

High-content imaging–based
functional precision medicine in
rheumatoid arthritis

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

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Declaration

I hereby solemnly declare that this thesis was written independently under the guidance of Giulio Superti-Furga and Leonhard X. Heinz, without further assistance of third parties, that other sources besides those cited were not used and that excerpts from the sources used – both content and verbatim quotations – are indicated as such.

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Abstract

Rheumatoid arthritis (RA) is a frequent, chronic and systemic autoimmune disease, characterized by persistent synovial inflammation, leading to cartilage and bone destruction and subsequently to invalidity of the patient (Smolen et al., 2018). Even though a treatment response can often be achieved in the course of current state-of-the-art treatment regimens, due to a lack of predicative biomarkers, their selection is mostly based on a suboptimal trial-and-error principle resulting in a significant individual and socioeconomic burden (Smolen et al., 2023; Uhlig, Moe, & Kvien, 2014). At the same time, there is a strong trend towards precision medicine (PM) where biologic characterization is used to stratify patients and predict clinical response (Delpierre & Lefèvre, 2023; Jameson & Longo, 2015; McAlister, Laupacis, & Armstrong, 2017). Emerging from these developments, functional PM (FPM) aims to predict an individual's drug response on the basis of direct assessment of drug effectiveness on primary material (Letai, Bhola, & Welm, 2022).

Driven by recent advancements in high-content imaging (HCI)-based FPM in hematology (Kornauth et al., 2022; Snijder et al., 2017) we aimed to translate this technology into an assay for the functional in-depth characterization of peripheral blood mononuclear cells (PBMCs) of patients with RA. We therefore developed an RA-specific *ex vivo* drug perturbation panel applied to a unique 2D monolayer model culture compatible with perturbation-free immunolabelling of the most important PBMC immune cell subsets. Additionally, a computational image analysis pipeline was developed that allowed for the cell type-specific assessment of immune cell abundances and morphological characteristics on a single cell level in a cell-to-cell contact-specific manner.

A total 690 million PBMCs of 65 patients with RA and 33 healthy controls were functionally characterized upon *ex vivo* stimulation with RA-specific drugs and reference stimuli. This allowed for the systematic characterization of immune cell responses to RA-specific drugs. By linking experimental with clinical information, we were able to identify PBMC phenotypes specific to various *in vivo* treatments of RA patients and ultimately could identify PBMC responses that were significantly associated with the current treatment as well as the change in RA disease activity.

The presented study therefore forms the fundamental proof-of-concept for the application of HCI-based FPM in rheumatic autoimmune diseases and lays the foundation for the application of this methodology in upcoming prospective clinical trials.

Zusammenfassung

Rheumatoide Arthritis (RA) ist eine häufig auftretende, chronische und systemische Autoimmunerkrankung die durch anhaltende synoviale Entzündung ohne adäquate Behandlung zu Gelenksdestruktion und Invalidität führt (Smolen et al., 2018). Obwohl mit aktuellen Therapiestrategien häufig ein gutes Therapieansprechen erzielt werden kann, fehlen spezifische Biomarker, die die Wirksamkeit verfügbarer Medikamente prognostizieren können. Dies führt zu einem 'trial-and-error' Prinzip der Therapiewahl und schließlich zu einer hohen individuellen und sozioökonomischen Krankheitslast (Smolen et al., 2023; Uhlig et al., 2014). Daraus hervorgehend entwickelt sich ein Trend zur Präzisionsmedizin (PM), in der eine genaue biologische Charakterisierung des individuellen Patienten zur Stratifizierung und Vorhersage des klinischen Ansprechens genutzt wird (Delpierre & Lefèvre, 2023; Jameson & Longo, 2015; McAlister et al., 2017). Daraus hervorgehend wird in der sogenannten funktionellen PM (FPM) versucht, eine Vorhersage des individuellen Therapieansprechens durch die Quantifizierung von Medikamenteneffekten auf primärem Material treffen zu können (Letai et al., 2022).

Aufbauend auf kürzlich erzielten Erfolgen im Bereich der Hämatologie (Kornauth et al., 2022; Snijder et al., 2017) durch den Einsatz von Hochdurchsatzmikroskopie-basierten FPM, entwickelten wir einen Ansatz zur funktionellen Charakterisierung von mononukleären Zellen des peripheren Bluts (PBMCs) für Patienten mit RA. Hierfür etablierten wir ein experimentelles System, das es erlaubt die wichtigsten Typen an Immunzellen *ex vivo* mit RA-spezifischen Medikamenten zu behandeln und deren Phänotypen (Abundanz, Morphologie und Zellinteraktionsverhalten) mittels HCI und computergestützter Bildanalyse zu untersuchen.

In Summe konnten 690 Millionen PBMCs von 66 Patienten mit RA und 33 gesunden Spendern funktionell charakterisiert werden. Dies ermöglichte es die Auswirkungen der *ex vivo* Medikamente systematisch zu charakterisieren sowie, durch die Verknüpfung von experimentellen mit klinischen Daten, Rückschlüsse auf die durch *in vivo* Therapien hervorgerufenen Effekte auf PBMCs zu ziehen. Schließlich konnten wir mit unserer Methode PBMC Phänotypen identifizieren, die signifikant mit der aktuellen Krankheitsaktivität sowie Therapieansprechen assoziiert waren.

Entsprechend stellt diese Studie den Machbarkeitsnachweis für die Anwendung von HCI-basierter FPM im Bereich rheumatologischer Autoimmunerkrankungen dar und dient als Basis für zukünftige prospektive klinische Studien.

Publications arising from this thesis

***Ex vivo* imaging-based high content phenotyping of patients with rheumatoid arthritis**

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Abbreviations

ACPAs	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ADA	Anti-drug antibody
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ANCA	Anti-neutrophil cytoplasmatic antibodies
APR	Acute phase reactants
ATP	Adenosine triphosphate
AZA	Azathioprine
b	Biologic
BF	Bright field
BiTE	Bispecific T-cell engager
bs	Biosimilar
CAR	Chimeric antigen receptor
CDAI	Clinical disease activity index
CDSS	Clinical decision support systems
CIA	Computational image analysis
CD	Cluster of differentiation
CDSS	Clinical decision support systems
CeMM	Research Centre for Molecular Medicine of the Austrian Academy of Sciences
CNN	Convolutional neural network
COX	Cyclooxygenase
cs	Conventional synthetic
CT	Computed tomography

CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAPI	4',6-diamidino-2-phenylindole
DAS	Disease activity score
DIP	Distal interphalangeal joint
DMARD	Disease-modifying antirheumatic drug
DRAQ5	Deep red anthraquinone 5
ECM	Extracellular matrix
EGA	Evaluator global assessment
ESR	Erythrocyte sedimentation rate
EULAR	The European Alliance of Associations for Rheumatology
FAP	Fibroblast activation protein
FAPi	FAP inhibitor
FISH	Fluorescence in situ hybridization
FLS	Fibroblast-like synoviocytes
FPM	Functional precision medicine
GC	Glucocorticoid
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HCI	High-content imaging
HCS	Healthy controls
HTI	High throughput imaging
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
i.v.	Intravenous

JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
LEF	Leflunomide
LORA	Late onset RA
MACE	Major adverse cardiovascular event
MCP	Metacarpophalangeal joint
ML	Machine learning
MOA	Mode of action
MRD	Minimal residual disease
MTX	Methotrexate
MUV	Medical University of Vienna
NGS	Next-generation sequencing
NIH	National Institute of Health
NSAID	Non-steroidal anti-inflammatory drug
NUDT15	Nudix hydrolase 15
OA	Osteoarthritis
PADI2	Peptidylarginine deaminase 2
PD-L1	Programmed death-ligand 1
PDX	Patient derived xenograft
PD-1	Programmed death-1 receptor
PGA	Patient global assessment
PIP	Proximal interphalangeal joint
PM	Precision medicine
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor- κ B ligand

RF	Rheumatoid factor (RF)
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
s.c.	Subcutaneous
SE	Shared epitope
SDAI	Simplified disease activity index
SJC	Swollen joint count
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SpA	Spondylarthritis
SSZ	Sulfasalazine
STAT	Signal transduction and activator of transcription
STM	Synovial tissue macrophage
TDM	Therapeutic drug monitoring
TJC	Tender joint count
TMRM	Tetramethylrhodamine methyl ester
TNF	Tumor necrosis factor
TNFi	TNF-inhibitors
TPMT	Thiopurine S-methyltransferase
Ts	Targeted synthetic
UA	Undifferentiated arthritis
VEGF	Vascular endothelial growth factor
WES	Whole exome sequencing
WGS	Whole genome sequencing
2D	Two dimensional
3D	Three dimensional

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1 Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease, associated with autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs), in which the innate and adaptive immune system, with contribution of stromal cells, drives and perpetuates persistent synovial inflammation. If untreated, this can lead to cartilage and bone destruction and subsequently to invalidity of the patient (Smolen et al., 2018).

1.1.1 Epidemiology and burden of disease

Numerous studies and reviews report the rising prevalence of autoimmune diseases, not only causing significant individual harm but also increasing burden to the global socioeconomic situation (Dinse et al., 2022; Lerner, Jeremias, & Matthias, 2015; Norris, Johnson, & Stene, 2020). However, gaining full understanding of the epidemiology of autoimmunity is difficult, as it is influenced by a multitude of factors such as genetic predisposition, exposome, psychosocial stress and lifestyle choices (Miller, 2023).

Epidemiological studies report RA to be amongst the most prevalent inflammatory arthropathies, with rates between 0.5 and 1% in the global adult population as compared to other inflammatory joint diseases such as juvenile idiopathic arthritis (JIA: 0.02%), psoriatic arthritis (PsA: 0.10–0.25%) and spondylarthritis (SpA: 0.9-1.7 %)(Carron, De Craemer, & Van den Bosch, 2020; Gabriel & Michaud, 2009; Martini et al., 2022; Scotti, Franchi, Marchesoni, & Corrao, 2018). The age adjusted overall female to male ratio of patients with RA is reported to be ~ 2.45:1 with individual register studies indicating higher ratios in younger and lower ratios in older patients (Black et al., 2023; Kvien, Uhlig, Ødegård, & Heiberg, 2006). Incidence rates of RA are associated with age with cumulative lifetime risk raising to 3.6% for women and 1.7% for men as compared to being < 1% for both groups at an age under 50 years (Crowson et al., 2011). Disease in patients with an early onset is associated with higher rates of autoantibody positivity whilst an onset above the age of 58 years is associated with higher clinical disease activity, more pronounced radiographic findings at baseline and higher systemic inflammation as quantified by erythrocyte sedimentation rate and serum-CRP levels (Innala et al., 2014). In addition to impact of disease on the individual patient, RA causes substantial socioeconomic burden comprising

predominantly of care and treatment-associated costs (drug costs, inpatient and outpatient care), absenteeism and work disability (Hsieh et al., 2020).

1.1.2 Genetic contribution and risk factors

Current understanding depicts RA as a multifactorial systemic autoimmune disease, that forms on a set of genetic and epigenetic risk factors upon the influence of known and unknown environmental factors (Smolen et al., 2018).

Despite the heterogenous clinical presentation, twin studies showed high rates of heritability of RA, suggesting genetic factors to contribute for ~60% of disease liability (MacGregor et al., 2000). The most prominent of those genetic contributors to RA is the “shared epitope” (SE), a conserved sequence in the HLA-DRB1 gene, encoding for one of three amino acid variants (QKRAA, QRRRA, or RRRRA) of the third hypervariable region of the HLA-DRβ1 chain (Gregersen, Silver, & Winchester, 1987). Another significant genetic variant associated with various autoimmune diseases (type 1 diabetes, RA, systemic lupus erythematosus (SLE), etc.) is the single nucleotide polymorphism (SNP) 1858C>T in the PTPN22 gene, increasing the risk of developing RA via altered T- and B-cell receptor signaling and type-I-interferon (IFN) response (Begovich et al., 2004; Mustelin, Bottini, & Stanford, 2019; Stanford & Bottini, 2014). Large scale genome-wide association studies (GWAS) have been able to identify a growing number of genetic variants linked to RA. Of importance, many of these risk variants are found in genes encoding for proteins involved in immune and inflammatory pathways, some of which are already well-established therapeutic targets (Ishigaki et al., 2022; Okada et al., 2014). Even though these analyses and the development of polygenic risk scores provide valuable insight into the genetic background of RA etiology, they have so far failed to fully explain disease susceptibility, stratify patient subpopulations or predict treatment response.

Beyond the genetic background, epigenetic processes such as DNA methylation, histone modification, chromatin remodeling and post-transcriptional regulation have been identified as crucial processes in the disease development of RA (Klein & Gay, 2015; Nemtsova et al., 2019; C. Yang et al., 2022). As a showcase, synovial fibroblasts, resident stromal cells of the synovial niche and important immunologic contributors, have been demonstrated to undergo significant epigenetic reprogramming, thereby adapting a proinflammatory invasive phenotype (Ai et al., 2018).

Additional risk factors for RA development and disease activity comprise of smoking, exposure to airborne pollutants, periodontal disease, obesity and socioeconomic status.

Despite its known adverse effect on the cardiovascular system, lung cancer and chronic obstructive lung disease, tobacco smoking, and especially the contained ingredient nicotine, is a known negative modulator of the innate and adaptive immune system (Sopori, 2002). In contrast, cigarette smoking is strongly associated with the development of ACPA-positive RA in the presence of the SE (Ishikawa & Terao, 2020). A meta-analysis by Sugiyama et.al. highlighted the increased risk of developing RA for 20 or more pack-years of smoking in men (odds ratio 2.31) and women (1.75) respectively (Sugiyama et al., 2010). In addition, active smoking status is associated with increased measures of systemic inflammation and clinical disease activity in RA patients and levels of APCAs were higher in patients who ever smoked as compared to patients who never smoked (Sokolove et al., 2016). Mechanistically smoking promotes the citrullination of proteins leading to the creation of autoantigens that trigger an immune response in an SE-restricted context (Klareskog et al., 2006). Other inhaled pollutants that are linked to RA development include silica and textile dust (Stolt et al., 2005; Too et al., 2016). Periodontal disease and alterations of the oral microbiome are linked to RA development and vice versa patients with higher disease activity also presented with more severe periodontitis (Rodríguez-Lozano et al., 2019; Zhang et al., 2015). Similarly, dysbiosis of the gut microbiome, e.g. increase of *Prevotella copri* and *Lactobacillus* species in early RA and depletion of *Hemophilus* species in active RA, is found in patients with preclinical and manifest RA (Zhang et al., 2015; Zhao et al., 2022). Reversely, various treatments for RA are associated with changing the gut microbiome (Zhao et al., 2022). Finally, meta-analysis confirmed the association of elevated body mass index with increased risk of RA development (Ohno, Aune, & Heath, 2020) and a low socioeconomic status was reported to be associated with poor outcome of RA (Dey et al., 2022).

1.1.3 Cellular and molecular pathophysiology

Current research describes RA development as a sequential process in which on a susceptible genetic and epigenetic background environmental factors trigger immunologic events that translate into a preclinical phase. This phase is characterized by cellular immune responses and generation of autoantibodies against self-antigens (e.g. citrullinated peptides), accompanied by a systemic increase in levels of cytokines and chemokines and clinical signs of autoimmunity. Ultimately synovial hyperplasia and immune cell infiltration occurs forming the pannus tissue that leads to cartilage and bone degradation and thereby joint destruction (Alivernini, Firestein, & McInnes, 2022).

Citrullination is the post-translational biological process of converting an arginine residue to a citrulline, catalyzed by enzymes called peptidylarginine deiminases. This modification results in the conversion of a positively charged to an uncharged amino acid side chain, yielding possible changes in protein conformation, subcellular localization, stability and protein-protein interaction propensities (Christophorou, 2022). Additionally citrullination can expose cryptic peptides, usually hidden from the immune system, via protein destabilization and altered protease cleavage leading to unique repertoire of citrullination-dependent autoantigens (Curran et al., 2023). Tobacco smoking is a key environmental factor that induces protein citrullination via the induction of peptidylarginine deaminase 2 (PAD2) in the lung (Makrygiannakis et al., 2008), leading to the post-translational modification of proteins such as vimentin, α -enolase and fibrinogen (Lugli et al., 2015). However, smoking may not be the only extrinsic factor causing excessive citrullination as oral dysbiosis, e.g. via *P. gingivalis*, was reported to induce ACPAs (Dissick et al., 2010) and citrullinated peptides from oral bacteria were recognized by ACPAs of RA patients (Brewer et al., 2023).

Antigen-presenting cells expose these self-antigens to T-cells via MHC class II molecules, particularly those containing the SE, as peptide citrullination was shown to foster the peptide MHC interaction, finally leading to autoantibody formation (Hill et al., 2003). Besides ACPAs, that target citrullinated peptides, autoantibodies associated with RA include RF, directed against the Fc portion of IgG, but also autoantibodies against other post-translational modifications like carbamylation and acetylation (Trouw, Rispens, & Toes, 2017). In a process called epitope spreading, that involves B- and T-cells, the repertoire of antibodies against self-antigens diversifies, thereby sustaining autoimmunity (Cornaby et al., 2015). The importance of this process was especially demonstrated for the early phase of disease progression from undifferentiated arthritis (UA) to RA, where patients that later evolved from UA to RA showed a more diverse repertoire of ACPAs at the time of disease onset as compared to patients who remained UA (van der Woude et al., 2010). In addition, studies have shown that autoantibodies display significant changes in their posttranslational modifications during RA disease development, i.e. an IL-23-TH17-cell-dependent reduction of Fc galactosylation, coinciding with the transition from seropositive arthralgia to symptomatic RA (Pfeifle et al., 2017; Rombouts et al., 2015).

However, the presence of autoantibodies alone is not enough to translate into manifest RA as RF positivity can be found in ~4% (Nielsen, Bojesen, Schnohr, & Nordestgaard, 2012; van Schaardenburg, Lagaay, Otten, & Breedveld, 1993) and ACPA positivity in ~1-3% (Hensvold et al., 2017; van Zanten et al., 2017) of the general population. One or multiple additional unknown triggers are required to translate autoantibody positivity into synovitis, characterized by the immune cell infiltration into the synovium and hyperplasia of the

synovial lining layer. The normal synovial membrane comprises of a multilayered structure of a 1-3 cell thick lining layer, formed by fibroblasts-like synoviocytes (FLS) and tissue-resident synovial tissue macrophages (STMs) sitting on top of a less dense sublining structure, containing FLS, STMs, blood- and lymph vessels and adipocytes (M. D. Smith, 2011). Functionally, the synovial membrane serves multiple roles in maintaining the joint. Firstly, via the production and absorption of synovial fluid, with its main components hyaluronic-acid and lubricin, providing lubrication to the joint. Secondly, supply of nutrients and metabolites for the avascular joint cartilage while at the same time clearing debris. And finally the formation of a non-adherent mechanical and immune-protective barrier (M. D. Smith, 2011). Whilst under normal conditions FLS maintain the synovial membrane structure by constant remodeling via a delicate homeostasis between the production of extracellular matrix and matrix-degrading enzymes, synovitis caused by RA leads to synovial hyperplasia and immune cell infiltration. During synovial inflammation STMs produce large quantities of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-8 (Kurowska-Stolarska & Alivernini, 2022) and FLS contribute by IL-6 production, secretion of matrix-metalloproteinases (MMPs) and exerting an invasive phenotype (Tsaltzkan & Firestein, 2022). At the same time infiltration of adaptive immune cells, such as T- and B-cells, occurs in the sublining compartment where they can form ectopic germinal centers leading to B-cell proliferation and antibody production (Humby et al., 2009; Timmer et al., 2007). Joint destruction occurs due to the invasive properties of the synovial pannus tissue infiltrating cartilage and bone. This invasive phenotype of the stromal tissue is mainly driven by RA-FLS that show an aggressive and infiltrating phenotype while producing large quantities of MMPs (Noss & Brenner, 2008). Bone destruction simultaneously occurs due to osteoclast activation and maturation which is driven by the complex proinflammatory cytokine milieu of the inflamed joint comprising of essential factors such as receptor activator of nuclear factor- κ B ligand (RANKL), TNF, IL-6 and IL-1 (Redlich et al., 2002; Takayanagi, 2007). It is this inflammatory signaling network that sustains disease and therefore yields a number of opportunities for targeted therapeutic intervention in the form of biologicals or small molecules that interfere with proinflammatory cytokines such as TNF or IL-6 (Smolen et al., 2018).

Important inflammatory cytokines such as IL-6 signal via the Janus kinase (JAK) - signal transduction and activator of transcription (STAT) cascade, which plays a critical role in RA (Malemud, 2018). Upon upstream receptor activation (e.g. by various interleukins, granulocyte-macrophage colony-stimulating factor (GM-CSF) or IFN- γ) activation of JAKs (JAK 1-3 and TyK2) activation leads to STAT phosphorylation, dimerization and translocation into the nucleus, promoting the expression of inflammatory mediators that

ultimately drive immune cell activation, synovial proliferation and thereby sustain the systemic and joint inflammation (O'Shea, Holland, & Staudt, 2013).

Altered JAK-STAT signaling is also crucial to exert the invasive proinflammatory phenotype of RA-FLS as JAK inhibition via Peficitinib and Tofacitinib reduced the production of growth factors, proinflammatory cytokines and MMPs and also attenuated the hyper proliferic phenotype in a three-dimensional model culture (Emori et al., 2020). Indeed, gaining comprehensive knowledge on the individual signaling components relevant to RA and deciphering the mechanism of dysregulation will not only help to understand disease pathogenesis but will also provide valuable novel targets for therapeutic intervention (Ding et al., 2023).

1.1.4 Disease manifestation and quantification of disease activity

In RA, systemic autoimmunity translates into synovial inflammation, leading to synovial hyperplasia and consecutive cartilage and bone destruction. The clinical representation of this immunological process involves chronic pain, soft swelling of the joints, often accompanied by morning stiffness and tenderness and decreased range of motion, and if untreated, irreversible joint destruction and disability of the patient (Grassi, De Angelis, Lamanna, & Cervini, 1998). Definite biomarkers are missing and the extent of disease activity and treatment response are primarily assessed with clinical scores, of which some include laboratory parameters that reflect systemic inflammation (Anderson et al., 2012).

Clinical presentation of RA can be heterogeneous and might share similarities with other arthritides, resulting in challenges for the rheumatologist when diagnosing individual patients. Therefore, no formal diagnosis criteria exist, but for the definition of homogenous populations for studies and trials, RA can be classified according to the 2010 ACR/EULAR classification criteria (Table 1) (Aletaha et al., 2010).

Table 1: Classification criteria of RA, adapted from (Aletaha et al., 2010): "Classification criteria for RA (score-based algorithm: add score of categories A–D a score of $\geq 6/10$ is needed for classification of a patient as having definite RA)" (Aletaha et al., 2010).

A. Joint involvement	
1 large joint¶	0
2–10 large joints	1
1–3 small joints (with or without involvement of large joints)	2
4–10 small joints (with or without involvement of large joints)	3
>10 joints (at least one small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least one test result is needed for classification)	
Normal CRP and normal ESR 0	0
Abnormal CRP or normal ESR 1	1
D. Duration of symptoms	
<6 weeks	0
≥ 6 weeks	1

Like other arthritides, RA often shows a typical pattern of symmetric joint involvement. This includes the proximal joints of the hands and feet (metacarpophalangeal joints (MCPs) and proximal interphalangeal joints (PIPs)) but spares the distal interphalangeal joints (DIPs). Additionally, wrists and ankles are often affected, as well as elbows, shoulders, knees and hip joints (Smolen et al., 1995). While RA does not affect other axial joints, synovitis of the atlanto-dental joint as part of the atlantoaxial joint can be found in about a quarter of early arthritis patients by MRI imaging, which can ultimately lead to subluxation and instability requiring surgical intervention (Carotti, Salaffi, Di Carlo, Sessa, & Giovagnoni, 2019; Magarelli et al., 2010). Affected joints appear with a soft, doughy swelling in the clinical examination and patients report them to be tender upon pressure during the examination. An additional symptom is the prolonged morning stiffness for often over one hour of the joints involved, regularly accompanied by a severe impairment of motion (Grassi et al., 1998). Radiological findings of the often-severe joint destruction in RA include pathognomonic erosions of the small joints, joint space narrowing, subluxations and deformities (Sommer et al., 2005). And there is a strong link between RA and osteoporosis, as mediators of local and systemic inflammation elevated in RA, such as pro-inflammatory interleukins and TNF, are known to promote differentiation and activation of osteoclasts via

various pathways resulting in reduced bone density (Llorente, García-Castañeda, Valero, González-Álvaro, & Castañeda, 2020).

No definitive biomarkers exist to monitor RA disease activity, therefore several scores have been developed to assess disease activity clinically with possible additional serological factors such as serum-CRP and erythrocyte sedimentation rate. Key components of clinical scoring is the standardized recording of the number of affected joints in the form of swollen (SJC) and tender (TJC) joint counts as well as the quantification of subjective disease activity on numerical rating scales by the patient and the healthcare professional (Anderson et al., 2012). The clinical disease activity index (CDAI) for example waives laboratory results for acute phase reactants (APR) and numerically adds a 28 joints SJC, a 28 joint TJC to the patient global assessment (PGA in cm on a 10 cm scale) and evaluator global assessment (EGA in cm) for a composite score that correlates well with scores that include APR like the disease activity score (DAS)28 and the simplified disease activity index (SDAI) (Aletaha et al., 2005; Aletaha & Smolen, 2007). CDAI thresholds for disease activity are 0 - 2.8: remission, 2.9 - 10: low disease activity, 10.1 - 22: moderate, 22.1 - 76: high for remission (Aletaha & Smolen, 2005).

For the purpose of clinical trials, the improvement in disease activity to a given intervention can be assessed in ordinal categories as unimproved or reaching the 20% (ACR 20), 50% (ACR 50) and 70% (ACR 70) threshold in improvement of TJC and SJC and 20% / 50% / 70% improvement of 3 of 5 ACR core set measures (PGA, EGA, pain, disability and APR) (Felson et al., 1995; Felson, Anderson, Lange, Wells, & LaValley, 1998). Other than the numeric thresholds of disease activity indices, ACR/EULAR defines Boolean remission criteria for RA as fulfilling all of the following: $SJC \leq 1$, $TJC \leq 1$, $CRP \leq 1$ mg/dL and $PGA \leq 2$ cm (measured on 10 cm scale) (Studenic et al., 2023).

