022) (1), suggest that there might be molecular and cellular similarities between these two conditions.

Mooradian et al. (2018) found that pretreatment levels of CXCL10, IL-17, and TGF-β1 (out of 1305 circulating proteins) could serve as biomarkers for prediction of ICI-IIA in patients who were treated with PD-1 inhibitors, CTLA-4 inhibitors, or a combination of both (334). This is interesting because CXCL10, IL-17, and TGF-β1 have all been implicated in the pathogenesis of RA (335–341). Serum levels of CXCL10 and IL-17 are elevated in RA and have been found to correlate various measures of disease activity (342–344). Furthermore, in Paper 2, an increase in synovial TGF-β1 abundance was observed in both ERA and LRA patients at baseline compared to HC (Aboo et al. unpublished – Paper 2, Supplementary Data 1) (202). Thus, the observation that pretreatment levels of these cytokines are predictive markers for ICI-IIA (334) could suggest that patients who develop ICI-IIA might have underlying preclinical RA that becomes unmasked, which is one of the proposed onset mechanisms of ICI-IIA (9,22,23). Contrarily, a study by Daoussis et al. (2020) measured TNFα, GM-CSF, IFN-g, IL-2, IL-4, IL-6, IL-10, IL-12 and IL-17, but found no significant upregulation in patients developing ICI-IIA (16). However, it is noteworthy to mention that Daoussis et al. (2020) measured post-treatment cytokine levels, which, as stated in Paper 1 was confirmed through correspondence with the authors (1,16). Consequently, these results cannot be compared directly with those of Mooradian et al. (2018).

When examining ICI-IIA at the synovial tissue level, our knowledge stems from case reports (25,26). Medina et al. (2021) reported a case where a 32-year-old male with metastatic recurrence was successfully treated with a combination of ipilimumab and nivolumab (25). However, the patient subsequently developed ICI-IIA, leading to discontinuation of ICI therapy and an examination of his synovial tissue biopsy that revealed infiltration of B cells, T cells, and macrophages (25).When they compared these histopathological findings with those of Dennis et al. (2014), the pioneering study on synovial histopathological phenotypes in RA, the biopsy exhibited striking similarities to lymphoid phenotype described in Dennis et al. (2014) (25,150). This is

also true when we compare the histopathological findings of Medina et al. (2021) with those of Humby et al. (2019), where the ICI-IIA in this case report appears to exhibit striking similarities with the lympho-myeloid pathotype in RA described by Humby et al. (2019) (25,132). Murray-Brown et al. (2020) reported a case where a 62-year-old male achieved oncological remission following treatment with nivolumab (26). However, following discontinuation of ICI therapy, the patient developed ICI-IIA that was nonresponsive to corticosteroids, MTX, and hydroxychloroquine (26). This led to examination of his synovial tissue biopsy that revealed infiltration of memory T cells, macrophages, but no B cells (26). When they compared these histopathological findings with those from three treatment-naïve RA patients, the biopsy exhibited similarities with RA in terms of immune cell infiltration phenotype, TNFα to IL-6 ratio, and hypervascularization and synovial hyperplasia (assessed through arthroscopy) (26). Based on these findings, the patient was treated with infliximab (a TNFi), which led to resolution of his synovitis and reduced CRP levels (26). Thus, preliminary evidence from case reports suggests there could be an overlap between ICI-IIA and RA, and exploring this further could have the potential to improve our understanding of ICI-IIA.