RA, as a chronic systemic inflammatory condition, is associated with a range of physical comorbidities, including cardiovascular disease, osteoporosis, malignancies, but also neuropsychiatric disorders like depression, anxiety as well as fatigue (Dougados et al., 2014; P. C. Taylor et al., 2021). Risk of death due to cardiovascular disease (including ischemic heart disease and cerebrovascular disease) is increased by ~50% in RA patients (Aviña-Zubieta et al., 2008) and therefore special care should be taken in the routine treatment of RA patients (Agca et al., 2017). Whilst various immunomodulatory treatments may affect the cardiovascular risk beneficially or adversely (Baoqi et al., 2021), the individual patient's cardiovascular conditions might limit treatment options due to contraindications for specific drugs (Konzett & Aletaha, 2024). Additionally, in comparison to the general population, RA is associated with a significantly increased rate of bone, joint, connective tissue and respiratory infections, even when adjusted for demographic and

lifestyle factors as well as comorbidities and (GC) co-medication (Doran, Crowson, Pond, O'Fallon, & Gabriel, 2002). The risk of serious infections is also higher for patients with RA as compared to patients with non-inflammatory rheumatic and musculoskeletal diseases (Bella et al., 2019) and bDMARD treatment increases the risk for opportunistic infections (Greenberg et al., 2010; Ozen et al., 2019). Meta-analysis demonstrated RA patients to have an ~10% increased risk for malignancies in general, particularly lymphomas (2-3 fold increase) and lung cancer (1.63 fold increase) as compared to the general population (Simon, Thompson, Gandhi, Hochberg, & Suissa, 2015). Patients with RA have a significantly reduced bone mineral density and two-fold increased frequency of osteoporosis as compared to the general population (Haugeberg, Uhlig, Falch, Halse, & Kvien, 2000). Chronic pain, defined as persistence or recurrence of pain sensation for over 3 months, is a major challenge in the care of RA patients, as pain is on the one hand a cardinal symptom of disease activity, but can on the other hand be caused by secondary conditions such as osteoarthritis and fibromyalgia (Mathias et al., 2021; Treede et al., 2019). Even though recommendations for screening approaches to prevent comorbidities in RA patients are scarce, EULAR emphasizes the importance of a structured assessment of risk factors and collection of data on coexisting conditions to consider in routine care (Baillet et al., 2016).

1.1.5 Current treatment approaches

Current state-of-the-art treatment regimens for RA aim to achieve remission or low disease activity in a treat-to-target approach (Smolen et al., 2010), by the administration of a variety of disease-modifying antirheumatic drugs (DMARDs), including the conventional synthetic (cs) DMARDs, biologic (b) DMARDs and their biosimilars (bs) as well as targeted synthetic (ts) DMARDs (Smolen et al., 2023).

The term csDMARDs summarizes a class of small molecule drugs, comprising of methotrexate (MTX), sulfasalazine (SSZ) and leflunomide (LEF). For MTX and SSZ the mechanism of action is poorly understood, whilst for LEF its active metabolite A77 1726 inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, that catalyzes the rate limiting step of pyrimidine de novo synthesis, thereby limiting the expansion of activated lymphocytes (Fox et al., 1999). Meta-analysis has confirmed MTX' effect on clinical response, improving the physical function and halting radiographic progression, whilst adverse effects (e.g. nausea, hepatotoxicity and bone marrow suppression) can be attenuated by folic acid supplementation (Lopez-Olivo et al., 2014). Similar benefits have been demonstrated for SSZ (Suarez-Almazor, Belseck, Shea, Wells, & Tugwell, 2000), the

daily dosage of 3 g, however can limit patient adherence and treatment duration (G. Kim, Barner, Rascati, & Richards, 2016).

bDMARDs and their biosimilars comprise of a heterogeneous group of antibodies and receptor constructs that target cytokines like TNF and IL-6, interfere with T-cell co-activation or deplete B-cells (J. A. Singh et al., 2009). TNF-inhibitors (TNFi) include infliximab, adalimumab, golimumab, certolizumab-pegol and etanercept, which all negatively interfere with TNF signaling, effectively reduce synovial inflammation, cartilage and bone destruction (Tracey, Klareskog, Sasso, Salfeld, & Tak, 2008). IL-6 plays a central role in RA pathogenesis, mediating pro-inflammatory immune responses via contribution to synovitis, osteoclast differentiation, B-cell maturation and Th17 differentiation (Pandolfi et al., 2020). Therefore blocking IL-6 receptor signaling by tocilizumab, a humanized IL-6 receptor blocking antibody and sarilumab, a human IgG1 antibody against the IL-6 receptor have proven as an effective treatment for RA (Huizinga et al., 2014; Smolen et al., 2008). Abatacept is a CTLA-4-IgG1 fusion protein, that mechanistically interferes with T-cell co-activation by binding to CD80 and CD86 on antigen-presenting cells, thereby inhibiting CD80/86-CD28 interaction, which is important for T-cell proliferation, differentiation and effector function (Alegre, Frauwirth, & Thompson, 2001; Cope et al., 2024). Rituximab, a chimeric antibody directed against CD20, which is expressed on pre- and mature B-cells, and was developed to induce B-cell depletion, amongst other mechanisms via antibody-dependent cytotoxicity, as treatment for B-cell malignancies (Pierpont, Limper, & Richards, 2018). Given their pivotal role in various autoimmune diseases, B-cells, and their depletion have been identified as an effective treatment strategy for RA in the combination with MTX or as monotherapy (Edwards et al., 2004; Lopez-Olivo, Amezcua Urruela, McGahan, Pollono, & Suarez-Almazor, 2015).

JAK inhibitors are currently the only representatives of the class of tsDMARDs approved for RA treatment, and are, in comparison to bDMARDs, administered orally (Di Matteo, Bathon, & Emery, 2023). JAKs mediate intracellular signaling of type I and type II cytokine receptors via STAT and some inhibitors show JAK-selectivity (filgotinib: JAK1, upadacitinib: JAK 1,2) whilst others are none selective (baricitinib, tofacitinib, peficitinib) (Bonelli et al., 2024). Even though interference with the JAK-STAT pathway proved effective in the treatment of various autoimmune diseases, adverse effects like frequent herpes zoster reactivation and the increased occurrence of malignancies and major adverse cardiovascular events (MACE) request increased caution in specific patient populations (Bonelli et al., 2024).

Due to the lack of DMARDs and their high anti-inflammatory and immunosuppressive potential, GCs have long been the cornerstone of RA treatment, however their significant long-term adverse effects limit their application to short-term pulse therapy. Their main

application in current state-of-the-art treatment regimens is in the form of short-term tapers as bridging in the combination of with DMARDs to control disease activity until the DMARD reaches its full effectiveness (Smolen et al., 2023). Their value for limiting structural disease consequences, however is highlighted by their capability to beneficially influence radiographic progression, especially in early arthritis (Kirwan, Bijlsma, Boers, & Shea, 2007). Even though low-dose GC administration might not suppress adrenal function, its long-term use is discussed controversially in the literature, particularly in the context of difficult-to-treat RA, as a clear dose dependency was demonstrated. Increased all-cause mortality was shown to increase in a dose-dependent way starting from 8-15 mg prednisone daily as well as upon reaching a cumulative dose of 40 g (del Rincón, Battafarano, Restrepo, Erikson, & Escalante, 2014). Of importance, the management of measures for the prevention of long-term GC adverse effects, such as osteoporosis, has to be part routine care by rheumatologists, especially if GC doses reach higher levels (Duru et al., 2013).

While non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their analgetic and anti-inflammatory propensities, their role in RA treatment is limited due to a lack of disease modification and in parts by their severe gastrointestinal and cardiovascular adverse effects (Crofford, 2013). Even though cyclooxygenase (COX)-2 selective drugs show benefits in terms of reduced rates of upper gastrointestinal events as compared to non-COX-selective NSAIDs, their application is associated with a significantly increased risk for cardiovascular events (Bombardier et al., 2000; Fitzgerald, 2004).

Novel targeted treatment approaches for RA facilitating alternative ways of B-cell depletion or targeting FLS directly have been demonstrated in case reports or have shown promising results in animal models in the following studies and are awaiting first applications in human. Beyond their application in the field of hematology, cell-based therapies including chimeric antigen receptor (CAR) T-cells targeting B-cells, have proven effective in various autoimmune diseases (Bhandari, Bhandari, & Bhandari, 2023; Schett, Mackensen, & Mougiakakos, 2023). For RA for example, Haghikia et al. recently reported the effectiveness of targeting CD19⁺ B-cells by fully human second-generation autologous CARs in a patient with RA and myasthenia gravis (Haghikia et al., 2024). Genetic engineering has led to the development of fourth-generation CARs, that beyond their capability to deplete B-cells can secrete antibodies (in this case against IL-6 and TNF), that have been demonstrated efficacious and safe in a set of three RA patients, previously refractory to treatment (Y. Li et al., 2025). Another approach to achieve B-cell depletion is the activation of CD3⁺ T-cells and consecutive lysis of B-cells via blinatumomab, a bispecific T-cell engager (BiTE), first applied in the treatment of B-precursor acute lymphoblastic leukemia (Topp et al., 2015). Its application for the treatment of multidrug-resistant RA was showcased in compassionate-

use study of 6 patients resulting in effective B-cell depletion, reduced T-cell activity and significant clinical improvement (Bucci et al., 2024). Given its specific expression by activated FLS at the site of inflammation, fibroblast activation protein (FAP) might represent a novel opportunity to target FLS directly (Bauer et al., 2006). PET-imaging of gallium 68-labeled FAP inhibitor (FAPI) showed significant correlation of FAPI uptake with clinical sites of inflammation and radiographic joint destruction (Luo et al., 2023) and has therefore been suggested as a promising target for CAR therapy (Aghajanian, Rurik, & Epstein, 2022). Beyond its diagnostic function, FAP has been demonstrated as a therapeutic target for radioligand therapy in an animal model of RA (Tseng et al., 2025). Due to the novelty of these approaches, their lack of validation in large scale cohorts and the absence of long-term efficacy and safety data, they are not yet reflected in current routine treatment guidelines (Smolen et al., 2023).

In terms of clinical routine disease management, the overarching goal of RA treatment is to achieve sustained remission or low disease activity in all patients by suppression of inflammation, alleviating pain, restoring mobility and preventing long term joint destruction and disability (Smolen et al., 2010). Frequent follow-up visits are important to monitor disease activity and assess treatment success – if no improvement is attained, treatment should be adjusted after 3 months, if the treatment target has not been met in 6 months, the therapy should be adapted (Smolen et al., 2023). Current EULAR treatment recommendations advise to immediately initiate therapy in patients diagnosed with RA with MTX and the addition of short-term GC tapers (Smolen et al., 2023). If contraindications to a csDMARD treatment with MTX are present (e.g. renal insufficiency or intolerance) therapy with SSZ or LEF should be established (Smolen et al., 2023). In the presence of poor prognostic factors (e.g. persistent high disease activity, high APR, high SJC, high levels of autoantibodies, presence of early erosions) a bDMARD / tsDMARD should be added, however risk factors, especially for JAK-inhibitor treatment should be considered (Smolen et al., 2023). If no poor prognostic factors are present, a second csDMARD can be considered (Smolen et al., 2023). Indeed, studies have demonstrated the superiority of combination treatment of csDMARD + bDMARD / tsDMARD, however if no csDMARD can be administered, anti-IL-6 / tsDMARD mono therapy might be superior than TNF-blockade / abatacept / RTX alone (Smolen et al., 2023). Upon failure of one bDMARD / tsDMARD, any other bDMARD / tsDMARD can be administered, even within the same class and in sustained glucocorticoid-free remission dose reduction of the cs / b / tsDMARD can be considered (Smolen et al., 2023).

1.1.6 Biomarkers

The current 2010 ACR/EULAR classification criteria for RA incorporate RF and ACPA status as well as the APRs ESR and serum CRP as relevant items for the classification of RA, however spare other possible genetic, transcriptomic, proteomic, lipidomic, socioeconomic or clinical imaging biomarkers (Aletaha et al., 2010). Similarly, the current treatment guidelines list the presence of autoantibodies (RF and/or ACPAs), high disease activity despite csDMARD treatment, failure of 2 or more csDMARDs, early erosions as well as a high SJC as poor prognostic factors (Smolen et al., 2023). However, neither of these known factors directly translate into the recommendation for a specific drug MOA, highlighting the need for the development and validation of novel biomarkers for patient stratification and treatment selection.

1.1.7 Treatment limitations and needs

Even though the introduction of cs-, b- and tsDMARDs and standardized treatment led to significant advances in the care of RA and remission can be achieved in most patients, there are still unmet needs when it comes to understanding pathophysiology, treatment response prediction and ultimately preventing disease rather than long-term immunosuppression (Smolen et al., 2018). The EULAR task force on the management of RA highlighted a comprehensive set of open questions along the treatment recommendations in the form of a research agenda in 2023 (Smolen et al., 2023).

Amongst and beyond these points, questions on the optimal approach to arthralgia and standard of care in a pre-arthritis state arise. Various clinical trials aimed to prevent disease onset via therapeutic intervention in at-risk cohorts as reviewed by Deane et al. (Deane et al., 2025). In the PRAIRI study, for example, individuals positive for RF and ACPAs but without signs of arthritis were treated with a single dose of 1000 mg RTX or placebo, leading to a delay of 12 months in arthritis development in the RTX-treated group (Gerlag et al., 2019). In two similar studies (APIPPRA and ARIAA) preventive intervention with abatacept was associated with reduced progression to RA (25% vs. 37% and 8% vs. 35%) as well as improvement patient-reported outcomes and subclinical inflammation in ultrasonography and MRI imaging (Cope et al., 2024; Rech et al., 2024). And even though the TREAT-EARLIER study reported no differences in the primary endpoint of RA development upon preventive MTX treatment the improved clinical outcomes in all these studies suggest an emerging role for therapeutic intervention in the pre-arthritis phase of at-risk individuals (Deane et al., 2025; Krijbolder et al., 2022).

The persistent need for GCs in routine treatment of RA (studies report that 30-60% of patients remain on long-term GC treatment (George et al., 2021)) highlights the importance of low-barrier access to cs- b- and tsDMARDs on a global scale (Sokka, Envalds, & Pincus, 2008). This even more in the context of striking evidence for long-term adverse effects (del Rincón et al., 2014), however knowledge on definite thresholds is still scarce. With the manifold of reports exploring various omics approaches for biomarker development, many of these studies yet lack validation in large scale cohorts. In addition, if these studies achieve to stratify patients and disease based on biological evidence, we might have to overthink our perception of RA as a single disease entity and rather see it as a common clinical endpoint (Stanich, Carter, Whittum-Hudson, & Hudson, 2009), allowing for subset-specific treatment regimens.

Even though the initiation of current DMARD treatment upon diagnosis is well defined (Smolen et al., 2023), only few studies investigate thresholds when to taper or withdraw an established immunosuppression (Tascilar et al., 2021). Initial reports, like the NOR-DRUM trial argue against the routine measurement of anti-drug antibodies (ADAs) / neutralizing antibodies in clinical practice (Syversen et al., 2021), however, there is little evidence for their value in objectifying loss of bDMARD efficacy. Similarly, tools to assist personalized dosing and support therapy adherence are needed that might optimize individualized care (Hebing et al., 2022).

Given the European Medicines Agency's and United States Food and Drug Administration's warning for adverse effects of JAK-inhibitors, restricting their application in specific patient populations, future research is needed if JAK specificity is of importance and what mechanisms drive these adverse events in detail (Bonelli et al., 2024). Furthermore, it is still unknown if a combination treatment of JAK-inhibitors and bDMARDs might be a safe and superior treatment option for RA as current evidence is scarce (Lignou et al., 2025). In addition, there is little data suggesting a specific sequence of bDMARDs to follow until a sufficient treatment response is found (Solomon et al., 2021), as available studies addressed this question rather from an economic point of view (Ghabri, Binard, Pers, Maunoury, & Caro, 2020).

Finally, beyond deciphering the pathomechanism and adequately treating the inflammatory component of RA, efforts have to be made for a sufficient recognition and treatment of secondary pain syndromes and OA in RA patients (Mathias et al., 2021). In summary, even though significant progress has been made in understanding and treating RA all these points highlight the still existing unmet need for informed personalized treatment of RA.

1.2 Precision medicine and functional precision medicine

Precision medicine (PM) and personalized medicine are two terms often used synonymously. However, whilst personalized and individualized medicine tailor diagnostic and therapeutic procedures to the individual patient's clinical presentation, PM aims to biologically characterize and stratify patients with similar clinical presentation to predict beneficial diagnostic and therapeutic procedures (Delpierre & Lefèvre, 2023; Jameson & Longo, 2015; McAlister et al., 2017).

Classic PM builds on the approach of static features like the presence of genetic factors, occurrence of mutations or expression of biomarkers to guide therapy (Collins & Varmus, 2015). Functional precision medicine (FPM), in contrast, aims to test primary material from individual patients for treatment response allowing informed decision making (Letai et al., 2022). Up to now, approaches to personalize treatment in RA employ various omics methods for drug selection, whilst therapeutic drug monitoring (TDM) aims to optimize drug efficacy and safety (S. D. Sharma & Bluett, 2024). Even though individual studies reported success, tailored designs and overfitting hinder the inference of general principles, therefore neither of these approaches has found its way into general diagnostic or treatment recommendations for RA (S. D. Sharma & Bluett, 2024).

1.2.1 Precision medicine

Overall precision medicine aims to facilitate knowledge on the individual patient's characteristics to guide therapeutic and diagnostic decisions, leveraging treatment success whilst minimizing adverse effects and treatment-associated risks. Informational data on the individual subject may comprise genetic, epigenetic and transcriptomic information, proteomic and metabolic readouts, and environmental as well as lifestyle information that can be used to generate predictions via conventional analysis strategies or machine learning (ML) (Denny & Collins, 2021).

Whilst pharmacogenetics aims to reveal how single genetic variants affect pharmacokinetics and pharmacodynamics of individual drugs, pharmacogenomics integrate broader genomic information and knowledge on the affected pathways to infer how a genetic background influences drug response (Pirmohamed, 2001; Weinshilboum & Wang, 2006). A classic example for pharmacogenetic testing to avoid toxicity is the quantification of the activity of CYP2D6, a member of the cytochrome P450 superfamily of enzymes, that is involved in the metabolism of ~20% of common drugs, including analgetics, antiarrhythmics, antidepressants, antipsychotics and β -blockers (Maréchal et al., 2008; C.

Taylor et al., 2020). Even though *CYPE2D6* genotyping and testing for CYP2D6 activity is labor-intensive and therefore costly, it is recommended by some guidelines for tamoxifen treatment and psychiatric medication in the context of precision medicine (Goetz et al., 2018; Pratt et al., 2021; C. Taylor et al., 2020). Another example is the assessment for genetic variants in the *DPYD* gene that encodes for dihydropyrimidine dehydrogenase, the rate-limiting enzyme in the metabolism of 5-Fluorouracil (Dean & Kane, 2012). In rheumatology, azathioprine (AZA), an immunosuppressive drug that's active metabolites rely on inactivation via thiopurine S-methyltransferase (TPMT), is the most prominent representative for clinically applied pharmacogenetics. A set of genetic TPMT variants are associated with reduced catalytic activity, resulting in accumulation of active drug metabolites, bearing the risk of myelosuppression and hepatotoxicity (Clunie & Lennard, 2004). Additionally, variants in the *NUDT15* gene, encoding for nudix hydrolase 15, an enzyme involved in the hydrolysis of thiopurine metabolites of AZA are associated with higher toxicity, especially in patients of Asian ancestry (Dean, 2012).

Making treatment decisions on the individualized mutational burden is a well-established concept in oncology and hematology and knowledge of the precise genetic background offers the possibility of mutation-specific treatments (Aleksakhina & Imyanitov, 2021). Prime examples therefore include the *KRAS* mutation status for colon cancer (Sveen, Kopetz, & Lothe), EGF-receptor mutations in lung cancer (Imyanitov, Iyevleva, & Levchenko, 2021) and *HER2/neu* overexpression for breast cancer therapy (Exman & Tolaney, 2021). The wide availability of next-generation sequencing (NGS) technologies has revolutionized research and found its way into routine clinical testing, allowing for the sequencing of a set of genes, the whole exome (WES) or even the whole genome (WGS). Especially smaller panels of genes (tens to hundreds of individual genes) allow for fast processing, high sequencing depth and easier interpretation but have to be curated for the specific clinical question (Malone, Oliva, Sabatini, Stockley, & Siu, 2020). Additionally, in the case of cancer, tumor heterogeneity, the question of whether the detected mutation is the real underlying driver and the availability of approved mutation-specific treatments limit the one-to-one translatability of sequencing results into personalized treatment (Damodaran, Berger, & Roychowdhury, 2015).

Even though systematic characterization of epigenetic landscapes yielded valuable insights into cancer pathogenesis and drugs targeting epigenetic modifications like HDAC inhibitors are well established as co-medication in hematology and oncology, evidence for successful personalized treatment approaches based on individual epigenetic profiling are scarce (Shanmugam, Rakshit, & Sarkar, 2022; T. Yang, Yang, & Wang, 2021). Quite in reverse,

currently applied and suggested biomarkers for the application of epigenetic drugs much rather rely on the genetic background and expressional analysis (T. Yang et al., 2021).

Transcriptional profiling by RNA sequencing (RNA-Seq) allows for quantitative assessment of gene expression signatures, reflecting a more functional assessment than DNA-based assays. These expression profiles can inform about dysregulated signaling networks beyond the underlying mutational burden and have for example found application in routine testing to predict treatment responses to PARP inhibitors (Lord & Ashworth, 2016), and further subclassify diffuse large B-cell lymphoma (Scott et al., 2014). Complementing DNA-based routine testing with RNA-Seq may therefore uncover additional variants with clinical significance and simultaneously expand knowledge on the functional effects of mutations in malignant diseases (D. Li et al., 2025). Indeed, transcriptional profiling has revealed specific signatures associated with autoimmune diseases, disease activity, risk of flares and treatment response (Nagafuchi, Yanaoka, & Fujio, 2022). Examples therefore include the increased IFN signature in SLE patients (Bennett et al., 2003) and reported better clinical response to anifrolumab, a monoclonal antibody that interferes with IFN signaling, in patients with a high IFN signature (Morand et al., 2020) as well as a CD8⁺ T-cell exhaustion signature associated with less flares in SLE and ANCA-associated vasculitis (McKinney, Lee, Jayne, Lyons, & Smith, 2015). For RA however, positive studies for PM driven by transcriptional profiling, relied on material from the site of inflammation, requiring primary material in the form of biopsies from synovial tissue, thereby limiting the broad applicability in the clinics (Nagafuchi et al., 2022).

For decades, proteomics, the large-scale study of proteins, especially via mass spectrometry, has contributed to biomarker development for PM, however has found only limited application in routine clinical applications - the development of multiplexed affinity-reagent-based assays, however led to a growing field in high throughput proteome analysis (Correa Rojo et al., 2021; Duarte & Spencer, 2016). Beyond its various roles in translational research, primarily analytical microarrays have found their way into routine clinical applications (Su et al., 2021). On the other hand, metabolomics studies have been applied for assessing toxicity and optimizing dosing as well as monitoring the individuals exposome – the summary of all health related exposures over a lifetime (Schmidt et al., 2021). A more concrete application of metabolomic profiling was demonstrated for triple negative breast cancer, allowing for the identification of distinct metabolic subgroups and revealing potential novel therapeutic targets (Xiao et al., 2022).

The quantitative and semiquantitative assessment of intracellular and cell surface proteins is well established in histopathology for disease classification and state differentiation, thereby indeed guiding treatment (McCourt, Boyle, James, & Salto-Tellez, 2013). Indeed,

the biggest benefit of immunohistochemistry is its wide availability of the technique and spatial resolution allowing for the assessment of protein expression analysis on cellular and even sub-cellular resolution in primary material from biopsies (McCourt et al., 2013)

A classic and well-established example therefore is the classification of breast cancer. Beyond other characteristics three essential markers are assessed in routine diagnostics, that represent valuable mechanistic anchor points for informed treatment regimens (Goldhirsch et al., 2013). The two hormone receptors, estrogen receptor and progesterone receptors play a crucial role in the development and progression of disease with the estrogen receptor in particular promoting cell proliferation and survival, however also representing a valuable target for treatment with selective estrogen receptor modulators such as tamoxifen (Harbeck et al., 2019). Immunohistochemistry is also used for the evaluation of HER2/neu, an epidermal growth factor family member, that is overexpressed in ~15% of breast cancer cases, and mechanistically promotes cell proliferation, differentiation and survival (Harbeck et al., 2019). Furthermore, it is exploited as a drug target for the monoclonal antibody trastuzumab, that interferes with HER2 signaling and can lead to cell death via antibody-dependent cytotoxicity (Spector & Blackwell, 2009), but also the antibody-drug conjugate trastuzumab-deruxtecan. Upon binding to the receptor, trastuzumab-deruxtecan is internalized, the chemical linker is cleaved in lysosomes and deruxtecan, that functions as a topoisomerase 1 inhibitor and interferes with DNA replication specifically in HER2 expressing cells, released (Modi et al., 2020).

A key immunohistochemical staining target that translates into guided treatment is the assessment for programmed death-ligand 1 (PD-L1) expression in biopsy material (Vranic & Gatalica, 2023). PD-L1 is a cell surface protein, that interacts with the programmed death-1 receptor (PD-1) on T-cells and negatively regulates T-cell and other immune cell responses, acting as an immune checkpoint in physiological conditions. Overexpression of PD-L1 in the tumor microenvironment can therefore be found in many cancers to evade the immune system, however, can also represent a valuable therapeutic MOA in the form of blocking antibodies against either PD-1 or PD-L1, so-called immune-checkpoint inhibitors. For that reason, immune checkpoint inhibition is a well-established cornerstone in the treatment of various cancers including solid tumors (e.g. melanoma, non-small cell lung cancer, urothelial and renal cell carcinoma) and hematological malignancies like Hodgkin and non-Hodgkin lymphoma (P. Sharma et al., 2023). Another immunological regulator that is not only targeted in autoimmune disease but also in immune-oncology is cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), that competes with the co-stimulatory molecule CD28, thereby negatively regulating T-cell responses. Blocking CTLA-4 with ipilimumab can result in a strong anti-tumor immune response and thereby significant survival benefits for

patients with metastatic melanoma (Hodi et al., 2010). As however only 20-40% of patients respond to immune-checkpoint inhibition therapy, immunohistochemical testing for marker expression is crucial to predict individual treatment response and avoid sometimes life-threatening immune-related adverse events such as colitis, nephritis, pneumonitis, arthritis, myositis, immune endocrinopathies and liver toxicity as well as rash and pruritus (Doroshov et al., 2021; Martins et al., 2019).

In non-solid hematological malignancies flow cytometric assays are routinely used in the clinics, providing valuable information for diagnosis, disease stratification and also guiding personalized treatment via therapeutic monitoring (Betters, 2015; Craig & Foon, 2008). Beyond other measures for diagnosis, the 5th edition of the world health organization's classification of myeloid and lymphoid malignancies heavily relies on flow cytometric immunophenotyping (Alaggio et al., 2022; Khoury et al., 2022). Beyond its capability to classify malignant disease and thereby guide treatment according to guidelines, a prime application for flow cytometry is the detection of minimal residual disease (MRD), allowing for the identification of small numbers of residual leukemic cells, thereby quantifying treatment response and predicting relapse probability (Modvig et al., 2021). In the field of autoimmunity, flow- and mass-cytometric immune profiling has been studied in various diseases, including SLE and multiple sclerosis (Böttcher et al., 2019; Nagafuchi, Shoda, & Fujio, 2019). Tanaka et al. for example identified three subgroups based on T-cell heterogeneity in active SLE patients, with the follicular T-cell-dominant group being most resistant to treatment (Tanaka, Kubo, Miyagawa, Iwata, & Nakayamada, 2019). However, in contrast to hematology and oncology, large prospective studies assessing the value of immunophenotyping for autoimmune diseases are missing.

Fluorescence in situ hybridization (FISH) is a cytogenetic routine technique that allows for the identification of specific genetic alterations and can provide valuable information on the underlying mechanism of disease, thereby providing a measure of risk stratification and guiding personalized therapy for solid tumors and hematologic malignancies (Chrzanowska, Kowalewski, & Lewandowska, 2020; Hu et al., 2014). A prototypic example is the application for the detection of the BCR-ABL fusion gene, a hallmark of chronic myeloid leukemia, qualifying the patient for a targeted therapy with a tyrosine kinase inhibitor such as imatinib (S. G. O'Brien et al., 2003). Similarly, FISH is employed for the identification of rearrangements resulting in the promyelocytic leukemia-retinoic acid receptor alpha fusion gene in acute promyelocytic leukemia. Here, treatment with all-trans retinoic acid, which leads to the proteolytic degradation of the fusion protein, promotes differentiation of the malignant cell clone (Lo-Coco et al., 2013; Sanz et al., 2019). Whilst immunohistochemistry is widely used for screening of HER2 amplification in breast cancer samples, FISH is still

considered the gold standard for assessing the HER2/neu status, thereby guiding therapy with trastuzumab (Ahn, Woo, Lee, & Park, 2020).

Clinical decision support systems (CDSS) are software implementations designed to improve individual treatment success by assisting clinical decision making and are categorized in knowledge-based and non-knowledge-based CDSS (Berner & La Lande, 2007; Bright et al., 2012). A typical example of a knowledge-based CDSS are systems monitoring for drug-drug interactions and dosing errors and are built on a curated database of which a software algorithm infers interaction and dosing data and highlights relevant information for the treating physician (Van De Sijpe et al., 2022). Non-knowledge-based rather rely on large training data sets and apply machine learning approaches (Berner & La Lande, 2007). Probably most widely applied is the screening for lung cancer on computer tomography (CT) images by various deep learning techniques (Masood et al., 2020; Thanoon, Zulkifley, Mohd Zainuri, & Abdani, 2023).

1.2.2 Precision medicine in rheumatoid arthritis

RA presents with diverse clinical manifestations and heterogeneous response to drugs, requiring personalized adaptations of treatment regimens to treat disease to target (Smolen et al., 2010). Even though current guidelines suggest somewhat standardized treatment of early RA, clinicians at the same time face the availability of a wide range of b- and tsDMARDs of various modes of action (MOA) as add-on medication to csDMARDs (Smolen et al., 2023). Even though many efforts have been made to identify clinical, serologic and radiographic predictors of drug response, no truly prognostic biomarker has been found to guide therapy, resulting in the still best practice of trial-and-error medicine (Lequerré, Rottenberg, Derambure, Cosette, & Vittecoq, 2019; Smolen et al., 2018).

Many clinical factors are associated with treatment response in RA, these include age, sex, disease activity but also life-style measures like obesity and smoking (Bergstra et al., 2018; Capelusnik & Aletaha, 2022; Innala et al., 2014; Mancarella et al., 2007; Poudel, George, & Baker, 2020; Söderlin, Petersson, & Geborek, 2012). As RA onset has a very distinct age distribution (Crowson et al., 2011), several studies investigated the treatment response to csDMARDs and TNF inhibitors in relation to age (Sugihara & Harigai, 2016). Patients above the age of 60 presented with a significantly higher baseline SDAI as compared to patients <45 years in the ESPOIR cohort and showed reduced clinical response to treatment in general (Krams et al., 2016) but also to TNFi treatment specifically in another study (Yamanaka et al., 2015). There is not only a strong sex bias for the development of RA (Kvien et al., 2006), but registry studies demonstrated, that female sex is also associated

with reduced treatment response as compared to male sex (Hyrich, Watson, Silman, & Symmons, 2006; Kleinert et al., 2012; K. B. Lauridsen et al., 2025). Indeed, high disease activity at baseline is often leading to radiographic progression (Smolen et al., 2020; van der Heijde, 2001), but was also associated with higher treatment response to tocilizumab (Kristensen et al., 2014; Maldonado-Montoro, Cañadas-Garre, González-Utrilla, Plaza-Plaza, & Calleja-Hernández, 2016). Whilst smoking is associated with increased risk of developing RA, especially in the presence of the SE (Sugiyama et al., 2010), smoking status also demonstrated as a significant discriminatory factor for treatment response to csDMARDs (Saevarsdottir et al., 2011), TNFi therapy (Hyrich et al., 2006; Saevarsdottir et al., 2011) and csDMARD + TNFi combination therapy (Levitsky et al., 2017), however not to RTX-treatment (Chatzidionysiou et al., 2019). Obesity (≥ 30 kg/m²) at baseline was associated with significantly lower clinical response as compared to overweight (25–29.9 g/m²) or normal weight (< 25 kg/m²) after 2 years of treatment in the Swedish pharmacotherapy trial (SWEFOT) (Levitsky et al., 2017). Furthermore, patients with obesity achieved lower remission rates upon 12 months of TNFi treatment (Gremese et al., 2013). Mechanistically, adipose tissue, in part via adipokines, contributes to systemic and local inflammation (Neumann et al., 2021). On the other hand, body mass can affect relative dosing of bDMARDs, as infliximab, for example, is dosed based on body weight, whilst adalimumab is administered a fixed dosage (Mould, 2015).

As the genetic component in RA development is well established (Ishigaki et al., 2022), pharmacogenomic and pharmacogenetic studies have been conducted in rheumatology, to ultimately utilize knowledge on the individuals genetic background for disease control (Babiker-Mohamed, Bhandari, & Ranganathan, 2024; Szekanecz et al., 2013). Even though GWAS identified a relatively large number of genetic variants that were associated with TNFi treatment response (Ferreiro-Iglesias et al., 2019; Massey et al., 2018; Sánchez-Maldonado et al., 2021), they so far failed to predict treatment response, driving current genetic research towards the development of integrative polygenic risk scores (Honda et al., 2022; C. M. Lewis & Vassos, 2020).

In a longitudinal study, treatment of RA with TNFi showed remodeling of the epigenetic landscape towards a state comparable with healthy controls and identified cell type-specific CpG sites associated with disease (Julià et al., 2022). Other studies, even though reporting significant associations of epigenetic remodeling at week 4 associated with MTX treatment response at month 6, however could not find differences in baseline methylation patterns differentiating good from poor responders (Nair et al., 2020). Indeed the aggressive epigenetic phenotype of FLS is known to contribute to joint inflammation, however, on top

of complicated sample acquisition, no prospective studies were conducted to assess if this phenotype is associated with treatment response (Ai et al., 2018).

Upon the emergence of cost-effective RNA-Seq technologies, transcriptional profiling was predicted to revolutionize patient stratification and thereby allow for treatment response prediction in RA (S. L. Smith, Plant, Eyre, & Barton, 2013). As with the analysis of epigenetic remodeling, in transcriptional profiling facilitating microarrays, treatment response is reflected by changes of gene expression related to immune cell function, however no differences were observed in baseline between responders and non-responders (Oswald et al., 2015). With the introduction of RNA-Seq, there is an increasing number of publications aiming to use transcriptional profiling for the identification of a preferential treatment mode of action, however in part with contradicting results. The lack of single gene transcriptional data to predict treatment response is for example highlighted by the controversial reports on CD39 expression quantification as a predictor to TNFi treatment response (Oliver et al., 2021; Spiliopoulou et al., 2019). Probably the most promising transcriptional predictor of selective bDMARD response is the type I IFN signature, that was associated with improvement upon treatment with tocilizumab (Sanayama et al., 2014), abatacept (Yokoyama-Kokuryo et al., 2020) and TNFi (van Baarsen et al., 2010), however was also a negative predictor for B-cell depleting therapy (Rateman et al., 2012; Sellam et al., 2014; Thurlings et al., 2010).

Beyond transcriptional profiling of peripheral blood immune cells, recent studies facilitated ultrasound-guided biopsies of synovial tissue to characterize primary material from the site of inflammation. In these studies, samples were not only stratified immuno-histologically based on cellular composition but also characterized on a transcriptional level for the identification of predictors of treatment (Bykerk, 2023; Humby et al., 2021; Myles J. Lewis et al., 2025; Rivellese et al., 2023; Rivellese et al., 2022).

The R4RA stratified, biopsy-driven multicenter, open-label clinical trial was designed to demonstrate the superiority of tocilizumab in patients with a B-cell-poor synovium as compared to RTX treatment in a cohort of patients that did not respond to TNFi before. Clinical assessment of treatment response was performed 16 weeks after randomized treatment with either 2 doses of 1000 mg RTX or a regimen of 8 mg of tocilizumab per KG of body weight intravenously every four weeks. Dichotomization into B-cell-rich or poor based on histology did not reveal a superior response to tocilizumab (higher rate of 50% reduction in CDAI) as compared to B-cell-depleting therapy. Stratification based on expression data, however, showed a significantly higher response rate for tocilizumab as compared to RTX (Humby et al., 2021). Consecutive in-depth analysis of the 164 samples and stratification according to semiquantitative immunohistochemical synovial pathotypes

(fibroid pauci-immune, diffuse-myeloid, and lympho-myeloid pathotype (M. J. Lewis et al., 2019)) revealed patients with a diffuse-myeloid and thereby B-cell-poor synovium to respond significantly better to tocilizumab than RTX. This was however not the case for the B-cell-poor fibroid pauci-immune or the B-cell-rich lymphoid pathotype. Interestingly, *in silico* deconvolution of RNA-Seq data identified patients with high T-cell infiltration into the synovium enriched in RTX responders whilst tocilizumab response was associated with the presence of macrophages and dendritic cells. Gene expression signatures associated with RTX response included humoral and lymphocytic modules, whilst patients that were refractory to all three MOAs showed an enrichment of a stromal / fibroblast gene signature. Finally, paired biopsies pre and post treatment were used to identify drug-specific changes of the synovium and ML was used to create a model predictive of response to treatment (Rivellese et al., 2022).

Many studies aimed to characterize PBMCs of RA patients by flow cytometry as a surrogate for the immunological niche of the synovium and use this material to stratify disease and predict treatment response, thereby avoiding limitations of biopsy-driven approaches. As a recently published showcase, Kubo et al. demonstrated immunologic diversity by quantitatively assessing 34 immune cell subsets in a cohort of 533 b/tsDMARD naïve patients with RA and 96 healthy controls (Kubo et al., 2025). This extensive immunoprofiling led to the identification of 5 distinct peripheral blood cell abundance phenotypes that were associated with differential response to TNFi, IL-6 blockers, abatacept or JAK inhibitors and allowed for the identification of a preferred b/tsDMARD MOA for 4 of the 5 clusters. The identified phenotypes were additionally validated in a cohort containing 183 b-/tsDMARD naïve RA patients (Kubo et al., 2025).

In a similar study design, the open-label, biopsy-driven randomized STRAP trial aimed to systematically assess the superiority of TNFi or IL-6 blockade over RTX in the presence of a B-cell poor synovium in a cohort naïve to bDMARD treatment. After biopsy, patients were immunohistochemically classified as B-cell-poor or B-cell-rich and randomized into 3 treatment arms (etanercept s.c. 50 mg weekly, tocilizumab 162 mg s.c. weekly or 2 doses of 1000 mg of RTX i.v.) for 16 weeks and treatment efficacy was assessed by ACR 20 response. However, also this study failed to meet its primary endpoint in demonstrating the superiority of etanercept or tocilizumab pooled over RTX treatment in patients with a B-cell-poor synovium. Vice-versa ACR 20 responses were not significantly different for either RTX or TNFi and anti-IL-6 pooled treatment in patients with a B-cell-rich synovium. In terms of structural damage to the joints however, the study found significantly higher radiographic progression as quantified by the Sharp van der Heijde score especially in the RTX-treated population with a B-cell-rich synovium (Rivellese et al., 2023). Deep transcriptional profiling

via RNA-Seq in combination with ML allowed for the generation of a model that predicted clinical response to either treatment with high precision (AUCs between 0.748 and 0.763) and models for RTX and tocilizumab response were cross validated with data from the R4RA cohort (Myles J. Lewis et al., 2025). Even though they failed to predict treatment response based on dichotomization into B-cell-rich and B-cell-poor synovium, these two clinical studies and their consecutive molecular profiling highlight the potential future value of ultrasound guided synovial biopsy in combination with expression profiling for treatment prediction. Therefore, additional studies are needed to validate the proposed predictive ML models especially in the context of difficult-to-treat patients and alternative treatment regimens such as JAK-inhibition.

The proteomic landscape at the site of inflammation but also systemically shows significant differences between patients with RA and healthy controls. Indeed, the most prominent components are well established biomarkers such as RF and ACAPs, that were shown to be associated with treatment response to various b- and tsDMARDs (Bird et al., 2019; Courvoisier et al., 2021; Genovese et al., 2020; Julià et al., 2021). Specifically, by pooling 16 observational registries with a total of 27583 patients Courvoisier et al. demonstrated that seropositivity was associated with increased remission and low disease rates (according to CDAI) upon RTX, abatacept and tocilizumab, but not TNFi treatment (Courvoisier et al., 2021). Similarly, a meta-analysis of four placebo-controlled phase II and III trials showed that RTX treatment led to a greater decrease in DAS28-ESR after 24 weeks of treatment in seropositive patients (Isaacs et al., 2013).

Metabolomic analyses to gain knowledge on RA have been conducted in various primary biological materials including peripheral blood, urine and synovial fluid (Kapoor et al., 2013; S. Kim et al., 2014; M. B. Lauridsen et al., 2010; L. Xu et al., 2022). Cuppen et al. for example identified a set of 88 metabolites that were associated with disease activity (DAS28, ESR or CRP) and built a predictive model combining metabolomics with clinical data to predict TNFi treatment response, that outperformed prediction on clinical data alone (Cuppen et al., 2016). Likewise, in a study by Sweeney et al., serum of active seropositive RA patients with a co-medication of MTX was assessed for metabolomic and lipidomic changes upon treatment with RTX in responders and non-responders to B-cell depletion. A set of 7 polar metabolites and 15 lipids was associated with ACR 20 response to RTX (Sweeney et al., 2016), however, none of the aforementioned studies has been validated in large scale cohorts and therefore no metabolic profiling strategies are incorporated in current treatment recommendations (Smolen et al., 2023).

On the one hand, large efforts have been made to optimize DMARDs dosing to maximize efficacy while minimizing adverse effects by TDM as dose-efficiency dependencies were

shown for several TNFi (Jamnitski et al., 2012; Jurado et al., 2017; Rosas et al., 2014; Takeuchi, Miyasaka, Inoue, Abe, & Koike, 2009; van den Bemt et al., 2013) and Tocilizumab (Arad & Elkayam, 2019; Kneepkens et al., 2017). On the other hand, up to two thirds of RA patients develop ADAs against bDMARDs leading to neutralization and enhanced degradation, ultimately reducing drug levels (Moots et al., 2017; Pizano-Martinez et al., 2023). Occurrence rates of ADA levels vary significantly among different bDMARDs – for TNFi they are reported to reach rates up to 67% for infliximab (Krintel et al., 2013), 28% for adalimumab (Bartelds et al., 2011), 6.5% for golimumab (Kay et al., 2008), 5.6% for etanercept (Dore et al., 2007) and 8.1% for certolizumab (Fleischmann et al., 2009) in studies with > 100 patients per treatment. bDMARDs of other MOA yield ADA rates up to 2% for tocilizumab (Burmester et al., 2017) and 12.3% for sarilumab (A. F. Wells et al., 2019), 4.1% for abatacept (Nash et al., 2013) and 10.6% for RTX (Wientjes et al., 2022). The occurrence of ADAs, which can be neutralizing and reducing available drug levels, lead to inferior clinical efficacy of bDMARDs (F. B. Vincent et al., 2013). Another application for ADAs was proposed to predict whether an individual would respond to a bDMARD treatment of the same class. Jamnitski et. al for example, investigated treatment response to etanercept in a cohort of TNF-naïve, TNFi non-responders with and without ADAs and found, that response rates to etanercept were significantly lower in patients with ADAs than in the other two groups (Jamnitski et al., 2011). TDM in inflammatory diseases to optimize efficacy and safety for infliximab was assessed in the randomized, parallel-group open-label NOR-DRUM trial. Even though less infusion reactions were reported for the TDM group (5 vs. 16), no difference in rates of clinical remission were detected at week 30 for RA or other inflammatory diseases, thereby providing evidence against TDM in routine use (Syversen et al., 2021). In summary, based on current evidence, EULAR does not recommend the proactive use of TDM or ADA monitoring in routine care, however, might be applied and provide benefit in specific clinical scenarios (Krieckaert et al., 2023). Furthermore, there is a call for future research to systematically assess the value of TDM and ADA screening on bDMARD efficacy, safety and health economics (Krieckaert et al., 2023).

Integration of clinical data with readouts from omics approaches and annotation of drug-specific responses have created large data sets that form the basis for the development of supervised and unsupervised ML models for patient stratification and prediction of treatment response. However, when focusing on clinical data alone, ML models failed to outperform manual model building using logistic regression in predicting the persistence to MTX in RA (Westerlind et al., 2021). Starting from initially 32 clinical variables, Miyoshi et al. narrowed the number of relevant parameters down to 9 and used these to build a neural network, that could predict the response to infliximab with 92% accuracy (Miyoshi et al., 2016). Even

though validating the model on a set of patients from two other hospitals, the study lacks an alternative arm for treatment response prediction to test the model against (Miyoshi et al., 2016). Koo et al. used baseline clinical data to predict 1-year remission in patients treated with various bDMARDs (multiple TNFi, tocilizumab and abatacept) however drug-specific prediction accuracy was only between 52.8 and 72.9% (Koo et al., 2021).

Given the genetic contribution to RA development, various studies incorporated patients' genic data and clinical features to generate even more complex datasets to train ML-based models for treatment response stratification. Gosselt et al. for example aimed to compare ML approaches (LASSO, random forest and XGBoost) to multivariable logistic regression in predicting insufficient response to MTX (DAS28-ESR >3) in a cohort of 355 early arthritis patients. Even though all four approaches performed similarly, logistic regression was the most sensitive and baseline DAS28 was the most important baseline feature (Gosselt et al., 2021). The importance of clinical features was also demonstrated in a study aiming to combine information on demographics, disease activity, treatment and genetic data in a Gaussian process regression model to predict treatment response, when SNPs only contributed little to the overall predictive power of the model (Guan et al., 2019).

With increased complexity multi-omics datasets, incorporating information beyond clinical and genetic baseline data, the analysis faces the challenge that features outnumber observations by far, even in large cohorts. As a showcase, in a very comprehensive multi-omics approach, a Swedish study aimed to identify predictive markers for TNFi response in a cohort of 39 female MTX non-responders, of which 23 did show and 16 did not show a response to TNFi treatment after 3 months by conventional and ML-based analysis (Yoosuf et al., 2022). The data collected at baseline and follow-up comprised of PBMC gene expression data, the abundance of PBMC immune cell subsets and plasma proteins as well as measures of clinical disease activity. The gene expression data, however, was highly affected by a single outlier sample necessitating removal. The remaining samples yielded *EPPK1*, coding for Periplakin 1, as one of the top hits predicting TNFi response, a gene, however, not enriched in another genomic and transcriptional analysis of TNFi response (Aterido et al., 2019). The longitudinal analysis indeed found significant changes in gene expression after treatment, however the flow cytometric analysis revealed neither significant differences upon treatment in non-responders nor factors predicting TNFi response. Also, the plasma protein analysis could not separate between responders and non-responders or identify factors predicting treatment response, but showed expected markers such as CRP, IL-6, SAA and MMPs reduced upon TNFi treatment. The group finally aimed to build linear models and kernel-based classifiers for TNFi treatment prediction based on collected data but could only find limited utility of these classifiers when using flow cytometric, protein

abundance and transcriptomic data and in accordance with other reports in the literature, the linear model based on clinical data outperformed the other classification approaches (Yoosuf et al., 2022). In a similar study, focusing on expression and methylation data, Tao et al. were able to build random forest models predicting a response to adalimumab and etanercept with a 79% - 85.9% accuracy (Tao et al., 2021). The small sample size and lack of a validation cohort in the first case, however, limit the informative value of such studies. Finally, Jung et al. used a previously published RNA-Seq dataset from synovial biopsies of ACPA positive arthralgia, undifferentiated arthritis and early arthritis, early and established arthritis patients as well as healthy controls (Guo et al., 2017) to apply a naïve Bayesian classifier for treatment prediction (Jung, Park, & Kim, 2021).

Given the variance of therapy adherence to DMARDs in RA patients, either intentional or unintentional, ranging from 30% - 80%, personalized educational and technological measures might significantly improve patient outcomes on individual but also population-wide level (van den Bemt, Zwikker, & van den Ende, 2012). Approaches therefore include the introduction of eHealth tools like apps and websites to monitor disease activity and therapy adherence as well as the aim to increase health literacy in general. However, only about 30% of patients use the internet to gain disease specific information and the rate of use of electronic tools to track measures of disease activity or treatment reminders was very low (Magnol et al., 2021). This is problematic as good health literacy is significantly associated with lower disease activity (Gorter, Bakker, Ten Klooster, Boonen, & Vonkeman, 2023).

1.3 Functional precision medicine

Indeed, the ever-growing understanding of pathophysiology and genetics of in particular malignant diseases gave rise to the development of mutant-specific treatments, significantly improving patient outcomes. However, even though large efforts have been made to comprehensively profile human cancers (e.g. in the human cancer genome atlas, a repository currently covering > 20,000 individual samples spanning 33 cancer types (Chang et al., 2013)), information on the genetic background alone is often not enough to predict or explain an individual's treatment response. This is for example highlighted by the relatively low response rates observed in the tumor-agnostic precision oncology NCI-MATCH trial, where nearly 6,000 patients were screened and 1,593 individuals were assigned to one of the 38 phase two sub studies (O'Dwyer et al., 2023). Even though actionable mutations were found in 37.6% of the patients only 12,7% were assigned to a treatment and the overall

response rate was only 79/765 patients, resulting in 7 of the initial 27 sub studies reporting a positive outcome (O'Dwyer et al., 2023).

To overcome this gap between genetic information and treatment response, pioneered primarily in oncology and hematology, FPM aims to predict an individual's response to a therapeutic intervention based on dynamic data obtained from directly assessing the effectiveness on live primary material such as tissue or cells rather than relying on the genetic background. To gain such functional data for the translation into guided therapy, a variety of technical assays can be employed, including various ex vivo models such as 2 dimensional (2D), 3 dimensional (3D), and microfluidics cell cultures, but also in vivo systems like xenograft models and in vivo response monitoring devices (Flobak, Skånland, Hovig, Taskén, & Russnes, 2022; Letai et al., 2022; Napoli, Figg, & Chau, 2022).

Conventional 2D cell culture systems for adherent and non-adherent cells come with the benefit of relatively low cost, their integrability into automated liquid handling and screening platforms and various scalable readouts like viability, morphology, differentiation, intercellular interaction but also gene and protein expression as well as metabolic response. Probably the biggest benefit of 2D cultures, when it comes to translatability into a clinical routine assay is the fast turnover time, as fast feedback is critical. The feasibility of reporting individualized drug responses within 7 days was demonstrated by Liebers et al. in the SMARTrial, when the primary endpoint was met, and profiling data was provided for 73 of 80 screened patients in time (Liebers et al., 2023). This simplicity is, however, accompanied by some disadvantages like lack of in vivo environmental factors and non-physiological culture conditions, thereby limiting the informative value of cellular responses (Letai et al., 2022).

For the reproducible and scalable quantification of ex vivo drug responses of patient-derived material, microtiter plates can be precoated with drug libraries by acoustic droplet deposition (Hadimioglu, Stearns, & Ellson, 2016), and viability of whole cell populations can be quantified by scalable luminescence-based assays for adenosine triphosphate (ATP) quantification in material from hematologic malignancies like acute myeloid leukemia (AML) (Malani et al., 2022) or solid tumors (e.g. head and neck squamous cell carcinoma (C. Xu et al., 2018)).

An early report of functional drug sensitivity profiling that directly translated into patient treatment is the study by Frismantas et al., in which they profiled 68 patients with drug-resistant acute lymphoblastic leukemia (ALL) with a curated palette of 60 drugs by various analytical techniques including ATP-based viability assays and treated one patient with T-cell ALL based on the ex vivo drug prediction. The guided dasatinib monotherapy led to

resolution of all lesions after 2 months and a disease control over 5 months (Frismantas et al., 2017).

The applicability of a 2D monolayer culture for the high-content microscopy-based quantification of immunomodulatory effects was spearheaded in a proof of concept study by members of the Superti-Furga lab (Vladimer et al., 2017). One major novelty described in this publication was the introduction of a perturbation-limited seeding, culture, fixing and staining protocol for PBMCs in microscopy-optimized 384-well microtiter plates that allowed for the quantitative assessment of PBMC cell-to-cell interaction. These cell type-specific interaction propensities were quantified in the form of an interaction score, with individual interactions scored based on proximity of ≤ 15 pixels of two nuclear centroids. In combination with the systematic perturbation with a curated set of 1402 common small molecule drugs, the system was used to map these drugs for their potential to alter PBMC cell-to-cell interactions (Vladimer et al., 2017).

This perturbation-limited monolayer assay was then applied by our group in a single-arm, open-open label, pilot study by Snijder et al. for the microscopy-based single-cell ex vivo drug response profiling of patients with late-stage hematological malignancies (Snijder et al., 2017). In detail, primary material (bone marrow aspirate, PBMCs, pleural effusion, ascites or homogenized lymph nodes) was incubated overnight in a drug library containing 139 drugs in two concentrations, immunolabelled for the target blast population according to pathologists' guidance and imaged on an automated high content microscope. Image analysis allowed for the identification of cancer cell-specific on target drug action. The approach was first validated in a retrospective cohort of 20 treatment-naïve AML patients, where response could be predicted with 88.1% accuracy. 48 patients with aggressive hematological malignancies were screened and guided treatment was later applied to 17 patients, in 12 of them significantly outperforming the previous treatment regimen, highlighted also by the increase of median progression-free survival from 5.8 to 22.6 weeks (Snijder et al., 2017). In a prospective follow-up study 143 patients with advanced aggressive hematologic malignancies were ex vivo tested for drug response and 56 were treated according to FPM guidance. Of the FPM-treated patients, 30 achieved a 1.3-fold longer progression-free survival as compared to the previous treatment regimen and 13 were progression-free after 12 months, validating the feasibility of the approach and demonstrating the superiority of the FPM guided treatment over routine care (Kornauth et al., 2022).