While studies on synovial tissue have indeed enhanced our understanding of RA, there is also cumulative evidence suggesting that histopathological analysis of synovial biopsies could be used to inform therapeutic choices in RA (127,128,130,132,145,345). Interestingly, by employing a similar approach, Murray-Brown et al. (2020) were able to successfully treat ICI-IIA that was nonresponsive to corticosteroids and csDMARDs (26). Thus, by leveraging the insights gained from synovial biopsies in the context of RA (127,128,130,132,145,345), we could potentially inform the clinical decision-making and improve the treatment of ICI-IIA (26). The potential of synovial tissue biopsies to inform clinical decision-making, however, becomes even greater when treatments are tailored based on transcriptomic signatures of synovial tissue, which has proven to surpass the conventional histopathological characterization in predicting treatment outcomes in RA (127,128). The next question to address is whether proteomics is as powerful as transcriptomics in analysing synovial tissue biopsies to subsequently elucidate pathological mechanisms in RA and predict treatment outcomes. In Paper 2, which also served as a "proof-of-concept" study, we were indeed able to answer some fundamental questions concerning RA (Aboo et al. unpublished – Paper 2) (202). Specifically, our study highlighted the potential of combining UGSB, proteomics and bioinformatics to elucidate the complex molecular landscape of RA at different disease stages and facilitated the identification proteomic determinants of synovial pathotypes (Aboo et al. unpublished – Paper 2) (202). Additionally, in Paper 3 (unpublished results) the combination of UGSB, proteomics, and bioinformatics was successfully able to identify biological pathways associated with disease activity and identify a biomarker signature of disease activity (Paper 3). Now that “the concept has been proofed”, the logical progression would be to utilize the combination of UGSB, proteomics and bioinformatics to study ICI-IIA. This task is very challenging when considering the

limited availability of synovial biopsies from ICI-IIA patients. However, our team at Odense University Hospital is currently establishing a well characterized ICI-IIA cohort comparable to the cohort in Paper 2. In the meantime, another opportunity might be to collaborate with researchers from larger medical centres, including Laura Cappelli from Johns Hopkins Medicine, who has established a large biobank containing blood and synovial fluid samples from ICI-IIA patients, but not synovial tissue biopsies. In Paper 2, we indeed imply that synovial tissue proteomics is far superior to plasma proteomics for elucidating the pathological mechanisms in RA, but this claim deserves a more nuanced discussion (Aboo et al. unpublished – Paper 2) (202). MS-based proteomics is indeed limited by the presence of a few very highly abundant plasma proteins, which hinders the identification and quantification of less abundant proteins (346–349). Consequently, MS-based plasma proteomics can only identify a couple of hundred proteins unless additional extensive sample preparation steps are employed to deplete highly abundant proteins, a process that inherently compromises reproducibility (346–350). However, proteomics methods based on proximity extension assays, such as Olink (351), or aptamers, such as SomaScan (352,353), deserve attention because these methods offer high sensitivity when analysing plasma, and can detect less abundant proteins without prior depletion of highly abundant plasma proteins (354–356). Thus, analysing the more readily available blood and synovial fluid samples using other proteomics technologies than MS-based proteomics could hold the key to understand ICI-IIA further at this point in time. That said, another interesting possibility could be to utilize the biomarker signature of disease activity in RA, that was identified in Paper 3 using MS-based proteomics, to investigate if this changes in response to ICI therapy, and whether it is more elevated in those who develop ICI-IIA. If this is true, then it could pave the way for early interventions and prophylactic treatments in patients undergoing ICI therapy (1). Nonetheless, one thing is certain regardless of the technology we employ: before advancing with further studies, as emphasized in Paper 1, we must establish well-defined diagnostic criteria and adopt a holistic approach to diagnosing ICI-IIA (1). Otherwise, we cannot establish valid endpoints for our biomarker discovery studies (1). Specifically, in Aboo et al. (2022) we stated *"We suggest that future studies should adapt general diagnostic workflows from rheumatological settings to ensure more valid endpoints. This includes a holistic approach with the integration of clinical findings, radiological findings, biopsies, questionnaires, and laboratory measurements. Rheumatology Common Toxicity Criteria (RCTC) have been proposed* (357)*, but since an ICI-IIA specific term is not listed in the scheme, the use of RCTC should be complemented by joint specific findings. Important points to consider when diagnosing rheumatic irAEs have also been proposed recently* (27)*, and especially the first point herein is worth considering in future studies: "Rheumatologists should be aware of the wide spectrum of clinical presentations of rheumatic and/or systemic immune-related adverse events that often do not fulfil traditional classification criteria of rheumatic and musculoskeletal diseases." – Cited from Kostine et al. (2021) (27).*" – Cited from Aboo et al. (2022) (1).

Building on this recommendation, integrating UGSB into the future diagnostic procedures for ICI-IIA may indeed provide valuable insights for establishing more reliable clinical endpoints. As already stated in the introduction, Orr et al. (2018) demonstrated, within the context of RA, that 71% of patients in DAS28CRP-defined remission still exhibited signs of synovial inflammation, and many patients with no so signs of synovial inflammation had a high DAS28CRP score (105). This raises questions about the sufficiency of relying on conventional clinical markers for diagnosis, not only in RA but in ICI-IIA as well.