The additional value of supervised ML for the classification of peripheral malignant blasts via cell morphology to complement cell surface marker-based identification was demonstrated on a dataset consisting of 390 individual samples from 289 patients with

various hematological malignancies (Heinemann et al., 2022). Based on the diagnostic markers, assigned by pathologists in the underlying studies, cells were classified as either healthy or malignant and a deep convolutional neural network (CNN) was trained to reproduce this dichotomic categorization solely on 4',6-diamidino-2-phenylindole (DAPI) and bright field BF images, making it robust against overfitting, e.g. for lineage specificity. In a post hoc analysis of 66 patients previously treated according to the cell lineage marker-based FPM guidance, the ML-based ex vivo drug predictions outperformed the marker-based FPM approach as well as the physicians' choice (Heinemann et al., 2022). Similarly, focusing on multiple myeloma specifically, Kropivsek et al. assessed 101 bone marrow samples from 70 patients microscopically in combination with ex vivo drug and immunotherapy treatment and complemented their data set with genetics, proteomics and cytokine profiling (Kropivsek et al., 2023). In a recent publication, Kazianka et al reported the first 55 patients of the multicentric, randomized controlled EXALT-2 trial and provided comparative data on treatment guidance by genomics, imaging-based and flow cytometric FPM. Whilst the median reporting time was shorter for FPM than genomics, actionable target identification was similar for genetic and microscopy based testing (65% and 64% respectively) and higher for flow cytometry-based FPM (86%) with an overall overlap of 60% of drug targets for genomics and FPM (Kazianka et al., 2025).

So far only a limited number of assays for the quantification of primary patient immune cell function have been established in routine clinical testing, such as IFN gamma release assays for the screening of previous contact with mycobacterium tuberculosis (Albert-Vega et al., 2018). This test is particularly valuable for detection of a latent tuberculosis infection in the context of an indicated b or tsDMARD treatment for RA and is therefore recommended by the EULAR in their recommendations for screening of chronic and opportunistic infections (Fragoulis et al., 2023).

In contrast to 2D culture systems, three-dimensional (3D) model cultures such as spheroids and organoids aim to resemble the physiological microenvironment and tissue architecture, allowing for less perturbed cell-to-cell interaction and gene expression behavior and may therefore reflect chemosensitivity better than 2D models (Imamura et al., 2015; Pampaloni, Reynaud, & Stelzer, 2007). These benefits, however, come with the downside of longer and more complex culture conditions, often leading to significantly culturing costs (Letai et al., 2022). Even though numerous studies are registered aiming to facilitate cancer organoids for treatment prediction, positive reports are relatively scarce, with a study by Pasch et al. as a rare showcase, where functional testing of patient derived organoids was translated into a personalized treatment of a patient with metastatic colorectal cancer (Pasch et al., 2019). Similarly, organoids comprising of various cells from the joint, including FLS,

monocytes, lymphocytes and chondrocytes are well established in the RA research field, however have not yet led to translational assays for FPM (Badillo-Mata, Camacho-Villegas, & Lugo-Fabres, 2022; Huang, Zhang, Lu, & Liang, 2023). In addition to, at least partially resembling the complex microarchitecture of the tissue by 3D cultures, the cultivation of primary material in microfluidics devices and chips adds the possibility of specifically engineering components of the microenvironment as well as the time-resolved assessment of cellular responses (e.g. to drugs or CAR-T cells) (Ayuso, Virumbrales-Muñoz, Lang, & Beebe, 2022). Even though applied frequently for the study of malignant diseases as well as the synovial niche in RA and promoted frequently as a valuable approach to FPM, no studies yet report the successful application of a microfluidics device for FPM (Ayuso et al., 2022; Gonçalves et al., 2022; Z. A. Li et al., 2023; Ma et al., 2018; Thompson et al., 2023; Zhai et al., 2024).

Patient-derived xenograft (PDX) models, that are especially applicable to cancer research and functional precision oncology, implant patient-derived tumor samples in immunodeficient host animals such as mice, zebrafish, pigs and rats. On the one hand, for the development of mutation-specific treatments, on the other for FPM as engraftment is associated with tumor reoccurrence and metastasis and xenograft bearing animals can be treated to assess drug efficacy. Even though these complex culture forms gained significant insight into cancer pathophysiology, and treatments of PDX correlated well with clinical response, evidence for the prospective prediction of treatment response is still missing (Blanchard, Brown, Ghazaryan, & Welm, 2025).

Abandoning patient-derived cellular model cultures at all, the non-invasive, real time in vivo assessment of biomarkers for disease activity, drug response prediction and treatment monitoring indeed would represent the ideal scenario for FPM. Advances in sensor and drug delivery technologies have allowed for recent developments in so-called closed loop systems resulting in personalized continuously monitored treatment regimens improving outcomes of diabetes in humans and cancer treatments in preclinical animal models (DeRidder et al., 2024; Templer, 2022).

Type I diabetes requires life-long supplementation with insulin to maximize time in normoglycemia and avoid long term effects of hyperglycemia causing microvascular (e.g. diabetic retinopathy, nephropathy and neuropathy), macrovascular cardiovascular and peripheral vascular diseases (Nathan et al., 1993). Subcutaneous glucose sensors are widely available and provide real-time data on glucose levels, thereby improving glycemic control and patient outcomes (Rodbard, 2017). The combination of continuous glucose monitoring with an insulin delivery system allowed for the development of a closed loop setup, with its superiority first demonstrated in a cohort of very young children with a mean

age of 5.6 years (Ware et al., 2022) and later in a more diverse cohort in the form of a bionic pancreas (Russell et al., 2022). Whilst classical chemotherapy dosage is calculated based on a patient's body surface area and weight, these dosing estimates do not factor in the individual's pharmacokinetics. DeRidder et al. published a first application of CLAUDIA, a closed loop system for the mass spectrometry-based quantification, automatic adaption and thereby close maintenance of chemotherapeutic drug levels in an animal model (DeRidder et al., 2024). However, current research goes far beyond the application of bionic sensors in closed loop systems, aiming to facilitate various synthetic biology approaches for FPM. These for example include the development of biomarker-triggered drug release systems but also genetically engineered cell-based closed loop systems to control metabolic and endocrine states as well as the immune system (Brunger, Zutshi, Willard, Gersbach, & Guilak, 2017; Stefanov & Fussenegger, 2022).

Even though fundamental improvements recently boosted the application of FPM in various medical fields, there are still numerous unmet needs when it comes to implementation into routine workflows. On the one hand, technically, pre-analytical sample handling introduces variability in approaches that are built on fresh intact primary material, even the most complex model cultures do not resemble the in vivo environment, and not all assays are scalable, especially in the context of combinatorial drug treatment. On the other hand, large scale prospective clinical trials demonstrating the superiority FPM for specific applications are still missing. Furthermore, therapy-limiting approval by regulatory bodies is substantially contributing to the high operational barriers currently restraining FPM applications in routine care (Letai et al., 2022; Napoli et al., 2022).

1.4 High-content high-throughput imaging

Microscopy-based studies of biological samples provided insights into tissue function in health and disease and the development of antibody-based techniques like immunohistochemistry and immunofluorescence imaging allowed for research on protein localization and function on a tissue, cellular and subcellular level. In addition to technical pitfalls that may occur during sample preparation, acquisition and manual selection of “representative” images, conventional imaging-based studies often report the findings subjectively descriptive or by semiquantitative scoring of phenotypes (North, 2006). With the arrival of digital image acquisition, electronic image processing and computational image analysis, high-content (HCI) and high-throughput imaging (HTI) emerged in academic and industry-driven life sciences to overcome the limitations of visual microscopy by adding objectiveness in image interpretation and reproducibility of analysis. HCI focuses on the automated acquisition of a multitude of functional and phenotypic parameters of cells in various conditions that were stained with fluorescently labelled antibodies or stained with organelle specific markers (Giuliano et al., 1997; Way, Sailem, Shave, Kasproicz, & Carragher, 2023). In contrast HTI aims to maximize the imaging speed and efficiency to gather a reduced set of features specific to the research question, often in the context of drug screening (Pegoraro & Misteli, 2017; Way et al., 2023).

1.4.1 Cell line-based model cultures

The range of biological material and forms of cell culture that are subject to HCI- and HTI-based studies can be diverse and the assay by itself tailored to fulfill the requirements of specific research questions. 2D assays with immortalized cell lines form the backbone of HCI and HTI because of their versatility, robustness and scalability, however selection of the correct cell line is crucial for drug screening and can significantly affect the observed phenotypes (Heinrich, Kumbier, Li, Altschuler, & Wu, 2023). The scientific power of HCI-based studies becomes apparent in differential drug response screening in wild type vs. cells with a particular mutation, which can reveal mutation-specific vulnerabilities. As an example, Mills et al. conducted an HCI-based study with consecutive image analysis to infer cytostatic and cytotoxic drug effects in 58 breast cancer cell lines with known mutational burden (Mills et al., 2022). To systematically probe into the protein function for cellular morphology and subcellular organelle localization, specifically the assembly of the nuclear pore complex, de Groot et. al used a library of 2281 CRISPR-Cas9 guide RNAs in combination with HCI (de Groot, Lüthi, Lindsay, Holtackers, & Pelkmans, 2018). Beyond the scope of screening for vulnerabilities in cancer cell lines, O'Brien et al. tested the

hepatotoxicity of 243 compounds by microscopy in human hepatocellular carcinoma cells (HepG2). In detail, they incubated cells for three days in various drug concentrations and performed a four-channel fluorescence staining to assess nuclear size and morphology, cellular calcium concentration, the mitochondrial membrane potential and the permeability of the plasma membrane. Assessment of these characteristics allowed for the detection of cytostatic as well as cytotoxic effects that reflected human toxicity with a sensitivity of 80% and specificity of 90%, highlighting the concordance of HCI-based toxicity screening (P. J. O'Brien et al., 2006).

1.4.2 Induced pluripotent stem cell cultures

Cell line-based models indeed present numerous benefits in high-content and high-throughput screening applications, however, are also accompanied with limitations, such as lack of physiological relevance and disease context, abnormal intracellular signaling and genetic drift as well as often immature phenotypes showing adaptation to 2D culture. The development of mammalian induced pluripotent stem cells (iPSC) allowed the research community to overcome some of these shortcomings in parts, leading towards more disease-specific cellular models. An early example is the study of iPSC-derived hepatocytes in combination with HCI for firstly the morphologic and phenotypic characterization of the cell population and secondly the imaging-based screening of drug toxicity with a library of 240 individual compounds, similar to the above mentioned study by O'Brien et al. (Sirenko, Hesley, Rusyn, & Cromwell, 2014). Further, Mioulane et al. developed an assay to study cardiotoxicity (apoptosis and necrosis) in a culture of pluripotent stem cell-derived cardiomyocytes, using HCI in combination with fluorescent dyes for nuclear morphology and cell membrane permeability as well as antibody-based staining for Caspase-3, labelling apoptotic cells (Mioulane, Foldes, Ali, Schneider, & Harding, 2012). Cationic cell organelle dyes, specifically the membrane potential-dependent mitochondrial dye tetramethylrhodamine methyl ester (TMRM) can be used to assess mitochondrial function in live cells (Ehrenberg, Montana, Wei, Wuskell, & Loew, 1988). In combination with iPSCs from patients with Parkinson's disease with mutations in the SNCA gene and healthy controls (HCs), Little et al. used HCI to assess mitochondrial morphology and function (Little et al., 2018).

1.4.3 Co-culture systems

Multicellular co-culture systems aim to further resemble the *in vivo* situation by adding multiple layers of complexity. These include consequences of paracrine and juxtacrine cell signaling, cell scaffolding in extracellular matrix (ECM), e.g. synthesized by co-cultured fibroblasts, that can result in enhanced cell function, as well as stroma-mediated drug resistance and the possibility to detect off-target effects. Furthermore, these model systems not only allow the study of naturally occurring cell-to-cell interactions but even the engineering synthetic interactions (Goers, Freemont, & Polizzi, 2014). The development of an HCI-based assay to study inhibitors of angiogenesis was described by Evensen et al., where endothelial cells are co-cultured with vascular smooth muscle cells resulting in the formation of tubular endothelial networks. The culture system furthermore allowed to assess anti-angiogenic drug effects in two distinct states, firstly in the phase of vascular endothelial growth factor (VEGF)-dependent angiogenesis and secondly, in mature, VEGF-independent networks (Evensen, Micklem, Link, & Lorens, 2010). Other fields that applied HCI for the quantitative assessment of phenotypes in co-culture systems include neuroscience for the role of astrocytes in neurotoxicity (Anderl, Redpath, & Ball, 2009) and stroma-mediated chemoresistance of cancers (Schuth et al., 2022). Finally, in rheumatology research, HCI was applied to systematically assess the effects of cytokine-induced milieu on the cell-to-cell interaction propensities of primary immune cells with FLS (Kugler et al., 2023).

1.4.4 3D model cultures

The impact of the modality of culture system was highlighted by the PREDECT consortium in an imaging and automated image analysis-heavy side-by-side comparison of various 2D and 3D culture formats for mechanistic studies in oncology and drug discovery (Stock et al., 2016). Yet potentially labor and time-intensive, recent imaging-based research shows a trend towards 3D spheroid and organoid (co-) cultures as well as microfluidic devices. Early adaptations of 3D culture systems for HCI include the co-culture of a luciferase-expressing prostate cancer cell line or a lung cancer cell line with human mesenchymal stem cells in phenol red-free ECM and medium on 96-well micro titer plates by Krausz et al. in a proof-of-concept cross platform study. Computational image analysis readouts included the total cell cluster area, mean and median cell cluster area, and the number of cell clusters and were compared to a fluorescence-based cell viability assay. The assay was evaluated in 3 runs on a set of 32 individual compounds in 4 concentrations demonstrating the feasibility of a high-throughput imaging-based screen for drug sensitivity in a 3D co-culture system

(Krausz et al., 2012). Focusing on the time-resolved interaction of cancer cells with immune cells, De Ninno et al. facilitate 2D and 3D microfluidics-based co-culture systems. Analysis of 2D bright field images as well as fluorescence images of 3D z-stacks, yielded insights into the complex cancer niche with the migratory potential and cellular interaction propensities of immune cells as readouts for immunomodulatory activities of anti-cancer drugs (De Ninno et al., 2021). For the study of the RA synovium in model cultures, Phillippon et al. recently described the imaging-based quantification of RA spheroids composed of FLS, umbilical vein endothelial cells as well as monocyte-derived macrophages. Z-stacks of spheroids cultured on 96-well U-bottom suspension micro titer plates were acquired by confocal microscopy and subsequently analyzed computationally, allowing for the quantification of spheroid outgrowth in various chemically perturbed conditions also demonstrating a more compact and interconnected network formed in the presence of RA-FLS (Philippon et al., 2023).

1.4.5 Cultures of primary material for ex vivo drug screening

Beyond mechanistic and drug screening studies, HCI showed particular potential for chemosensitivity screening in patient-derived solid and liquid biopsies in the context of FPM. A prime example is the above mentioned EXALT study conducted in a collaboration between the MUV and CeMM with the goal to provide informed personalized preferential treatment decisions for patients with leukemias and lymphomas (Kornauth et al., 2022; Snijder et al., 2017). Another HCI-based approach to assess chemosensitivity of primary ALL samples was published by Rowland et al., adding mesenchymal stromal cells to the culture and testing the efficacy of 40 anti-leukemia drugs. However, in this approach, the reduction of fluorescent markers to a DNA dye, rather than staining with cell type-specific surface markers as demonstrated in the EXALT study, limits the application to sorted cell populations or samples with very high blast fractions and at the same time losing the possibility to assess off target effects (Rowland et al., 2023). Finally, Haladik et al. recently expanded the approach of HCI-based drug screening in combination with integration of genetic and epigenetic data for FPM to primary samples of patients with pediatric AML (Haladik et al., 2025).

1.4.6 Methods of labelling

Methodologically, the spectrum of techniques to mark structures of interest and make them detectable in HCI-assays covers a wide range of pan-cell and organelle-specific fluorescent

dyes, fluorescently labelled antibodies and genetically encoded marker proteins, and can be extended by labelling-free techniques such as brightfield and phase contrast microscopy and augmentation methods like virtual microscopy.

Small molecule-based labelling

Small molecule fluorescent probes form the staining basis in HCI and HTI imaging experiments, as they are easy to apply and yield high fluorescence intensities (Choi, Lee, Park, Lee, & Lee, 2021; Zhu et al., 2024). The range of such probes include whole cell stains such as various cell trackers that diffuse into cells and are then activated and retained upon chemical cleavage (Lulevich, Shih, Lo, & Liu, 2009) and unspecific amine-reactive protein stains applied to fixed and permeabilized cells to label the whole cell area allowing morphologic analyses (Morales, Schafer-Hales, Marcus, & Belfield, 2008). Beyond that, organelle-specific probes enable researchers to study individual cellular components. Probably the most widely applied are bisbenzimidazole dyes such as DAPI and Hoechst 33342 for labelling of DNA in the UV / blue spectrum in HCI and conventional fluorescence microscopy assays (Bucevičius, Lukinavičius, & Gerasimaitė, 2018). Since excitation in this spectrum can cause autofluorescence, is associated with shallow optical penetration depth and may result in UV-induced phototoxicity, a number of chemical modifications of Hoechst dyes and the deep red anthraquinone 5 (DRAQ5) have been developed to allow for DNA staining in longer wavelengths (Bucevičius et al., 2018; Ortner et al., 2021). Another typical counterstain to visualize the cytoskeleton in fixed cell experiments are the fluorophore conjugates of the bicyclic heptapeptide phalloidin of *Amanita phalloides*, commonly known as phalloidin, that binds filamentous actin (Mazloom-Farsibaf et al., 2021). Chemically lipophilic, amphiphilic or membrane-anchored fluorescent constructs can be used to stain biological membranes (Filipe, Moreno, & Loura, 2020), whilst strictly lipophilic dyes enrich in lipid droplets (Fam, Klymchenko, & Collot, 2018). In contrast, other organelle-specific dyes facilitate the membrane potential of specific organelles to accumulate e.g. in mitochondria (Chazotte, 2011; Gökerküçük, Tramier, & Bertolin, 2020) or use ion trapping to label lysosomes, with some probes even allowing to assess lysosomal acidification via pH-dependent ratiometric emission (Duan, Tong, Fu, & Chen, 2023).

Combining fluorescent dyes in HCI and HTI protocols has led to development of comprehensive cell labelling strategies, called cell painting assays, applicable to large-scale compound and genetic perturbation studies (Seal et al., 2025). The first protocol published facilitates a combination of six fluorescent probes and was applied to detect cellular phenotypes in a screen for biological activity of 1600 small molecule compounds on a human osteosarcoma cell line by Gustafsdottir et al. and later in a larger screen of 30,616 compounds by Bray et al. (Bray et al., 2016; Gustafsdottir et al., 2013). Other publicly

available large scale cell painting data sets include the RxRx3 data set, a CRISPR-Cas9-based phenotypic KO screen by Fay et al. (Fay et al., 2023) and data from the JUMP cell painting consortium aiming to screen 116,750 unique compounds as well as the effects of gene over-expression (12,602 genes) and knockout (7,975 genes) (Chandrasekaran et al., 2023).

Antibody-based labelling

Complementary to small molecule fluorescent probes, fluorescently conjugated antibodies largely extend the target spectrum, enabling the precise localization and quantification of specific proteins and their modifications. Classic immunofluorescence staining can either be performed indirectly, where a primary, antigen-targeting antibody is detected with a secondary fluorophore-labelled antibody or directly with a targeting antibody that is tagged with fluorophores or conjugated to fluorescent proteins (Im, Mareninov, Diaz, & Yong, 2019). Among many, applications for antibody-based protocols in HCI and HTI assays include the staining for phosphorylated H2AX and computationally assessing the area of H2AX positive foci as a proxy for DNA damage (S. Kim, Jun, Kim, Jeong, & Lee, 2011) and nuclear translocation assays for transcription factors. Examples in the field of rheumatology where I could contribute are the quantification of NF- κ B nuclear translocation in a study investigating the role of mTOR for FLS function (Thomas Karonitsch et al., 2018) and the translocation of IRF5 and NF- κ B in a study deciphering the function of TASL as the immune adaptor for signaling of endosomal toll-like receptors (Heinz et al., 2020). Finally the ex vivo chemosensitivity studies of the EXALT trial and the recent study by Haladik make use of the specificity of fluorescently labelled antibodies to specifically detect the immunophenotype of the malignant cell sub-population (Haladik et al., 2025; Kornauth et al., 2022; Snijder et al., 2017).

Genetically encoded labelling

Genetically engineered proteins, e.g. those tagged with fluorescent proteins such as green fluorescent protein (GFP), allow researchers to directly assess protein localization and dynamics in real time in live cells without fixation/chemical permeabilization or perturbation. Alternative approaches include the use of a reporter gene, where a fluorescent protein is induced under specific conditions (Boutros, Heigwer, & Laufer, 2015) or the expression of genetically engineered calcium indicators (N. Wu, Nishioka, Derecki, & Maher, 2019).

Augmented microscopy

To overcome the need for staining or ectopic expression of fluorescent proteins altogether, recent research has developed an approach termed augmented microscopy or digital /

virtual staining. In this approach, neural networks are trained on paired datasets to identify cellular and subcellular features from brightfield or phase contrast images in a label-free way. Whilst this approach eliminates the risk of phototoxicity, reduces cost and time and allows for unlimited multiplexing, thereby enabling full-scale longitudinal studies, a specific model may also be prone to effects like algorithmic hallucination, lack generalizability and may fail to unravel biologic novelty (Fanous, Pillar, & Ozcan, 2023; Ounkomol, Seshamani, Maleckar, Collman, & Johnson, 2018; Sullivan & Lundberg, 2018).

1.5 Computational image analysis

Computational image analysis (CIA) has transformed research, translating visual observations into reproducible quantitative data, allowing for consecutive stringent statistical analysis, offering the opportunity for large scale experimental approaches that would be impossible to address by human hand (Eils & Athale, 2003; Sbalzarini, 2016).

In comparison to manual scoring of imaged biological phenomena, CIA, especially via pipeline-based software implementation provides the benefit of scalability, when hundreds of images can easily be analyzed locally on researchers' computers, whilst large scale studies of thousands to millions of images from HCI and HTI experiments often require specific computational infrastructure like high-performance computing or cloud-based solutions (Bray et al., 2017; Luik, Rosas-Bertolini, Reits, Hoebe, & Krawczyk, 2024). This standardization of an image analysis pipeline, applied to all images in exactly the same way, results in a high level of reproducibility and precision by elimination of the subjectivity of the human eye and brain, leading to large results data sets and enormous statistical power (Eliceiri et al., 2012). However, at the same time CIA can produce highly complex data with thousands of individual measurements per cell. Readouts include parameters such as intensity measurements on a picture and object level, detailed morphological characteristics and cellular heterogeneity even in a spatial context of cell distribution and allowing for the quantification of proximity and cell-to-cell interactions. The combination of these benefits leads to the ability to detect novel hidden phenotypes that would have been too subtle to the human eye (Eils & Athale, 2003; Eliceiri et al., 2012; Sbalzarini, 2016).

1.5.1 Evolution of biological image analysis

Whilst CIA software exists as commercial products, major developments were made by research institutions providing open-source applications to the scientific community. The following examples represent three types of widely used applications: FIJI, a stand-alone software focused to run locally for interactive single image analysis allowing for additional custom plugin development (Schneider, Rasband, & Eliceiri, 2012); QuPath, an interactive, machine learning-based tissue classification tool, optimized to handle large file sizes and most commonly applied in digital pathology (Bankhead et al., 2017); and CellProfiler, a modular, pipeline-based application focused on scalability and reproducibility on large-scale imaging data sets (McQuin et al., 2018).

Development of ImageJ started in the 1970s as a s Pascal-based program at the United States' National Institute of Health (NIH) and became popular as NIH Image before it was rewritten in the system-agnostic language Java and distributed as ImageJ (Schneider et al., 2012). Ever since the user interface hardly changed, but with the release of Fiji, a distribution of ImageJ2, bundled with numerous plugins, researchers were provided with an even more powerful software, allowing for the fast implementation of new image analysis algorithms into ImageJ (Schindelin et al., 2012). Even though the application of ImageJ in research fields beyond biology and medicine such as geology, astronomy and history demonstrate the scientific versatility of the application, the vast majority of publications facilitating ImageJ stem from life science-related fields (Schroeder et al., 2021). The applicability of ImageJ ranges from relatively easy tasks like the quantification of bands on western blots (Gallo-Oller, Ordoñez, & Dotor, 2018) and counting individual cells (Grishagin, 2015) to complex tasks like particle tracking in multidimensional imaging data sets that are achieved via plugins like TrackMate (Tinevez et al., 2017).

QuPath, short for quantitative pathology, was released in 2016 and published in 2017 by Bankhead et al. with the aim to provide an open-source application for digital pathology focusing on whole slide analysis and being capable of handling the often extremely large file sizes of these images (Bankhead et al., 2017). One of its key advantages is the intuitive graphical user interface, that allows researchers to interactively train classical machine learning models such as random forests, random trees and support vector machines but also supports deep learning models like WSInfer (Bankhead et al., 2017; Kaczmarzyk et al., 2024). QuPath is mostly used for the assessment of brightfield and haematoxylin and eosin histopathology as well as immunohistochemically stained specimens as reviewed by Humphries et al. (Humphries, Maxwell, & Salto-Tellez, 2021).

CellProfiler was first published in 2006 by Carpenter et al, providing an open-source program for high-throughput image analysis (Carpenter et al., 2006). CellProfiler provides a graphical user interface and allows researchers to build modular pipelines to address scientific questions like cell counting and morphologic analysis of identified objects. Over its major releases, CellProfiler gained significant functionality like the processing of 3D stacks and integration of deep learning (McQuin et al., 2018). Besides the above-mentioned large scale genetic and drug perturbation studies (Bray et al., 2017; Chandrasekaran et al., 2023) and the description of cell painting (Bray et al., 2016; Gustafsdottir et al., 2013), all described CeMM- & MUV- based studies of ex vivo drug screening for FPM facilitated CellProfiler to extract cellular features for consecutive analysis (Haladik et al., 2025; Kartnig et al., 2025; Kornauth et al., 2022; Snijder et al., 2017).

1.5.2 Modular steps in an image analysis pipeline

After data acquisition and file handling, CIA usually comprises of a number of consecutive steps including image pre-processing, segmentation and feature extraction, to translate the information contained within the image into a numerical readout for statistical analysis (Sbalzarini, 2016; Uchida, 2013). The following chapters summarize general approaches of CIA and exemplify several key steps also detrimental for our study.

Image pre-processing

Native or raw images come in different sizes and shapes and often harbor imperfections and artifacts. Color images, for example, may have to be split into grey scale single channel images in the first place to retrieve the information of individual stainings. Technical artifacts in imaging data range from shot and sensor noise, uneven illumination to distortion necessitating image pre-processing to optimize consistency and can enhance or suppress feature, all together making them more exploitable by algorithms (Y. I. Wang, 2007). To reduce granularity in microscopic images, algorithms for denoising and smoothing are often the first step in an image analysis pipeline by applying gaussian filtering which can also cause the softening of edges or median filtering which is particularly useful to remove dark or bright pixels while better preserving sharp edges (Iwabuchi, Kakazu, Koh, & Charles Harata, 2014; Uchida, 2013). Uneven illumination of the field of view or technically constrained vignetting may limit consecutive segmentation approaches calling for appropriate illumination correction to remove brightness gradients affecting the whole image and therefore consecutive quantifications (S. Singh, Bray, Jones, & Carpenter, 2014) **(Figure 1)**. To remove uneven illumination, autofluorescence and other low-frequency variations, especially in low exposure fluorescence images, additional background correction e.g. via a rolling ball filter may be applied by subtraction or division to enable better consecutive thresholding (Helmuth, Burckhardt, Greber, & Sbalzarini, 2009; Sternberg, 1983). Additionally, unavoidable distortions in the optical pathway may be present in the image set that can be addressed to reduce this bias and individual features can be enhanced or suppressed mathematically (Y. I. Wang, 2007). Finally, images are often normalized or rescaled to even out staining and exposure fluctuations and for better algorithm performance with stable gradients (Anghel et al., 2019).

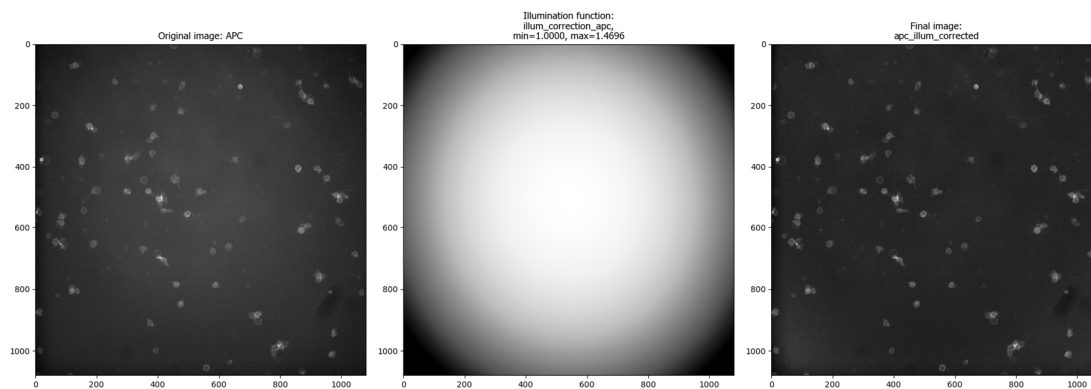


Figure 1: Calculation and application of illumination correction function to reduce optical artifacts: Left: Raw greyscale image of the APC channel showing a concentric brighter center and dimmer edges (vignetting); Middle: Image correction function calculated by polynomial fit; Right: Division of the original image (left) by the correction function (middle) results in an image corrected for uneven signal intensity. Exemplary visual control output from a CellProfiler-based image analysis of PBMC monolayer culture.

Object identification

Whilst identification of individual objects of interest in microscopy images might be easy to the trained human eye, this task might be challenging to implement in a CIA pipeline to generate reproducible precise results (Y. Wang et al., 2024). The most basic and widely used approach for image segmentation and identification of individual (seed) objects like fluorescently labelled nuclei is water shedding (Malpica et al., 1997; L. Vincent & Soille, 1991) (**Figure 2**). In detail, in a first step, a threshold is applied to the pre-processed images (e.g. a DAPI grey scale image) to create a binary mask differentiating foreground cells from background. In a second step the distance of every foreground pixel is calculated to the closest background pixel and based on these distance values local maxima are detected that function as seeds for individual objects. The then inverted image, it can be imagined as 3D landscape, is “flooded” and the line where “waters” from individual seed points meet is called the watershed which is used to dissect clumped or touching objects. Finally, the individual objects are labelled for immediate quantification or to serve as seed points for the identification of objects in other channels (Malpica et al., 1997; L. Vincent & Soille, 1991) (**Figure 3**). From here on, other regions of interest can be identified either by expanding or subtracting a specified number of pixels from the diameter of the object (e.g. for the consecutive quantification of a peri-nuclear region) or differences of intensity of another staining of interest are used to define individual cells (Jones, Carpenter, & Golland, 2005)

(**Figure 4**). Several of such steps can be combined to add or subtract individual objects from one another to identify cell compartments such as the cytoplasm or a membrane compartment (Carpenter et al., 2006).

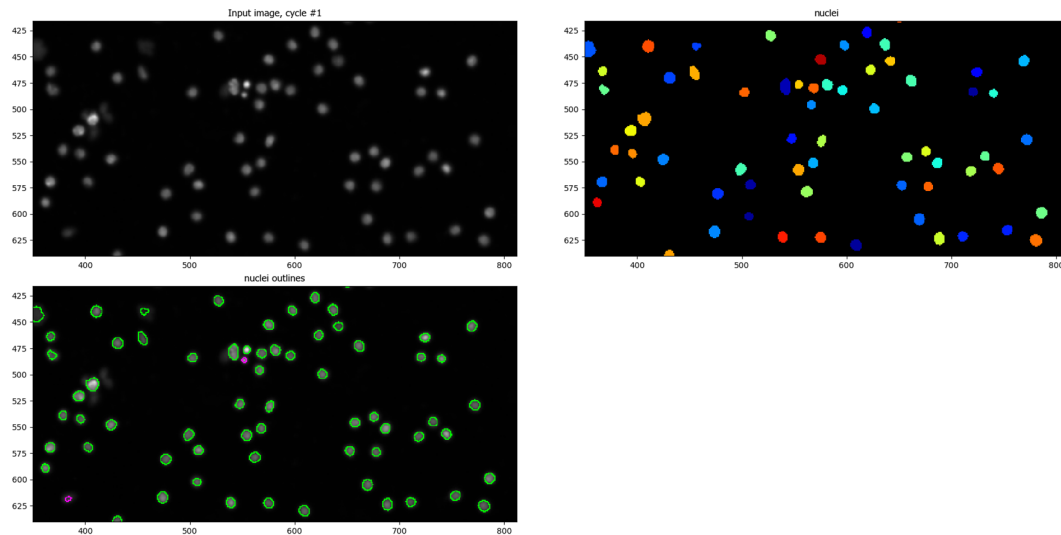


Figure 2: Image segmentation – identification of primary objects (nuclei): Top left: image of DAPI stained nuclei in a 2D culture of PBMCs; Top right: image of individual nuclei detected; Bottom left: image of outlines of individual nuclei detected that were in range (green) or out of range (pink) of the set limits for nuclear size. Exemplary visual control output from a CellProfiler-based image analysis of PBMC monolayer culture.

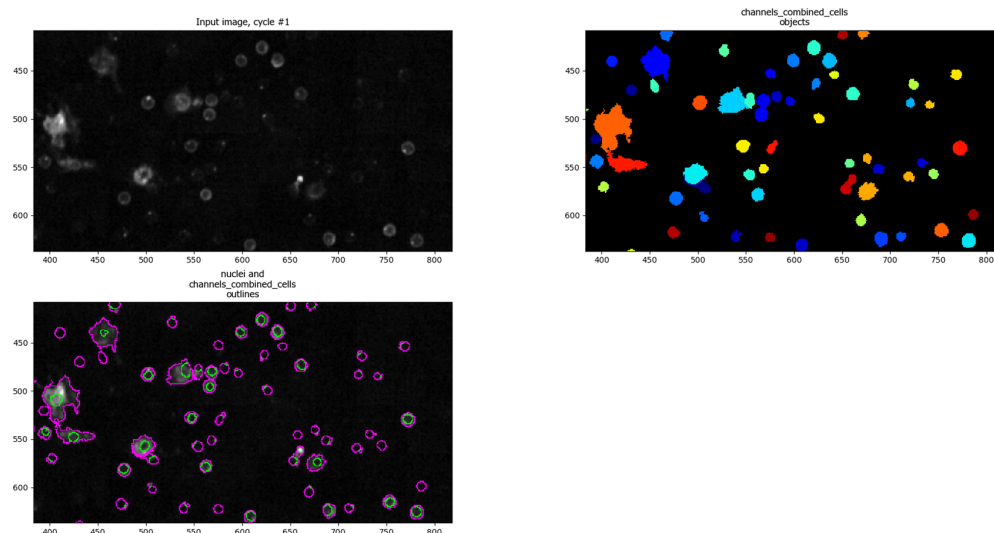


Figure 3: Identification of cells for morphological analysis. Top left: greyscale summation picture of 3 individual channels labelling cell surface markers; Top right: individual objects are identified via a Voroni-based method according to intensity of the immunolabelling; Bottom left: outlines of the primary seed objects (green) and individual cells (pink). Exemplary visual control output from a CellProfiler-based image analysis of PBMC monolayer culture.

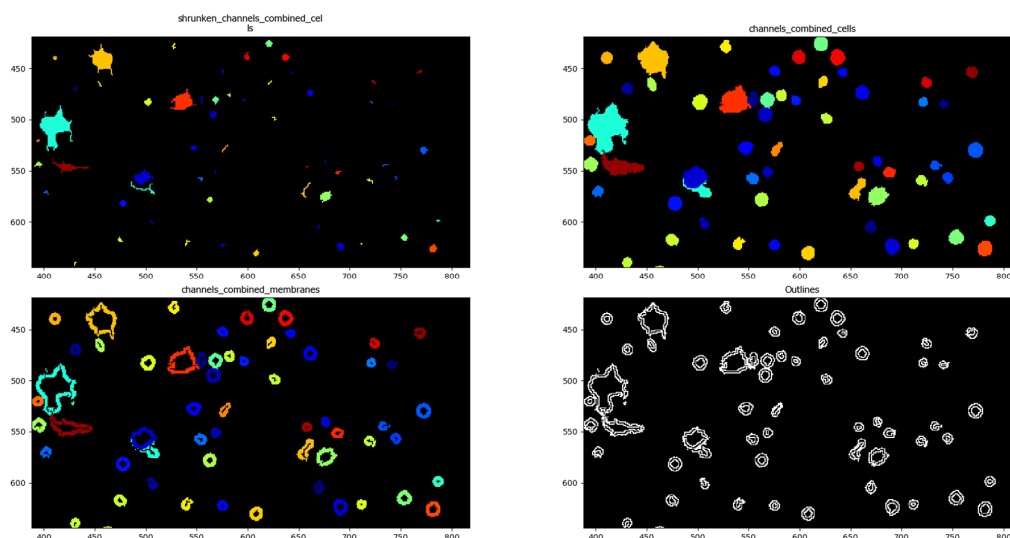


Figure 4: Identification of cell membranes for consecutive staining intensity quantification: Top left cells were shrunk by three pixels; Top right: Individual identified

cells; Bottom left: The membrane compartment as defined by subtraction of shrunken cells from original cells; Bottom right: Outlines of individual cell membranes for consecutive membrane quantification. Exemplary visual control output from a CellProfiler-based image analysis of PBMC monolayer culture.

Object quantification

The final goal of the CIA is the quantification of individual objects in their number, morphology, intensity propensities including texture as well as spatial relationship for statistical analysis (Sbalzarini, 2016). Morphological aspects of individual cells include area (2D) and volume (3D), their perimeter, eccentricity and roundness as well as how elliptical they appear (Bourn, Daly, Huggett, Braybrook, & Rivera, 2025; Chen, Zhao, Wu, Yao, & Zhang, 2012). By quantification of individual pixels of a selected staining, intensity measurements can be inferred, which may include the mean and median and standard deviation of intensities (e.g. for the quantification of protein abundance in a specific compartment) but also allow to measure granularity and more complex mathematical representations of intensity distributions within objects like Zernike features (Khorin, Dzyuba, Chernykh, Butt, & Khonina, 2025; T. Wu et al., 2023). Finally, 2D and 3D data sets yield spatial information on the single pixel but also object level that can be quantified. Typical measurements include the calculation of the centroid position, symmetry of organelles in a cell or cells in a tissue specimen but also the distribution of cells in a given region of interest and how cells are distributed to one another by quantifying proximity or identifying touching objects (Ruzette, Kozlova, Cruz, Muranen, & Nørrelykke, 2025; Vladimer et al., 2017) (**Figure 5**).

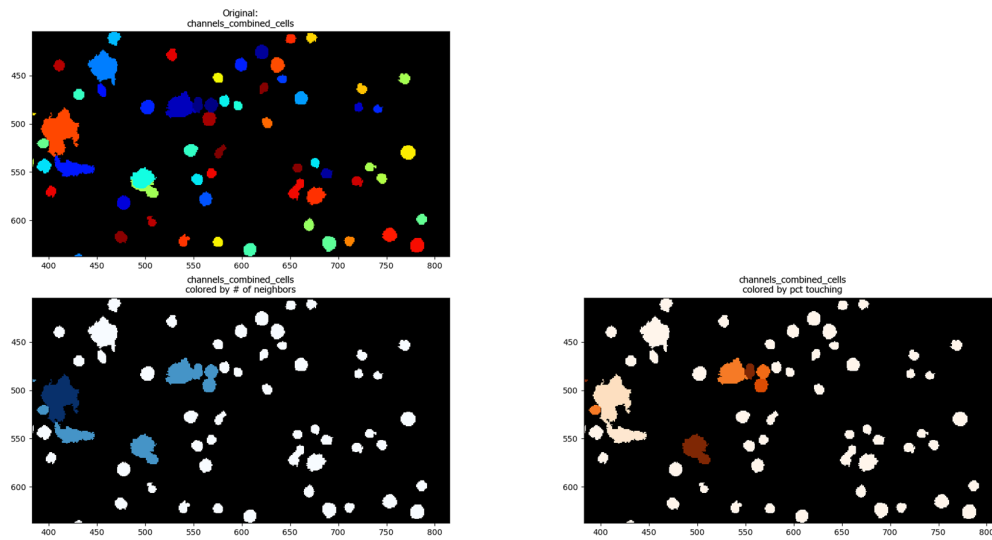


Figure 5: Quantification of spatial information for the assessment of cell-to-cell interaction propensities: Top left: Individual cells as identified from nuclear seed objects; Bottom left: Visual representation of the quantification of the number of touching cells, color coded for the number of interaction partners; Bottom right: Visual representation of the quantification of the percentage of the perimeter of an individual object that is in contact with another object. Exemplary visual control output from a CellProfiler-based image analysis of PBMC monolayer culture.

1.6 Aims of this thesis

The presented thesis had the scientific goal to establish an imaging-based high-content phenotyping assay in combination with *ex vivo* drug stimulation as an FPM approach to RA to overcome the suboptimal “trial-and error principle” for treatment selection in RA.

In the course of this study, we specifically aimed to:

- Establish a robust screening platform for the microscopic assessment of PBMC phenotypes upon *ex vivo* treatment with RA-specific drugs and immune stimuli.
- Develop a computational image analysis pipeline for the automated quantification of cell type abundances, cell morphology and cell-to-cell interaction.
- Identify PBMC phenotypes in patients with RA and HCs that are associated with *ex vivo* drug and immune stimulus perturbation.
- Extract cellular phenotypes that reflect the *in vivo* medication of patients with RA.
- Identify cellular features that are associated with RA disease activity.
- Demonstrate the feasibility of a high-content high-throughput microscopy assay in combination with *ex vivo* drug and immune stimulus treatment as a valuable approach to FPM in rheumatic diseases.

2 Results

2.1 Prologue

The development of biomarkers assisting personalized medicine and methods overcoming the suboptimal trial-and-error principle currently employed for optimal treatment selection for autoimmune diseases represents an unmet need in the field of rheumatology.

To address this, the following work represents a consequent continuation and expansion of efforts for HCI-based FPM conducted in the lab of Giulio Superti-Furga towards systemic autoimmune diseases such as RA. Extending its successful application in hematocology, we could develop the scientific framework for the systematic investigation of immunologic *ex vivo* phenotypes of PBMCs from patients with RA, thereby contributing to the ultimate goal of personalized medicine and informed decision making for preferential treatment selection in the field of autoimmunity.

At the same time, the expertise on the most extensive application of fluorescence microscopy-based assays, CIA and bioinformatics presented within this PhD thesis was vital for numerous complementary studies where I could contribute. This covers research in the field of rheumatology (Boeszoermenyi et al., 2023; Brandstetter et al., 2019; Heinz et al., 2020; Thomas Karonitsch et al., 2018; T. Karonitsch et al., 2024; Kugler et al., 2023), solute carriers (Bensimon et al., 2020; Girardi, Agrimi, et al., 2020; Girardi, César-Razquin, et al., 2020; K.-C. Li et al., 2021; Moskovskich et al., 2019; Sedlyarov et al., 2018) and in studies related to hematologic malignancies (Bigenzahn et al., 2018; Haladik et al., 2025; Pemovska et al., 2021).

The presented proof-of-concept study forms the foundation for future prospective clinical trials currently conducted in the framework of international collaborations (Horizon Europe SQUEEZE, IHI Autopix) aimed at further demonstrating the clinical utility of our method.

Ex vivo imaging-based high content phenotyping of patients with rheumatoid arthritis



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Summary

Background High content imaging-based functional precision medicine approaches have been developed and successfully applied in the field of haemato-oncology. For rheumatoid arthritis (RA), treatment selection is still based on a trial-and-error principle, and biomarkers for patient stratification and drug response prediction are needed.

Methods A high content, high throughput microscopy-based phenotyping pipeline for peripheral blood mononuclear cells (PBMCs) was developed, allowing for the quantification of cell type frequencies, cell type specific morphology and intercellular interactions from patients with RA (n = 65) and healthy controls (HC, n = 33). Samples were exposed to a curated set of RA-specific small molecules, biologicals and reference stimuli for 24 h to assess *ex vivo* drug effects. Data on *ex vivo* PBMC phenotypes were integrated with information on patients' *in vivo* medication and disease activity.

Findings The unbiased data from in total 6.9e8 individual cells were collected and allowed for the identification of PBMC phenotypes specific to disease activity as well as *in vivo* and *ex vivo* treatment. The arrayed *ex vivo* drug perturbation enabled the systematic characterization of drug effects, clustering by mode of action and uncovered morphologic alterations associated with biologic disease-modifying anti-rheumatic drug (DMARD) treatment. Individual *in vivo* treatment regimens translated into altered immune cell abundances in patients with a comedication of conventional synthetic DMARDs when compared to HCs. Global integration of PBMC characteristics led to clustering of patients according to disease activity and correlation with clinical data.

Interpretation The application of the developed screening tool demonstrates a technical proof-of-concept for feasibility of a functional precision medicine approach to the *ex vivo* immunophenotypic characterisation of patients with RA.

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Keywords: Rheumatoid arthritis; High-content microscopy; Drug profiling; Biomarker; Precision medicine

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease with the hallmark of persistent synovial inflammation resulting in chronic pain, cartilage, and joint destruction and if untreated permanent disability of the patient.¹ While treatment to target has improved patients' disease courses significantly over the last decades, the given medications are heterogenous,

consisting of conventional synthetic (cs) disease-modifying antirheumatic drugs (DMARDs) (methotrexate, leflunomide and sulfasalazine) and biological (b) DMARDs with various molecular targets (TNF, IL-6, CD20, CTLA-4) and targeted synthetic (ts) DMARDs, with the additional use of glucocorticoids and non-steroidal anti-inflammatory drugs.^{2–4} Despite this substantial therapeutic advancement, a high number of

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Research in context

Evidence before this study

Despite constant progress in the treatment of rheumatoid arthritis (RA), a significant number of patients do not reach clinical remission. In state-of-the-art practice, drug choice still follows a trial-and-error principle, primarily due to a lack of meaningful biomarkers. This highlights the necessity for novel personalised treatment approaches that assist clinicians to stratify patient cohorts and to optimise treatment selection. High content imaging (HCI)-based assessment of cellular phenotypes of primary cells in response to *ex vivo* drug exposure has recently been pioneered as an approach to functional precision medicine (FPM) in the field of haematology allowing to predict drug effectiveness *ex vivo*.

Added value of this study

We developed an HCI-based pipeline for the quantitative assessment of cellular phenotypes including cell identity,

morphology and cell-to-cell interaction in primary peripheral blood mononuclear cells (PBMCs) from patients with RA and healthy controls. In combination with arrayed *ex vivo* drug perturbation and the patient-specific clinical information on *in vivo* treatment and disease activity, this screening tool provided new phenotypic insights into drug action and disease. Taken together, the study demonstrates a technical proof-of-concept for the feasibility of an FPM approach to RA.

Implications of all the available evidence

Here, we translated an HCI-based FPM approach for the identification of cellular phenotypes into a tool for drug response profiling and patient stratification in autoimmune research. This approach lays the foundation for the future application of FPM within more controlled prospective clinical trials in RA and possibly other autoimmune diseases.

patients still do not reach clinical remission and treatment decisions are often based on a trial-and-error principle.^{1,3} A variety of individual genetic or molecular biomarkers have therefore been evaluated in the past for a more personalised treatment approach.⁶⁻⁹ Even though this development is encouraging, there has been limited success to implement these methods into routine care.⁴

Recently, a series of scientific advancements have successfully introduced the concept of functional precision medicine (FPM), an approach pioneered in the field of oncology.¹⁰ There, the primary material from patients is tested *ex vivo* for drug effectiveness to assist physicians' choice for optimal treatment selection. The most versatile FPM approach has involved high-content high-throughput microscopy, a powerful phenotyping method of cellular model cultures, enabling diverse applications such as drug screening, target identification and arrayed genetic investigations.^{11,12} The advantage of this methodology lies in the simultaneous acquisition of multidimensional parameters on a single cell level. These include quantification of protein abundance and localisation, organelle and cell morphology as well as individual cell-to-cell contacts. Beyond its application in cell cultures, the implementation in primary human material, including peripheral blood mononuclear cells (PBMCs), is an emerging opportunity for the functional in-depth characterisation of individual donors, thereby providing a new approach for diagnosis and prediction of treatment response. Clinical application of microscopy-based FPM to optimise treatment selection based on *ex vivo* drug responses was already successfully demonstrated in exploratory studies and clinical trials in haemato-oncologic malignancies.¹³⁻¹⁷ In addition, its

of drugs via monitoring of immune cell function has been shown in PBMCs.^{18,19}

Since many inflammatory disorders manifest systemically and multiple cell types contained in PBMCs act as major drivers of disease, we hypothesised that an imaging-based FPM approach to this primary material would allow for the in-depth characterisation of patients with RA and *in vivo* and *ex vivo* RA-specific drug responses. In the present study, we established the experimental protocol for the systematic perturbation and efficient characterisation of PBMCs from patients with RA and healthy controls by high-content microscopy. This enabled us to quantitatively assess a comprehensive set of parameters, including cell type frequencies, intercellular interactions, and morphologic characteristics on a single cell level and in a cell-to-cell context dependent manner.

Based on the established screening tool, we successfully assessed the effects of arrayed *ex vivo* perturbation of PBMCs with a curated set of RA-specific small molecules and biologics, and identified distinct drug effects on cellular morphology and intercellular interaction of specific immune cell subsets. Taking advantage of the various treatment regimens present in the RA cohort, we could also detect the imprint of individual *in vivo* patient medications on PBMC phenotypes. Furthermore, we demonstrated the feasibility to test the personalised response to RA drugs *ex vivo* for each individual donor. Beyond the effects of *ex vivo* and *in vivo* treatment on PBMCs, we systematically identified phenotypes associated with high disease activity. We detected the impact of disease, the effects of *in vivo* patient treatment and *ex vivo* drug perturbation in primary samples from individual donors, thereby demonstrating the successful FPM characterisation of a

rheumatic disorder. The setup was sensitive enough to clearly identify characteristics of RA-derived immune cell populations associated with disease activity, when compared to healthy controls.

Taken together, our study serves as a proof-of-concept for the utility of this screening tool as a promising approach to personalised treatment identification for RA and likely other autoimmune diseases.

Methods

Study procedure

Blood samples were acquired between 08:00 and 12:00 am and patients had to meet the 2010 ACR-EULAR classification criteria for rheumatoid arthritis and clinical disease activity was assessed by physicians at sampling based on clinical disease activity index (CDAI).²⁰

Ethics

The study was approved by the ethical commission of the Medical University of Vienna (EK Nr: 2030/2017). Patients and healthy controls had to sign written informed consent prior to participating in the study and were recruited from the outpatient clinic of the University Clinic for Internal Medicine III, Department of Rheumatology of the Medical University of Vienna. There was no active patient involvement in this study.

Ex vivo monolayer culture

PBMCs were isolated from 18 ml heparinized blood by density gradient centrifugation using Lymphoprep™ (STEMCELL technologies) following the manufacturers protocol. Cells were cultured at a density of 2e4 cells per well in CellCarrier Ultra 384 well plates (PerkinElmer) pretreated with selected drugs in Roswell Park Memorial Institute (RPMI) 1640 Media (Gibco) supplemented with 10% autologous serum.

Ex vivo drug treatment and stimulation

Compounds and stimuli for *ex vivo* drug treatment were selected according to current treatment approaches for rheumatoid arthritis and comprised of 3 csDMARDs (methotrexate (2 µM), leflunomide (5 µM) and sulfasalazine (25 µM)), 5 bDMARDs (etanercept (10 µg/ml), certolizumab-pegol (50 µg/ml), tocilizumab (20 µg/ml), abatacept (250 µg/ml) and rituximab (1 µg/ml)), 3 tsDMARDs (tofacitinib (128 nM), baricitinib (5 nM), filgotinib (10 nM)), 2 glucocorticoids (prednisolone (500 nM) and dexamethasone (127 nM)), 2 non-steroidal anti-inflammatory drugs (naproxen (434 µM) and diclofenac (34 µM)), 5 immunostimulatory reference stimuli (IL-2 (500 U/ml), CD3-CD28 T cell activator (25 µl/ml), IL-10 (10 ng/ml), IL-17 (5 ng/ml) and LPS (10 ng/ml)) as well as dimethyl sulfoxide (DMSO). Used drug concentrations were based on reported peak plasma concentration and cells were cultured for 24 h in native conditions and under artificial proinflammatory

stimulation with 10 nM recombinant human TNF (300-01 A, Peprotech).

Immunostaining

Monolayer cultures were fixed and permeabilised by overlaying the monolayer culture with 0.5% paraformaldehyde and 0.15% Triton X-114 in phosphate-buffered saline (PBS) and incubated for 15 min at room temperature. Consecutive washing was performed on a PerkinElmer JANUS Mini Liquid Handler with PBS 1x and all samples were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich D9542) and the following cell surface markers for 24 h in overlapping staining panels: CD3-APC (BD Biosciences, #345767; 1:500), CD4-PE (BD Biosciences #555347; 1:1000), CD8-FITC (BD Biosciences #345772; 1:500), CD11c-AF488 (Thermo Fisher #MHCD11C20; 1:500), CD11c-APC (Beckman Coulter #B01680; 1:500), CD14-PE (Thermo Fisher #12-0149-42; 1:1000), CD19-FITC (Beckman Coulter #A07768; 1:500), CD56-PE (Beckman Coulter #A07788; 1:1000). For the comparison of formaldehyde fixed cells with live cells, immunostaining of live cells was performed for 2 h at 37 °C with the indicated antibody concentrations in media and nuclei were counterstained with Hoechst 33342 (1:1000, Thermo Fisher #62249).

High content imaging and automated image analysis

Stained plates were allowed to equilibrate to room temperature prior to imaging and microscopy was performed at 20x magnification on a PerkinElmer Opera high content imaging system in confocal mode (excitation 405 nm, 488 nm, 561 nm, 640 nm; emission 435 nm–480 nm, 500 nm–550 nm, 570 nm–630 nm, 650 nm–760 nm, 2-fold binning). Automated image analysis was performed using CellProfiler version 3.1.9.²¹ Nuclei were identified based on local maxima from the DAPI signal and cells were identified based on the merged cell surface marker signal. Identified individual cells were quantified for cell surface marker intensities, morphology and cell-to-cell interaction (Fig. 1b). Cell eccentricity was defined as the ratio of the distance between foci of the ellipse and its major axis length and cell compactness as the mean squared distance of the object's pixel from the centroid divided by the area.²¹

Automated cell classification

Automated cell scoring was performed based on median cell membrane intensity by fitting a bi-Gaussian distribution on log2-transformed intensity values in unstimulated DMSO wells. Individual cells were defined as surface marker positive when the median intensity was greater than the $\mu_1 + 4\sigma_1$ of the bi-Gaussian model.