As previously emphasized, utilizing synovial biopsies and pathotype classification can provide valuable insights into RA, and preliminary evidence from case reports suggests there could be an overlap between ICI-IIA and RA (25,26). Thus, synovial biopsies and pathotype classification could also serve as a useful tool for the diagnosis of ICI-IIA and the establishment of robust clinical endpoints for future biomarker discovery studies. However, our findings from Paper 2, adds another layer of complexity to this discussion. Proteomic analysis of UGSB samples from untreated ERA patients found that synovial pathotypes might reflect degrees of divergence from HC. Specifically, that the pauci-immune fibroid pathotype - characterized by a non-immune-centric nature and no signs of inflammation - was the least different from HC. Interestingly, a correspondence letter from Buch et al. (2020), suggested that this pauci-immune fibroid pathotypes might not always represent “true RA” but post-inflammatory scarring and/or coexistent OA (358). When we consider that the pauci-immune fibroid pathotype is refractory to csDMARDs and bDMARDs (128,132,359), together with its reported frequency in untreated ERA patients ranging from 19-27% (130,132,359), it aligns perfectly with the proportion of RA patients (20-30%) who are refractory to all treatment options (360). This further supports the hypothesis that these pauci-immune fibroid cases may not represent 'true RA' (358) and could possibly account for the treatment-resistant RA population. Indeed, if they have a non-immune-centric pathology, inhibiting the immune system will not improve their condition. If future studies can validate the hypothesis that the pauci-immune fibroid pathotype does not represent “true RA”, then the integration of synovial biopsies and subsequent pathotype classification into the diagnostic workflow could refine our perception of synovial pathotypes even further, and subsequently help us establish even more reliable clinical endpoints for ICI-IIA, but also in RA contexts.

So far, the discussion has focused on how insights from RA can be used to improve the understanding and treatment of ICI-IIA. But how can insights from ICI-IIA be utilized to gain insights into RA? RA develops slowly over the course of several years (32,361) which makes tracing the molecular and cellular changes from a healthy to a diseased state very challenging (1). ICI-IIA, on the other hand, develops within a much shorter timeframe (11,362) and in controlled settings, subsequently allowing patients to serve as their own controls (1). Thus, by collecting synovial tissue biopsies and blood samples from patients receiving ICI therapy, we can study the molecular and cellular changes in patients who develop ICI-IIA and patients who do not develop ICI-IIA (1). This could potentially shed light on the temporal development of RA, provided that ICI-IIA and RA share pathological mechanisms, and provided that ICI-IIA is not simply unmasking of preclinical RA. Studying the molecular and cellular changes from a healthy to a diseased state in a similar way, is very difficult in the context of RA and would require substantial resources and efforts. Biobanks offer a possible solution to this challenge because they contain samples that date many years back. Nonetheless, a significant limitation of biobanks is their lack of synovial tissue biopsies, which are pivotal for understanding RA pathology. Although a few biobanks, like the Dansk Rheuma Biobank, might contain synovial tissue biopsies, they are very unlikely to date back to a time when the patients were healthy. Therefore, at present, ICI-IIA might represent one of the most promising possibilities to elucidate the temporal dynamics of RA (beside animal models), which can help us gain insights into RA and pinpoint early disease drivers (1).

CHAPTER 4. CONCLUSION

This thesis highlights the potential of synovial tissue biopsies, proteomics, and bioinformatics to gain insights into the complex pathological mechanisms of RA. Employing a similar approach might also prove valuable as we progress to study the complexities of ICI-IIA. Nonetheless, this task requires multidisciplinary collaborations between rheumatologists, oncologists, pathologists, researchers from various fields, bioinformaticians, and patients. Preliminary evidence suggests that ICI-IIA and RA might share some pathological similarities. If this proves to be true, then RA can help us gain insights into ICI-IIA, and vice versa, ICI-IIA can help gain insights into RA. This could potentially lead to major breakthroughs in the treatment of both conditions and redefine the future of patients suffering from inflammatory arthritis. However, only time and collaborative efforts can truly validate this statement.

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