Statistics

Due to the exploratory nature of this study and the unknown size of phenotypic effects to be encountered, a

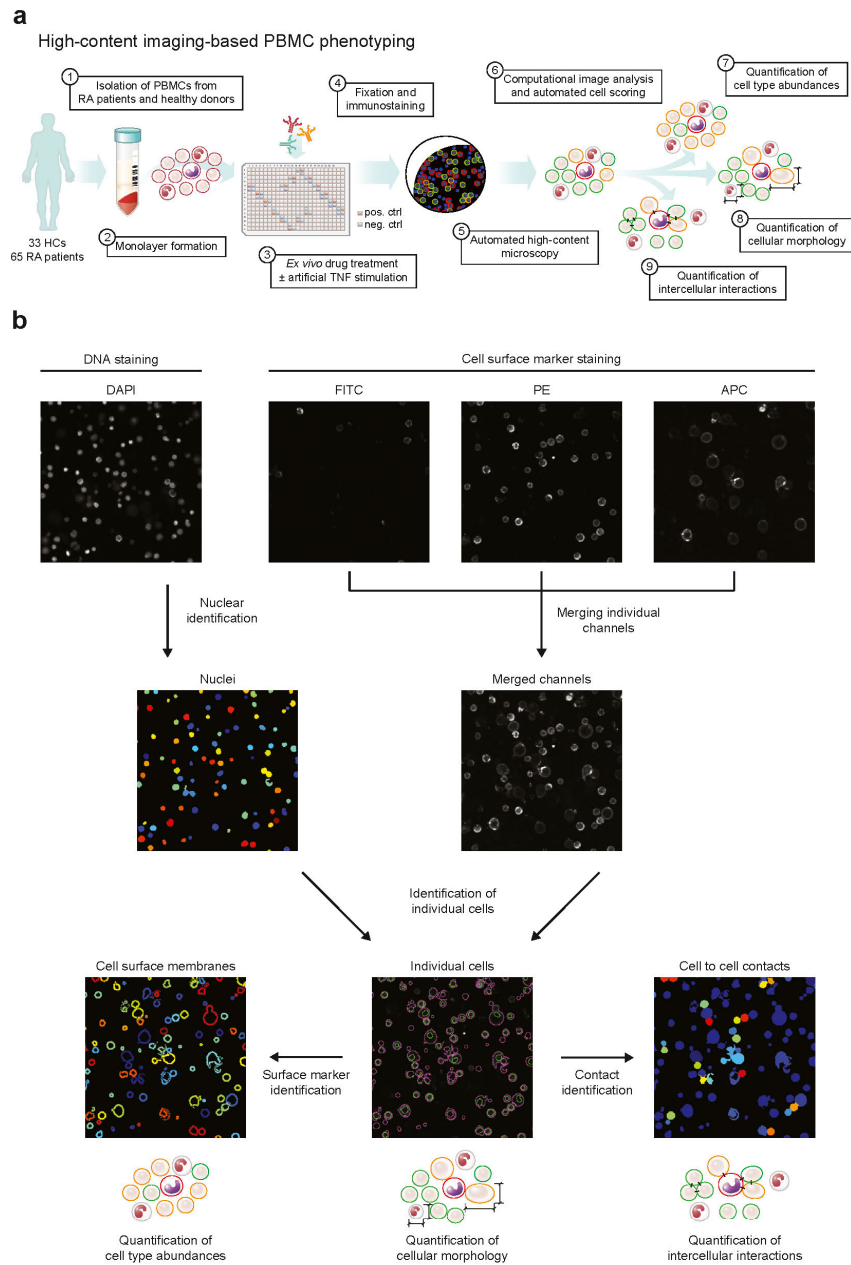


Fig. 1: Ex vivo profiling of PBMCs. (a) Experimental setup. PBMCs from patients with RA and healthy controls (HCs) were isolated by density gradient centrifugation and incubated for 24 h with immunomodulatory drugs and controls. Cells were fixed and stained without perturbing

power analysis for sample-size estimation was not performed. Instead, sample size was determined solely by economical and ethical considerations.

Statistical analysis was performed in R version 4.0.3²¹ facilitating the following packages: *tidyr*, *data.table*, *foreach*, *doParallel*, *mixtools*, *M3C*, *ComplexHeatmap*, *eulerr*, *ggrepel* and *viridis*.

Normality was assessed by Kolmogorov–Smirnov test and for selected comparisons visually by Q–Q plot, statistical significance by a two-sided Welch's t-test and multiple hypothesis correction was performed according to Benjamini and Hochberg. For paired t-tests, normality of differences was evaluated by Kolmogorov–Smirnov test. Unpaired t-tests were used for the comparison between individual donors or groups of donors and paired t-tests were applied to assess *ex vivo* treatment effect sizes in individual donors as described in the figure legends. Boxplots are presented in the style of Tukey where bars indicate the median, boxes indicate the first to third quartiles, the top whisker extends from the hinge to the largest value no further than $1.5 \times$ interquartile range (IQR) from the hinge and the bottom whisker extends from the hinge to the smallest value at most $1.5 \times$ IQR of the hinge. Individual data points are presented as dots on top of every boxplot and lines indicate paired samples, when measurements come from the same donor. Correlations were assessed using Spearman's rank correlation coefficient due to the non-normal distribution of clinical measurements of disease activity and 95% confidence intervals were calculated by Fisher z-transformation.

Role of funders

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Results

A high-content high-throughput imaging pipeline for phenotypic profiling of PBMCs from patients with RA

To introduce an imaging-based approach for diagnosis and therapeutic monitoring of rheumatic diseases, we

Disease activity	HCs	Low	Moderate	High
N	33	36	4	25
Female (%)	18 (54.5)	24 (66.67)	3 (75)	19 (76)
Male (%)	15 (45.5)	12 (33.33)	1 (25)	6 (24)
Mean age in years (SD) for women	53.11 (18.40)	55.92 (17.99)	44.00 (27.07)	56.16 (17.72)
Mean age in years (SD) for men	55.87 (22.83)	57.08 (14.15)	49.00 (NA)	55.50 (16.73)
In vivo treatment (%)				
Untreated		5 (13.9)	0 (0)	9 (36)
csDMARD		14 (38.9)	1 (25)	10 (40)
bDMARD		7 (19.4)	2 (50)	2 (8)
csDMARD-bDMARD combination		10 (27.8)	1 (25)	4 (16)

Patients with RA according to disease activity and healthy controls (HCs). N, number of samples; SD, standard deviation; cs, conventional synthetic; DMARD, disease-modifying antirheumatic drug; b, biologic.

Table 1: Participant characteristics.

established a two-dimensional monolayer model culture of PBMCs that allows for the *ex vivo* phenotypic profiling of primary material in response to a variety of small molecule and monoclonal antibody-based drugs (Fig. 1a). PBMCs of patients with RA and healthy controls (HCs) were isolated from heparinised blood by density gradient centrifugation and incubated for 24 h on a drug-precoated 384-well microplate. This curated set of *ex vivo* drug treatments consisted of 3 csDMARDs (methotrexate, teriflunomide and sulfasalazine), 5 bDMARDs of differing mode of action (certolizumab-pegol, etanercept, tocilizumab, abatacept and rituximab), 3 tsDMARDs (tofacitinib, baricitinib and filgotinib), 2 glucocorticoids (prednisolone and dexamethasone), 2 non-steroidal anti-inflammatory drugs (NSAIDs) (diclofenac and naproxen) and 5 reference stimuli (CD3-CD28 T cell activator, IL-2, IL10, IL17 and LPS). Subsequently, cells were fixed and fluorescently labelled with minimal monolayer perturbation and imaged by automated high content microscopy. This newly established arrayed approach allowed the simultaneous screening of multiple perturbations indicating its potential as a tool for disease identification and treatment monitoring.

We then established a CellProfiler²³ and R²² based image analysis pipeline to retrieve data on a single cell level. Quantification of surface marker intensities in combination with automated cell type scoring allowed for identification of immune cell subsets and the analysis of cell morphological parameters such as cell area, cell eccentricity and cell compactness. In addition, the

the monolayer culture and imaged. Automated image analysis was performed and information about cellular abundance, morphology and intercellular interaction was retrieved. (b) Computational image analysis. Nuclear staining was performed with DAPI and cell surfaces were labelled with surface marker-specific fluorescently-labelled antibodies. Illumination correction was applied to surface marker images and images were then merged. Cells were identified with nuclei as seeds based on the merged surface marker images and cell surface membranes were identified by shrinking cells by 3 pixels. Cell surface marker intensities were quantified on native images for cell type identification, cellular morphology was measured on individual cells and cell-to-cell contacts were quantified based on touching neighbouring cells. DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

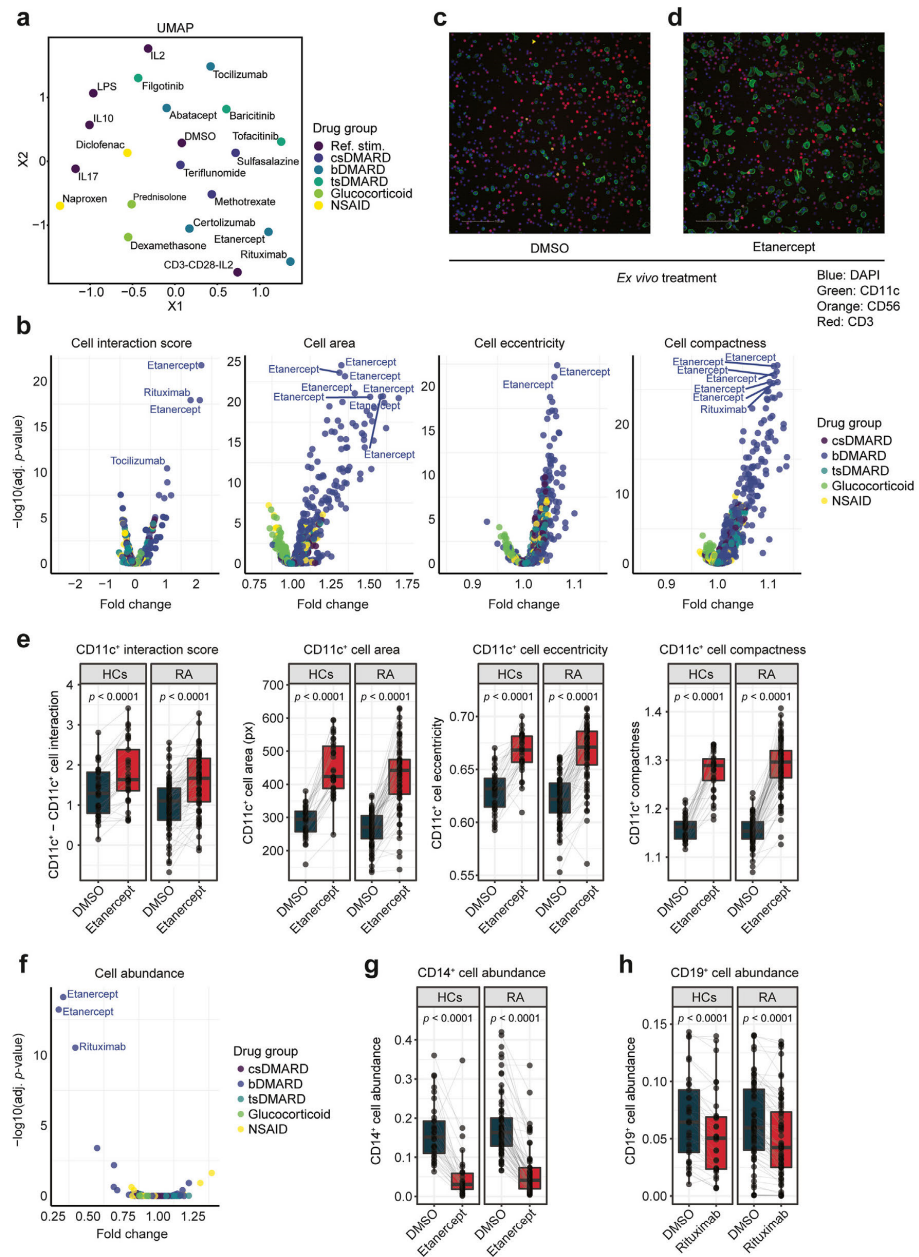


Fig. 2: Ex vivo immunomodulatory drug treatment affects cellular interaction, morphology and cell abundance of PBMCs from patients with RA and HCs. PBMCs from patients with RA and HCs after ex vivo treatment for 24 h. (a) UMAP dimensionality reduction of

method enabled the identification of intercellular interactions to quantify cell-to-cell contact-dependent morphological parameters. Frequencies of cell type-specific contacts were numerically assessed by an automated interaction scoring (Fig. 1b).

The established PBMC profiling pipeline was applied to a cohort of 65 patients with RA meeting the 2010 ACR-EULAR classification criteria and 33 HCs (Table 1). 46 of the patients with RA and 19 of the HCs were female and the average age was 55.5 and 54.4 respectively. Of the 65 patients 14 were untreated, 25 received medication with cDMARDs, 11 with bDMARDs and 15 a combination treatment of csDMARD and bDMARDs (Table 1).

The unbiased data from in total 6.9e8 individual cells were collected by high content microscopy and subsequently characterised in our automated high throughput analysis pipeline. In detail, the arrayed perturbation layout led to 42 individual *ex vivo* treatment conditions that, in combination with overlapping staining panels, resulted in the quantification of 13,188 distinct features for each individual donor. Validating our overall approach, the method successfully detected well established immunologic phenomena such as lymphoblast formation upon T cell activation in a dendritic cell contact-specific resolution as well as LPS-induced clustering and morphologic changes in CD14⁺ cells (Fig. S1a–g). Importantly, key parameters, including cell surface marker expression, cell morphology, and cell-cell interactions were comparable under formaldehyde fixed and live conditions (Fig. S2).

These proof-of-concept results confirm the performance of our high content microscopy-based pipeline to detect and quantify known immunologic phenotypes, thereby highlighting its potential use to further identify PBMC phenotypes specific to drug treatment and RA disease activity.

Effects of *ex vivo* drug treatment

First, we aimed to systematically assess the effects of *ex vivo* drug treatment and indeed found distinct impact of individual compounds on morphological features and interaction propensities of PBMCs of patients with RA

and HCs. Confirming the utility of our approach, uniform manifold approximation and projection (UMAP) revealed clustering of drugs of similar mode of action in patients with RA (Fig. 2a) and HCs (Fig. S3a). Reference stimuli such as T cell activator or LPS most potently modulated cellular interactions and morphology (Figs. S3b–d and S4a–c).

When focusing on RA-specific drugs, bDMARDs exerted the most significant *ex vivo* treatment effects (Fig. 2b). As example, exposure to the TNF-targeting biological etanercept resulted in pronounced changes in morphology and cell-to-cell interactions (Fig. 2b–d). Among multiple affected PBMC characteristics, *ex vivo* etanercept treatment led to CD11c⁺-to-CD11c⁺ cell interactions and distinct changes in CD11c⁺ cell morphology when compared to DMSO ($p < 0.0001$, paired Welch's t-test) (Fig. 2e). The increase in cell size was accompanied by significant changes in cell eccentricity and cell compactness resembling a more elongated phenotype in the presence of etanercept ($p < 0.0001$, paired Welch's t-test) (Fig. 2e and Fig. S5a and b). In contrast to cell eccentricity, CD11c⁺ cell area distribution per donor showed a bi-modal distribution upon *ex vivo* treatment with etanercept suggesting that the observed change in cell size is restricted to a distinct cellular sub-population (Fig. S5a and b). We also found lower CD14 staining intensity upon *ex vivo* etanercept treatment, resulting in reduced numbers of cells classified as CD14-positive ($p < 0.0001$, paired Welch's t-test) (Fig. 2f and g). These CD14^{dim} cells presented with morphological changes consistent with those observed in CD11c⁺ cells (Fig. S5c). Beyond effects on cellular morphology and serving as proof-of-concept for the versatility of our approach, *ex vivo* treatment with rituximab reduced the abundance of CD19⁺ B cells after 24 h of incubation ($p < 0.0001$, paired Welch's t-test) (Fig. 2h).

PBMC phenotypes associated with *in vivo* patient treatment

Encouraged by the results regarding *ex vivo* drug perturbation, we next assessed the impact of *in vivo* patient treatment regimens on our cellular model

ex vivo drug effects shows clustering of compounds by drug class. Dots represent individual *ex vivo* drug treatment conditions. (b) *Ex vivo* drug treatment of PBMCs from patients with RA ($n = 65$) affects intercellular interaction as quantified by interaction score and cell morphology, colour coded for class of *ex vivo* drug treatment. (c & d) *Ex vivo* treatment with the TNF inhibitor etanercept (d) has distinct effects on cellular morphology in CD11c⁺ cells. (CD11c green, CD3 red, CD56 orange, DAPI blue, 20x magnification) as compared to DMSO (c). (e) *Ex vivo* treatment with the TNF inhibitor etanercept affects CD11c⁺ cell-to-cell interaction as well as CD11c⁺ cell morphology from patients with RA ($n = 65$) and HCs ($n = 33$). (f) *Ex vivo* drug treatment of PBMCs from patients with RA ($n = 65$) affects cell type abundances, colour coded for class of *ex vivo* drug treatment. (g) *Ex vivo* treatment with the TNF inhibitor etanercept reduces the number of cells classified as CD14⁺ in PBMCs from patients with RA ($n = 65$) and HCs ($n = 33$). (h) *Ex vivo* treatment of PBMC with rituximab significantly affects B cell abundance in patients with RA ($n = 65$) and HCs ($n = 33$). (b, f) Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg. (e, g, h) Lines indicate matching donors and significance was assessed by a paired t-test. UMAP, uniform manifold approximation and projection; Ref. stim., reference stimuli; cs, conventional synthetic; DMARD, disease-modifying antirheumatic drug; b, biologic; ts, targeted synthetic; NSAID, non-steroidal anti-inflammatory drug; DAPI, 4',6-diamidino-2-phenylindole; adj., adjusted; px, pixels.

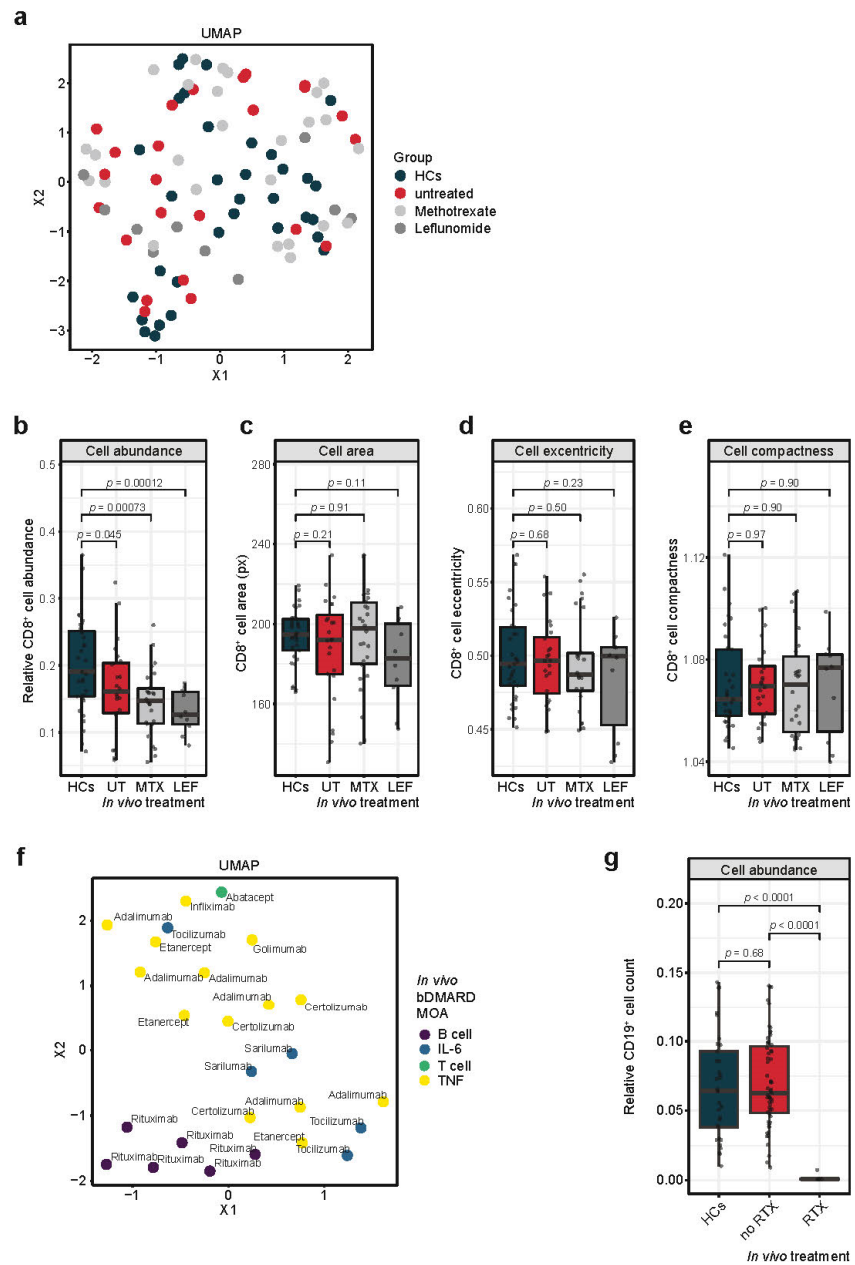


Fig. 3: In vivo patient medication affects ex vivo PBMC features. (a–e) PBMCs of HCs ($n = 33$), patients without csDMARD treatment ($n = 27$) and individuals with a comedication of methotrexate ($n = 26$) or leflunomide ($n = 10$) were assessed for differences in cellular abundance,

culture. We therefore compared cell abundances, cell morphological features and cell-to-cell interaction propensities in PBMCs of patients with a defined medication with those measured in HCs.

First, we focused on the patient subset that is under *in vivo* csDMARD treatment with methotrexate or leflunomide (Fig. 3a). The most significant finding related to *in vivo* treatment when comparing HCs to patients with methotrexate treatment was the reduction in CD8⁺ T cell abundance ($p = 0.00073$, Welch's t-test) (Fig. 3b). Even though this trend of reduced CD8⁺ cell abundance was already visible in the untreated sub-cohort, it only reached statistical significance under conditions of *in vivo* methotrexate ($p < 0.00073$, Welch's t-test) or leflunomide ($p < 0.00012$, Welch's t-test) treatment. However, differences in abundance were not associated with morphological changes ($p > 0.05$, Welch's t-test) (Fig. 3c–e). Additionally, *in vivo* leflunomide ($p = 0.029$, Welch's t-test), but not methotrexate ($p = 0.98$, Welch's t-test) treatment, was significantly associated with increased abundance of CD14⁺ cells, without affecting cell area ($p = 0.98$, Welch's t-test), eccentricity ($p = 0.72$, Welch's t-test), or compactness ($p = 0.61$, Welch's t-test) (Fig. S6a–d).

Next, we focused on PBMC phenotypes associated with bDMARD treatment according to the drugs' mode of action. UMAP dimensionality reduction revealed distinct clustering of individual patients with a bDMARD co-medication of rituximab (Fig. 3f). Indeed, our method was sensitive to sufficiently detect significantly lower CD19⁺ B cell counts in patients with co-treatment of rituximab ($p < 0.0001$, Welch's t-test) as compared to HCs and patients without a rituximab co-medication ($p < 0.0001$, Welch's t-test) (Fig. 3g).

Together, our methodology successfully allowed to identify distinct PBMC phenotypes related to *in vivo* medication as exemplified by the detection of altered T cell specific morphology features upon csDMARDs as well as prominent impact of bDMARD treatment, such as B cell depletion, in the cohort of patients with RA.

Identification of disease-specific phenotypes

To this end we exposed PBMCs from HCs and patients with RA with various treatments to our curated set of RA-specific drugs and various immunogenic reference

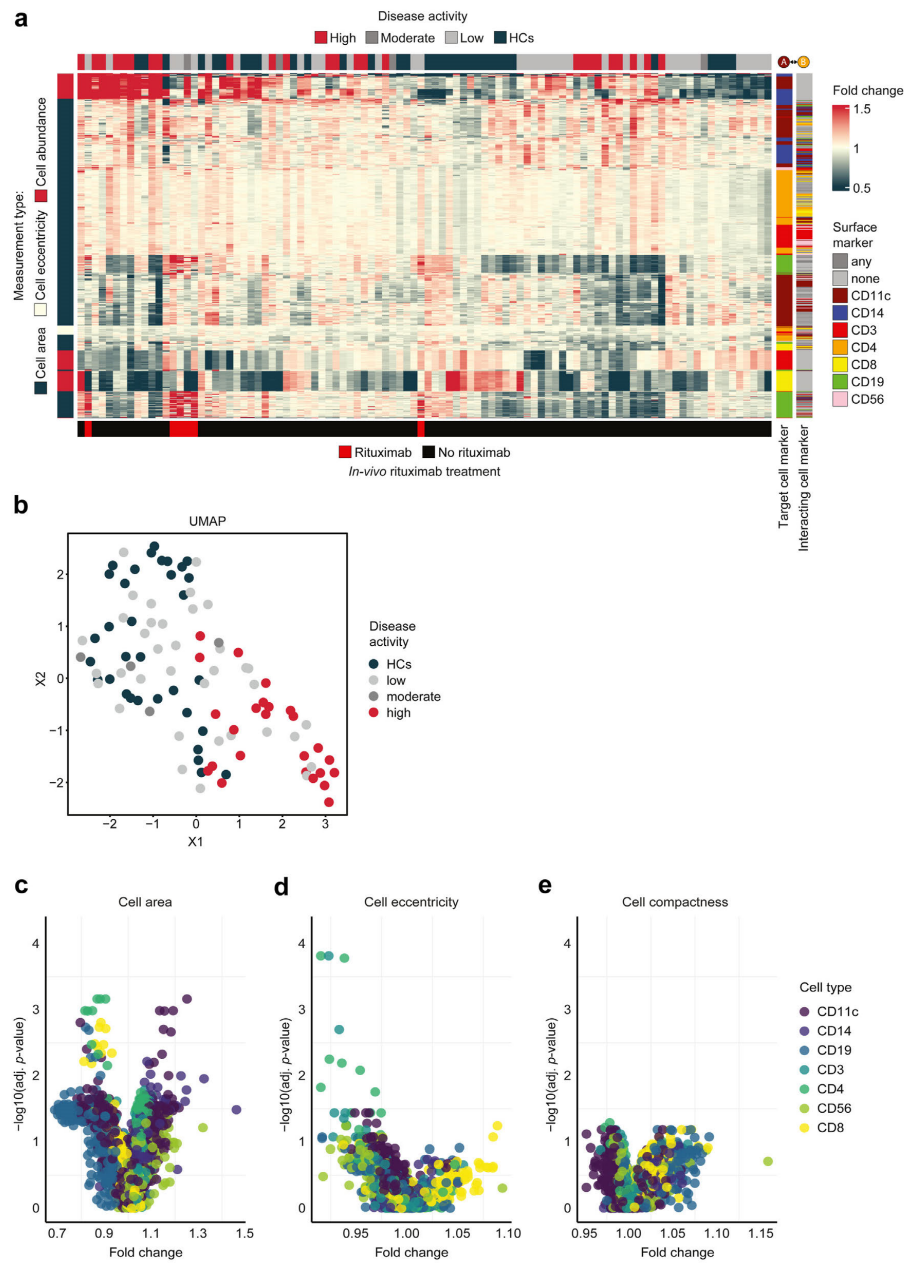
stimuli and assessed differential cellular *ex vivo* and *in vivo* treatment responses.

To identify PBMC phenotypes associated with disease, we derived a comprehensive set of parameters significantly (p adj. < 0.05 , Welch's t-test) altered in patients with high disease activity (Fig. 4a). Their representation as a hierarchically clustered heatmap enabled the visualisation of individual measurements per single donor and the multidimensional annotation according to disease activity (top), type of measurement (cell area, eccentricity and abundance; left), *in vivo* treatment (e.g. rituximab, bottom) and quantified cell type in a contact-dependent manner (target cell to interacting cell, right). To further visualize the significant parameters distinguishing high diseases activity patients from HCs, we applied dimensionality reduction by UMAP to this highly complex data. We indeed observed clear separation between HCs and patients with high diseases activity (Fig. 4b), unrelated to other evaluated variables such as treatment, rheumatoid factor or donor sex (Fig. S7).

Ranking the individual measurements by significance, we identified alterations of cellular morphology, specifically cell area and eccentricity (Fig. S8a), as the most important determinant of high disease activity in various immune cell subsets (Fig. 4c–e and Fig. S8b). Interestingly, we did not identify cellular responses to the *ex vivo* treatment with RA-specific drugs among the most discriminating conditions, but rather found differential cellular responses to the set of reference stimuli as the most distinguishing conditions (Fig. S8c).

The most prominent differential morphologic responses were observed in various T cell subsets, known drivers of systemic inflammation in RA (Fig. 4c–d and Fig. S8a and b). Indeed, we saw significant reduction of CD4⁺ and CD8⁺ T cell area upon *ex vivo* stimulation with T cell activator (CD4⁺: $p < 0.0001$, CD8⁺: $p < 0.0001$, Welch's t-test) (Fig. 5a and Fig. S8d), accompanied by alterations in eccentricity and compactness in patients with RA of varying disease activity when compared to HCs (Fig. 5a–c and Fig. S8d). To evaluate whether this observation is affected by *in vivo* patient treatment with csDMARDs, we compared untreated patients with high disease activity with those under treatment with methotrexate or leflunomide. No significant differences were found across tested groups, indicating that the above

morphology, and interaction in unstimulated and untreated wells. (a) Uniform manifold approximation and projection (UMAP) of all individual measurements. (b) CD8⁺ T cell abundance in healthy controls and in the respective groups. Dots represent individual donors with the indicated csDMARD treatment. (c–e) Cell morphological characteristics of CD8⁺ T cells in the respective groups. (f) UMAP of individual measurements from patients with a bDMARD comedication, colour represents the indicated mode of action (B cell depletion $n = 6$, IL-6-targeting $n = 5$, inhibition of T cell co-activation $n = 1$, TNF targeting $n = 14$). Dots represent individual donors with the indicated bDMARD treatment. (g) CD19⁺ B cell abundance in HCs ($n = 33$) and patients with ($n = 6$) and without rituximab treatment ($n = 59$) as a comedication. (b–e, g) Welch's t-test, p values as indicated. px, pixels; HCs, healthy controls; UT, untreated; MTX, methotrexate; LEF, leflunomide; MOA, mode of action; RXT, rituximab.



phenomenon is dominantly driven by disease state rather than the specific csDMARD treatment ($p > 0.05$, Welch's t-test) (Fig. S8e).

To extend our observation of cellular phenotypes specific to RA beyond those found in high disease activity, we tested for significantly altered features between low disease activity patients and HCs (p adj. < 0.05 , Welch's t-test). Whilst differences were less pronounced, we consistently found altered CD8⁺ cell abundance among the most prominently differentiating feature of both high ($p = 0.0070$, Welch's t-test) and low ($p = 0.0025$, Welch's t-test) disease activity RA as compared to HCs (Fig. S9a–e). To further address the question of *ex vivo* phenotypes specific to disease activity, we compared high and low disease activity patients with RA and found, cell-to-cell interactions of CD11c⁺ and CD14⁺ cells among the most significantly altered (p adj. < 0.05 , Welch's t-test) (Fig. S10a–c). Specifically, CD11c⁺ and CD14⁺ cell clustering was reduced in patients with high disease activity but not in patients with low disease activity ($p > 0.05$, Welch's t-test) as compared to HCs in DMSO ($p = 0.024$, Welch's t-test) control and upon *ex vivo* treatment with LPS ($p = 0.0036$, Welch's t-test) (Fig. S10d and e).

We finally assessed the correlation of PBMC phenotypes with the clinical activity index (CDAI) at the timepoint of sampling and the change of CDAI to the follow-up visit. As shown for disease activity classes, we found cell morphological changes of CD4⁺ cells upon *ex vivo* stimulation with T cell activator among the measurements with the highest correlation to CDAI (cell area: $R = -0.45$, $p = 0.0027$, cell eccentricity: $R = -0.48$, $p = 0.0013$, cell compactness: $R = -0.41$, $p = 0.0068$, Spearman's rank correlation coefficient) (Fig. 5d–f) and swollen and tender joint count (Fig. S11a and b). Beyond that, we also observed a correlation with the change of CDAI (Δ CDAI) to the following visit (cell area: $R = 0.53$, $p = 0.0027$, cell eccentricity: $R = 0.37$, $p = 0.046$, cell compactness: $R = 0.36$, $p = 0.051$, Spearman's rank correlation coefficient) (Fig. 5g–i). Other features correlating with changes in disease activity included CD11c⁺ cell eccentricity and

compactness, but not area, in *ex vivo* etanercept treatment conditions (Fig. S12a and b).

These results demonstrate the identification and characterisation of disease-specific phenotypes amongst which differential T cell responses to stimulation were found to be the most discriminating factors between HCs and patients with RA of varying disease activity.

Discussion

Knowledge on the diverse cellular and molecular features specific to rheumatoid arthritis is key for diagnosis, personalised treatment and disease monitoring, not only for the individual patient, but also to alleviate the associated socioeconomic burden.²³ Motivated by the introduction of imaging-based FPM as a novel approach to *ex vivo* drug response profiling in immunological studies^{18,24} and as a powerful tool for treatment selection in haematologic malignancies,^{13,15,16} we developed a pipeline for the multiparametric assessment of PMBCs from patients with RA and healthy controls.

In our study we characterised a palette of RA-specific small molecules and biologicals with different modes of action as well as defined control stimuli in an innovative *ex vivo* model culture system facilitating high content microscopy. Using this screening tool, we successfully detected known RA-related features associated with individual treatments including cell-to-cell contact-dependent lymphocyte activation as well as B cell depletion upon *ex vivo* rituximab treatment.^{25,26}

Importantly, our platform also allowed us to discover novel features related to individual drug treatment such as etanercept-induced morphological changes in monocytes. Furthermore, we identified distinct treatment-induced cell type-specific effects on T cell abundance and monocyte morphology and, in line with the *ex vivo* data, a strong effect of B cell-depleting therapy.²⁶ These findings therefore form the foundation for future studies mechanistically addressing the biology and action of anti-rheumatic drugs.

and *ex vivo* conditions between patients with RA with high disease activity ($n = 25$) and HCs ($n = 33$) for all study participants (rows). Individual donors are represented as columns and are clustered hierarchically. Heatmap colours indicate the fold-change from the mean value of the HC cohort for each individual measurement. Top annotation: Colour indicates the individual donor's disease activity (HC, high, moderate or low disease activity). Left annotation: Colours indicate the type of measurement (cell area, cell eccentricity or cell abundance). Right annotation: Type of cell-to-cell interaction of the individual measurement indicating the quantified cell type "A" in respect to the interacting cell type "B". Bottom annotation: Colour indicates the *in vivo* co-medication with (red) or without rituximab (black) as an example for the annotation of all samples with clinical information. (b) Uniform manifold approximation and projection (UMAP) of parameters of PMBCs from all *in vivo* and *ex vivo* conditions differentiating the compared groups of Fig. 4a with an adjusted p value < 0.01 (Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). Dots represent individual donors. (c–e) Peripheral blood mononuclear cell (PBMC) cell area of HCs ($n = 33$) and high disease activity patients with RA ($n = 25$) were treated in various *ex vivo* conditions for 24 h. (c) Cell area measurements per condition were assessed for differences between groups and are presented as fold change from HCs, colour coded for cell type. PBMC cell eccentricity (d) and cell compactness (e) in the respective cohorts. (c–e) Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg. HCs, healthy controls; adj., adjusted.

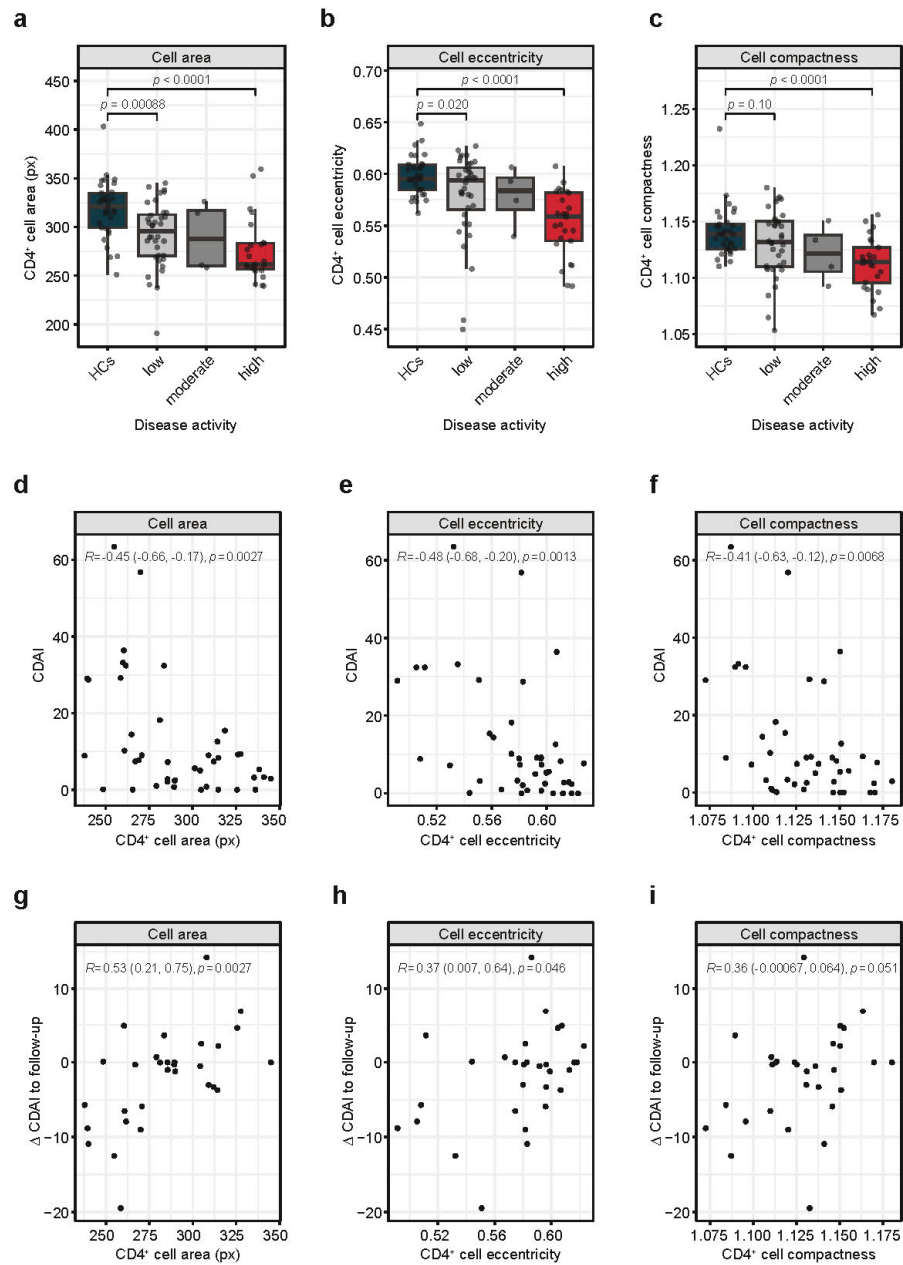


Fig. 5: Cell morphology in patients with RA and healthy controls (HCs). (a–c) Cell morphological characteristics of CD4⁺ cells of HCs ($n = 33$), and patients with low ($n = 36$), moderate ($n = 4$) and high ($n = 25$) RA disease activity upon CD3-CD28 T cell activator treatment, dots indicate

The microscopy-based assessment of morphologic features of immune cells provides a unique opportunity to identify disease state-specific cell morphologic phenotypes such as the reduced T cell reactivity upon activation in patients with RA. This observation most likely represents previously reported findings as a consequence of sustained autoimmunity-driven differential cell activation.²⁷

While our approach proves as a powerful tool for *ex vivo* immunophenotyping of patients' cells, it indeed only captures a short time window of disease and treatment and therefore does not reflect the *in vivo* pathophysiological setting, potentially missing factors contributing to disease. Specifically, most RA drugs exert their impact over a longer period than the one observable by our method and might for example require drug metabolism for their long-term disease-modifying activity. Additionally, the limited number of drugs, stimulatory conditions, and use of circulating immune cells, which do not represent the primary site of disease, may not reflect RA in its complexity. Especially the limited number of cell surface markers used to distinguish immune cell populations leave space for further improvements of the application of our technique in the future. This could be achieved by leveraging on more advanced staining, imaging and analysis techniques such as multiplexed imaging and machine learning-based cell profiling.^{28–30} Beyond the technical limitations, the low number of patients, in combination with the cohort diversity in terms of disease duration, disease activity, treatment regimens and further potentially unknown confounding factors, further restrict the pathophysiological relevance of our study.

Awareness of the aforementioned limitations is essential for future technical improvements. These include a more refined characterisation of PBMCs based on cell- and activation-specific markers in combination with cell type-specific stimuli, as well as the integration of key cells from the synovial microenvironment. On the analysis side, open-source tools enable exploratory research,^{21,22} however for further clinical implementations the development of a set of tailored and standardised procedures and analysis pipelines will be required.

Even though recent developments, such as the use of synovial biopsies as a stratification tool for RA, have led to an advancement for preferential treatment selection,^{8,9,31,32} neither genetics, serological biomarkers

nor radiographic or clinical scoring approaches have yet stratified patient cohorts comprehensively. As our newly developed method is minimally invasive and sampling of liquid biopsies easy to implement into routine care, the presented tool effectively complements and potentially synergises with the previously mentioned possibilities for disease assessment.

Promising future applications of our approach comprise the identification of at-risk cohorts as well as longitudinal monitoring of treatment responses. Ideally, the application of our technique would allow to detect RA-specific phenotypes in pre-symptomatic individuals and enable functional precision medicine approaches for treatment selection, monitoring of treatment efficacy and early detection of treatment failure. The presented data and the herein determined effect sizes for PBMC phenotypes therefore form the foundation to evaluate these objectives in future clinical trials where our tool could be implemented.

The presented pilot study lays the foundation for functional precision medicine outside the oncological domain. The study also represents a new showcase for the application of high content microscopy as a powerful tool for the investigation of immune system-related disorders. The approach should thus represent an as yet unexploited opportunity to accomplish disease stratification and improve treatment strategies for rheumatoid arthritis and likely other autoimmune diseases.

Contributors

FK, NM, NK, LH and GSF designed the study. FK recruited participants. FK, NM and NK were involved in designing and performing experiments. FK, UG and LH analysed the data. FK, MB, LH, DA and GSF wrote the manuscript. All authors read and approved the final version of the manuscript.

Data sharing statement

Data is available upon reasonable request. Anonymised data will be available upon request.

Declaration of interests

FK received support for attending conference from UCB Pharma GmbH. MB receives grants from Alphasigma. UG: declares no conflicts of interest. NM declares no conflicts of interest. NK is a co-founder of Alcyte GmbH (now Exscientia), member of the executive leadership team of Exscientia and owns shares of Exscientia PLC; in addition, NK is an inventor on several patents for pharmacoscopy issued to Exscientia. DA received grants, speaker fees, or consultancy fees Abbvie, Amgen, Galapagos, Eli Lilly, Sanofi, Janssen, Merck, Novartis, Pfizer, and Sanofi. LH declares no conflicts of interest. GSF is a co-founder of Alcyte (now Exscientia) and owns shares of Exscientia PLC; in addition, GSF has a patent for pharmacoscopy issued to Exscientia.

individual samples. (e and f) Correlation of CD4⁺ cell area (left), cell eccentricity (middle) and cell compactness upon 24 h *ex vivo* CD3-CD28 T cell activator treatment with clinical disease activity index (CDAI) at the timepoint of sampling (n = 43). (g–i) Correlation of CD4⁺ cell area (left), cell eccentricity (middle) and cell compactness upon 24 h *ex vivo* CD3-CD28 T cell activator treatment with change in CDAI between the timepoint of sampling and the CDAI recorded at the follow-up visit (n = 30). (a–c) Welch's t-test, p values as indicated. (d–i) Spearman's rank correlation coefficient R is indicated with its 95% confidence interval (in parentheses) and its statistical significance (p value). px, pixels, CDAI, clinical disease activity index.

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Appendix A. Supplementary data

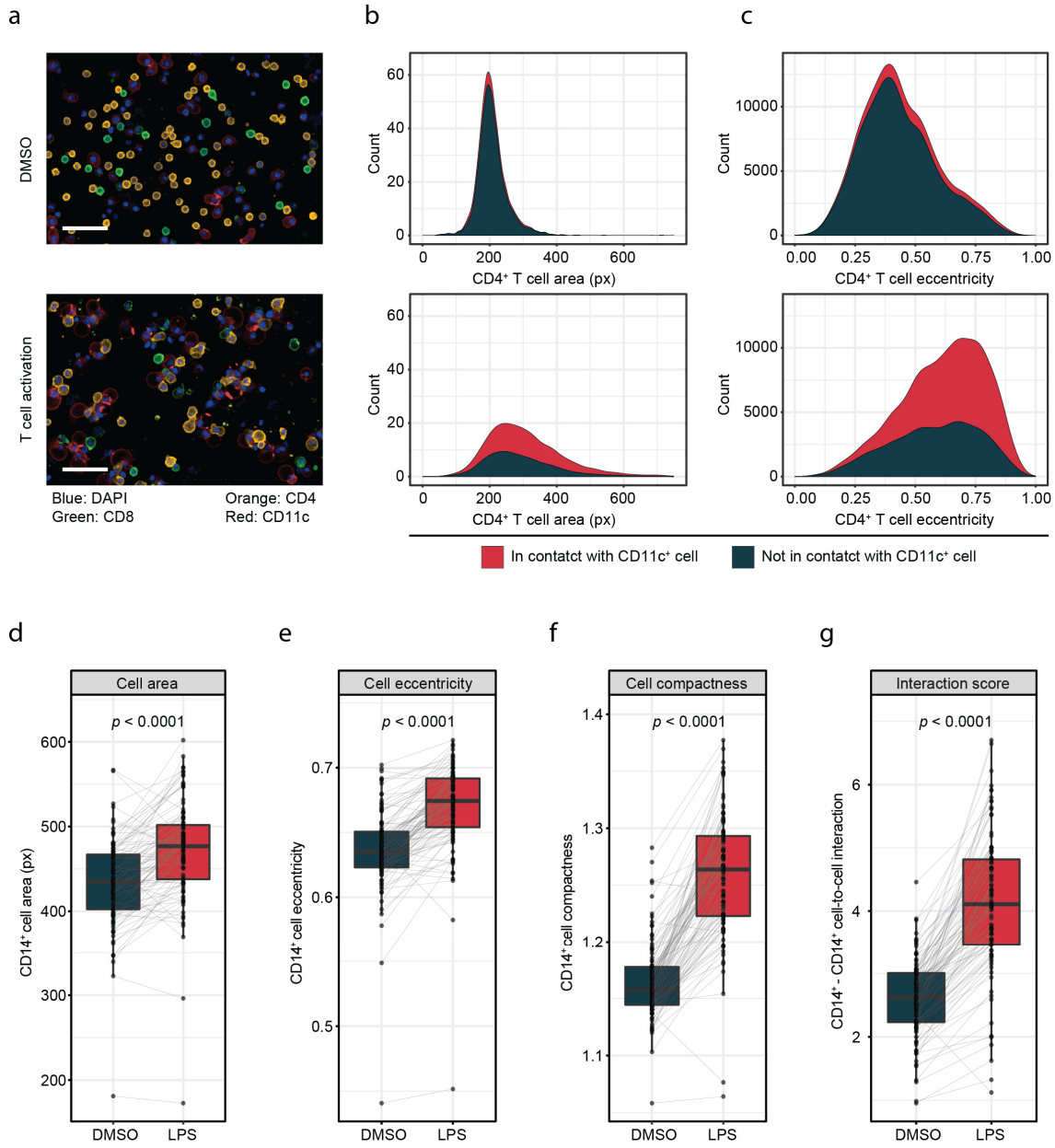
Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2024.105522>.

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SUPPLEMENTARY MATERIAL

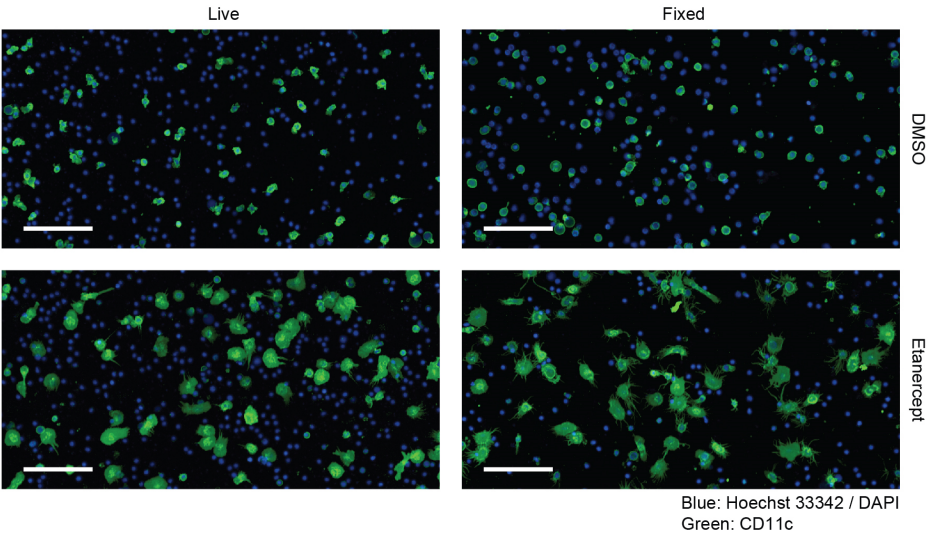
Supplementary Figure 1



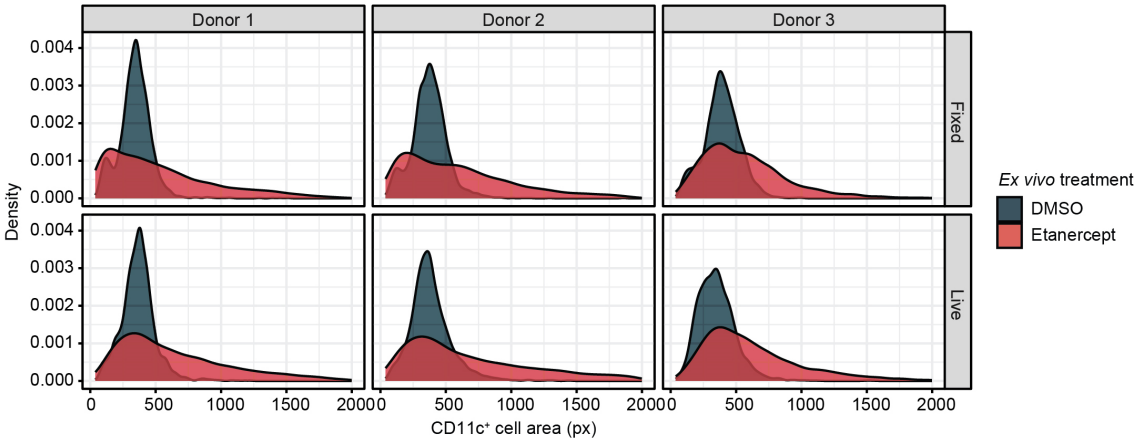
Supplementary Figure 1. Validation of known immunologic phenomena upon *ex vivo* stimulation. (a) Representative images of a peripheral blood mononuclear cell (PBMC) co-culture. DMSO (top) and T cell activation (bottom) depict enlarged and elongated CD4⁺ cells in a CD11c⁺ contact-specific manner upon T cell activation after 24h. (CD8 green, CD11c red, CD4 orange, DAPI blue, scale bar represents 50μm). (b-c) Computational image analysis for the numeric assessment of cell morphologic changes in a cell-to-cell contact-specific manner showing effects on (b) cell area and (c) cell eccentricity upon *ex vivo* treatment with DMSO (top) and after 24h of T cell activation (bottom). (d-f) *Ex vivo* quantification of CD14⁺ cell morphology features of PBMCs treated for 24h with DMSO or LPS. (g) *Ex vivo* quantification of CD14⁺-to-CD14⁺ cell interactions in PBMC samples treated for 24h with DMSO or LPS. (d-g) Lines indicate matching donors and significance was assessed by a two-sided paired t-test. DAPI, 4',6-diamidino-2-phenylindole.

Supplementary Figure 2

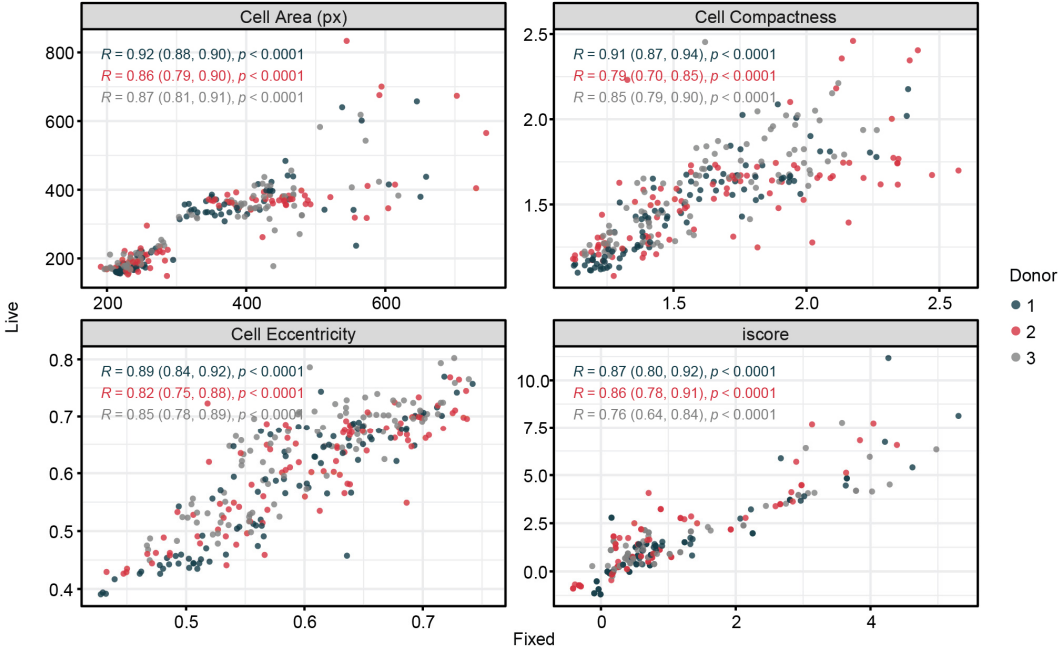
a



b

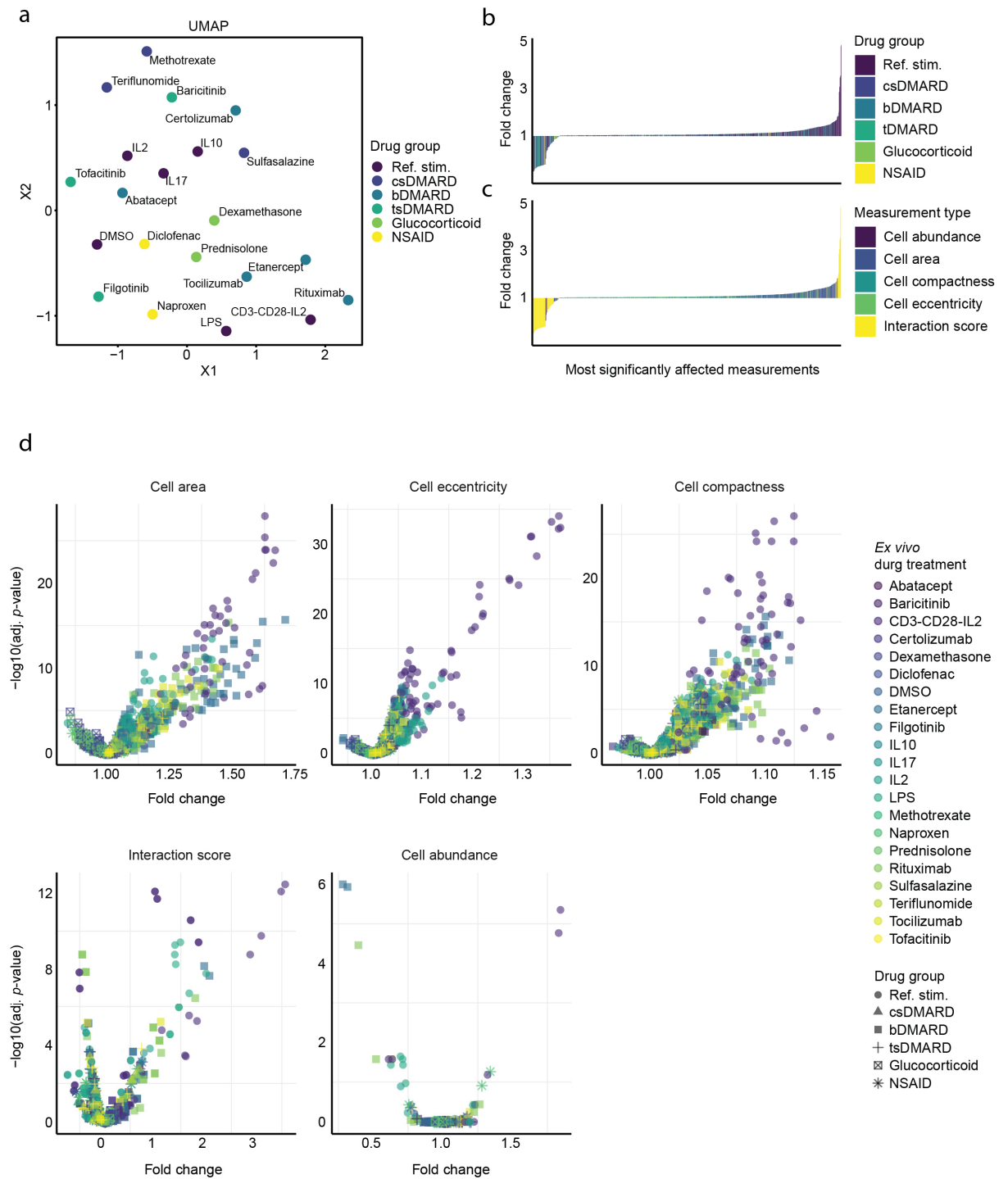


c



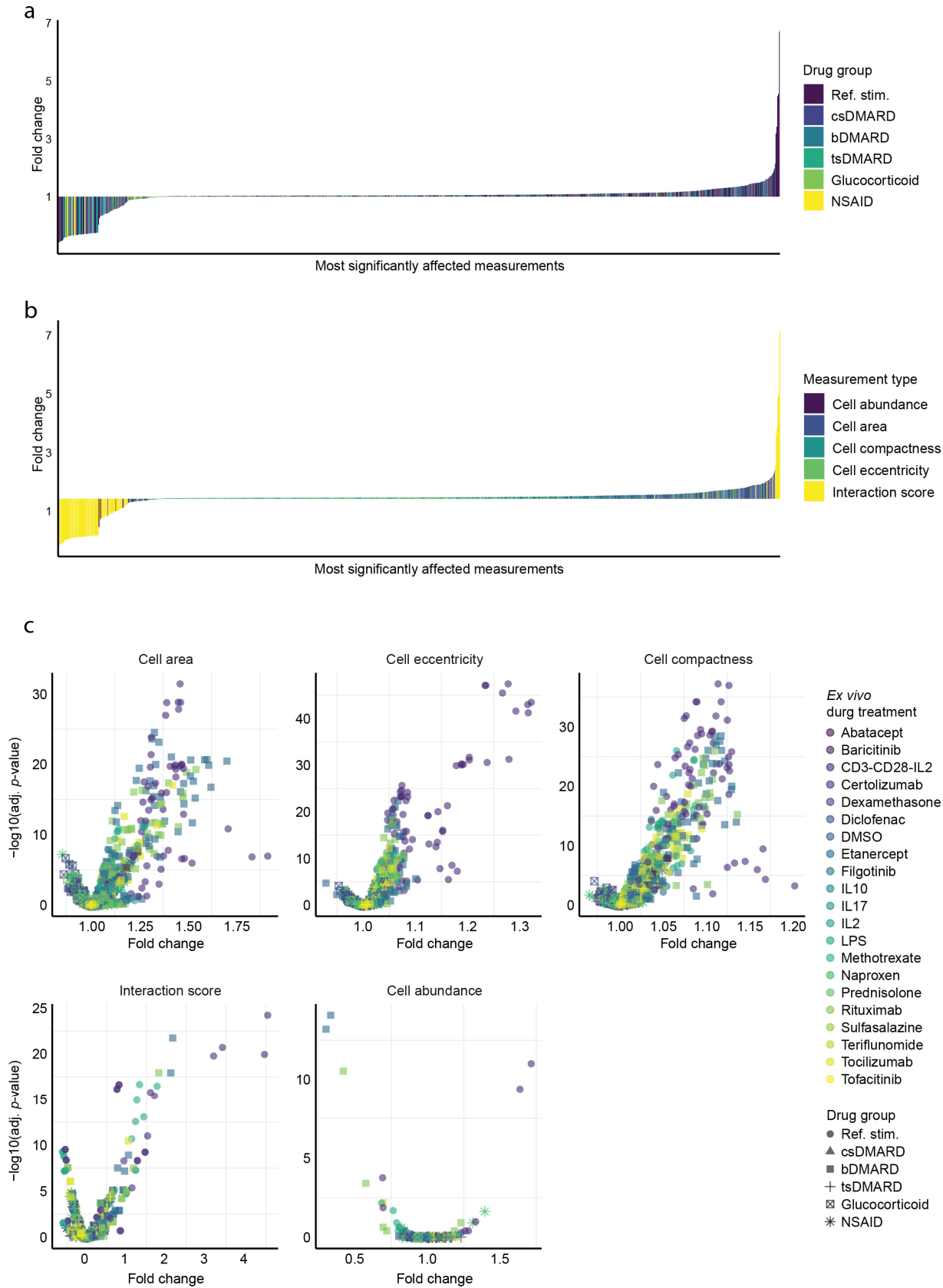
Supplementary Figure 2. Comparison of cell morphology. **(a)** Representative images of a PBMC co-culture. Cells were imaged live after 2h of immunostaining and counterstaining with Hoechst 33342 (left) or after staining as described in the methods (right) after 24 hours of *ex vivo* treatment with DMSO (top) or etanercept (bottom). (CD11c green, Hoechst 33342 / DAPI blue, scale bar represents 100µm). **(b)** Computational image analysis for the numeric assessment of CD11c⁺ cell area (px) upon 24h *ex vivo* treatment with DMSO or etanercept in three individual donors stained as described in Fig. S2a. **(c)** Correlation of cell morphological parameters and interaction scores of live stained and fixed cells for all 3 donors from Fig. S2b. Dots represent individual measurements, colour coded for indicated donor. Correlation was assessed by Spearman's rank correlation coefficient *R*, indicated with its 95% confidence interval (in parentheses) and its statistical significance (p value). DAPI, 4',6-diamidino-2-phenylindole; px, pixels.

Supplementary Figure 3



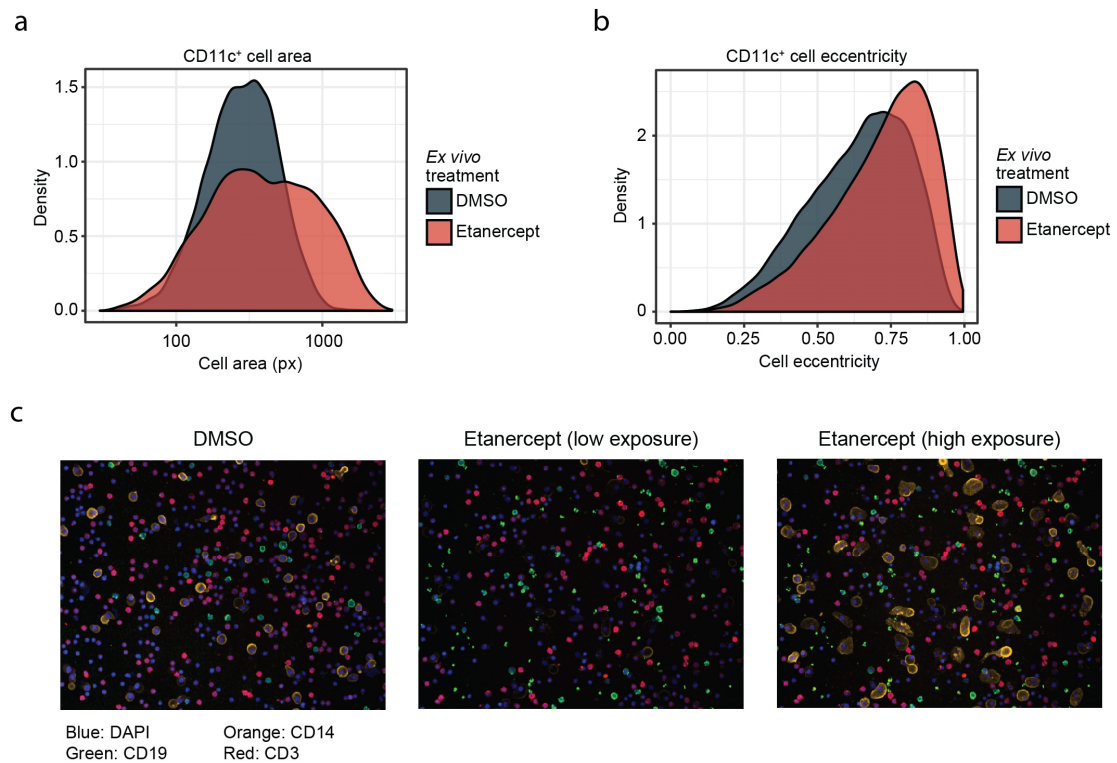
Supplementary Figure 3. *Ex vivo* drug treatment affects cellular interaction, morphology and cell abundance in healthy controls (HCs, n=33). (a) UMAP dimensionality reduction of *ex vivo* drug effects shows clustering of compounds by drug class. Dots represent individual *ex vivo* drug treatment conditions. (b) Most significant *ex vivo* drug effects colour coded class of *ex vivo* treatment (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). (c) Most significant *ex vivo* drug effects colour coded by type of measurement (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). (d) *Ex vivo* drug treatment of PBMCs from HCs affects PBMC cell morphology, cellular interaction and cell abundance, coded for class and individual drug of *ex vivo* treatment. (Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). UMAP, uniform manifold approximation and projection; Ref. stim., reference stimuli; cs, conventional synthetic; DMARD, disease-modifying antirheumatic drug; b, biologic; ts, targeted synthetic; NSAID, non-steroidal anti-inflammatory drug; adj., adjusted.

Supplementary Figure 4



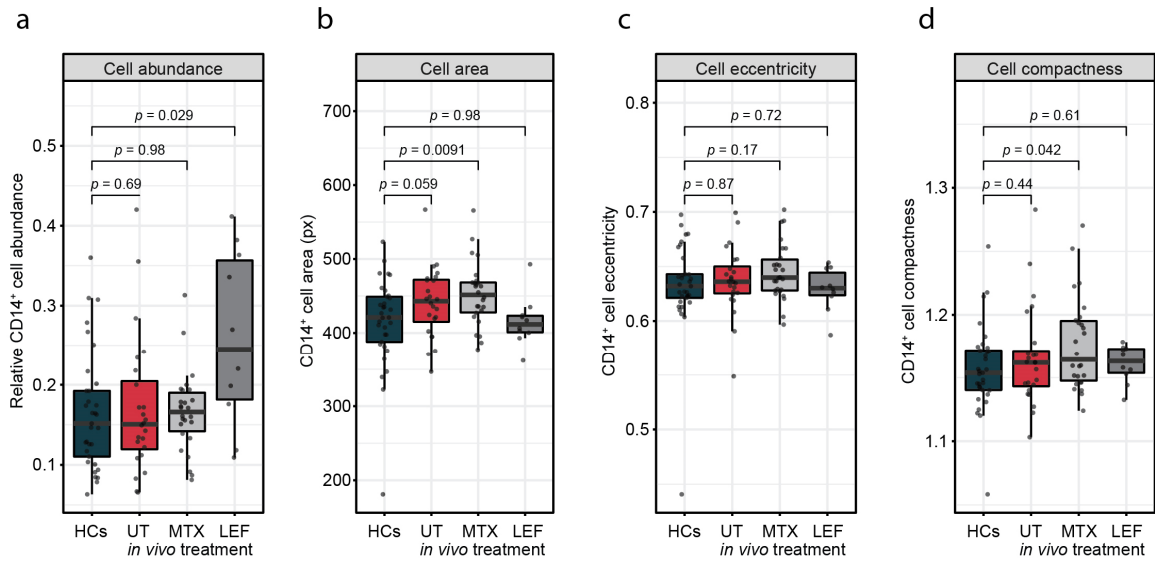
Supplementary Figure 4. *Ex vivo* drug treatment affects cellular interaction, morphology and cell abundance of PBMCs from patients with rheumatoid arthritis (RA) (n=65). (a) Most significant *ex vivo* drug effects colour coded class of *ex vivo* treatment (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). (b) Most significant *ex vivo* drug effects colour coded by type of measurement (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). (c) *Ex vivo* drug treatment of PBMCs from HCs affects PBMC cell morphology, cellular interaction and cell abundance, coded for class and individual drug of *ex vivo* treatment (Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). Ref. stim., reference stimuli; cs, conventional synthetic; DMARD, disease-modifying antirheumatic drug; b, biologic; ts, targeted synthetic; NSAID, non-steroidal anti-inflammatory drug; adj., adjusted.

Supplementary Figure 5



Supplementary Figure 5. *Ex vivo* immunomodulatory drug treatment affects monocyte morphology. (a) Distribution of CD11c⁺ cell area in a representative sample in DMSO control wells and in etanercept-treated PBMCs after 24h of *ex vivo* treatment. (b) Distribution of CD11c⁺ cell eccentricity in a representative sample in DMSO control wells and in etanercept-treated PBMCs after 24h of *ex vivo* treatment. (c) Representative images of a PBMC co-culture. DMSO (left) etanercept low exposure (middle) and etanercept high exposure (right) (CD19 green, CD3 red, CD14 orange, DAPI blue, 20x magnification). px, pixels; DAPI, 4',6-diamidino-2-phenylindole.

Supplementary Figure 6



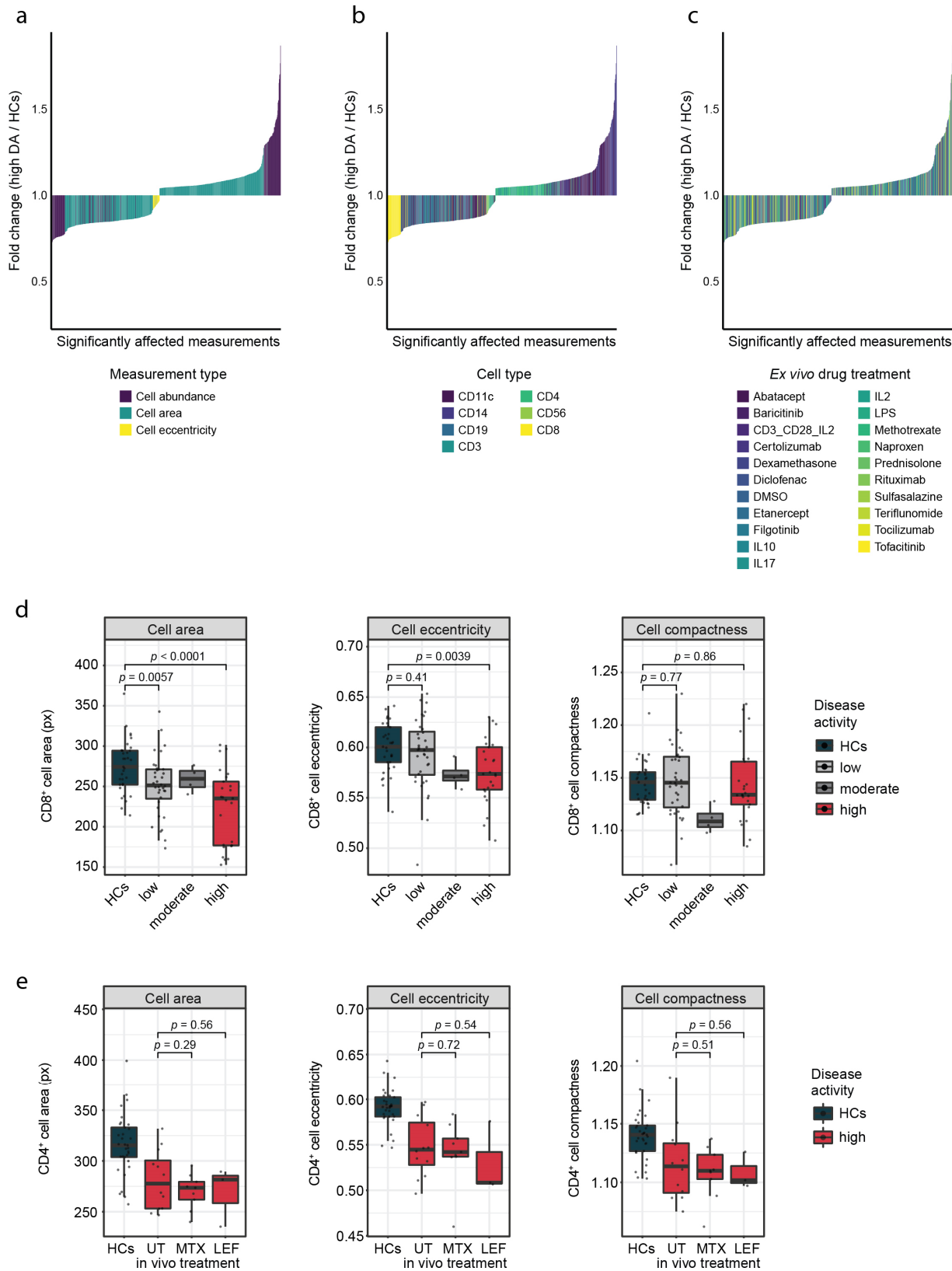
Supplementary Figure 6. *In vivo* patient medication affects PBMC characteristics. (a) CD14⁺ cell abundance in HCs (n=33), patients with no csDMARD (n=24) and patients with a comedication of methotrexate (n=26) or leflunomide (n=10). **(b-d)** CD14⁺ cell morphological characteristics in the respective cohorts of (Fig. S6a). **(a-d)** Welch's t-test. px, pixels; HCs, healthy controls; UT, untreated; MTX, methotrexate; LEF, leflunomide.

Figure 2 consists of five UMAP plots (a-e) showing the relationship between treatment and donor sex. The plots are arranged in a 2x3 grid, with the last cell empty. The x-axis for all plots is X1 and the y-axis is X2.

- a** UMAP plot showing the relationship between donor sex (female in red, male in blue) and treatment groups (HCs, csDMARD, bDMARD, tsDMARD, cs + bDMARD, cs + tsDMARD, untreated). The plot shows a clear separation between female and male donors, with female donors generally having higher X2 values.
- b** UMAP plot showing the relationship between treatment groups (HCs, csDMARD, bDMARD, tsDMARD, cs + bDMARD, cs + tsDMARD, untreated). The plot shows a clear separation between treatment groups, with HCs generally having higher X2 values.
- c** UMAP plot showing the relationship between treatment groups (HCs, csDMARD, bDMARD, tsDMARD, cs + bDMARD, cs + tsDMARD, untreated). The plot shows a clear separation between treatment groups, with HCs generally having higher X2 values.
- d** UMAP plot showing the relationship between treatment groups (HCs, csDMARD, bDMARD, tsDMARD, cs + bDMARD, cs + tsDMARD, untreated). The plot shows a clear separation between treatment groups, with HCs generally having higher X2 values.
- e** UMAP plot showing the relationship between donor sex (female in red, male in blue) and treatment groups (HCs, Methotrexate, Leflunomide, Sulfasalazine, untreated). The plot shows a clear separation between female and male donors, with female donors generally having higher X2 values.

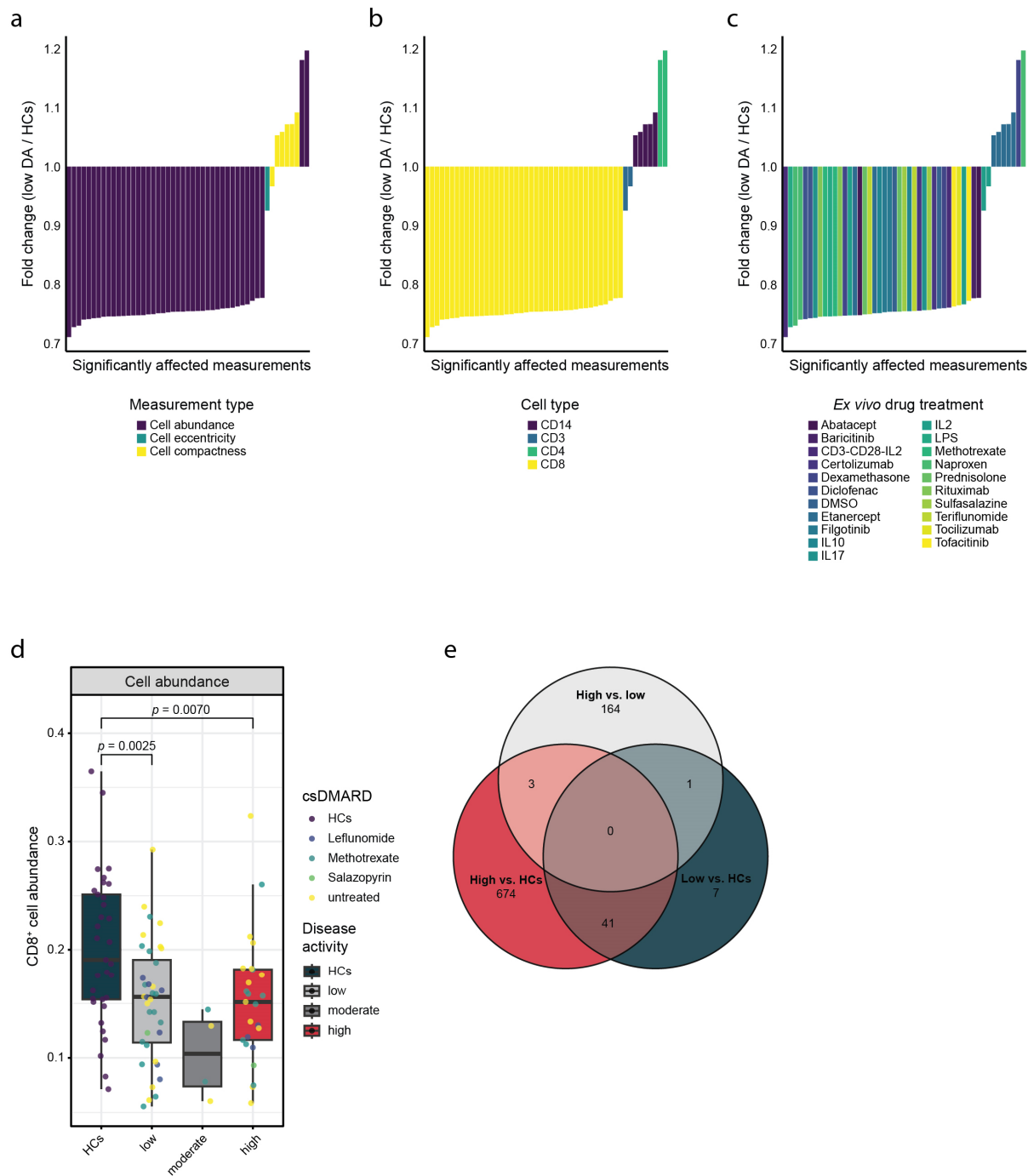
Supplementary Figure 7. Colour coding of clinical characteristics of individual donors on the UMAP projection from Fig. 4b. (a) *in vivo* treatment (b) *in vivo* csDMARD treatment (c) *in vivo* bDMARD treatment (d) rheumatoid factor positivity (> 15.9 IU/ml) (e) donor sex. Dots represent individual donors colour coded as described above. UMAP, uniform manifold approximation and projection; HCs, healthy controls; cs, conventional synthetic; DMARD, disease-modifying antirheumatic drug; b, biologic; ts, targeted synthetic.

Supplementary Figure 8



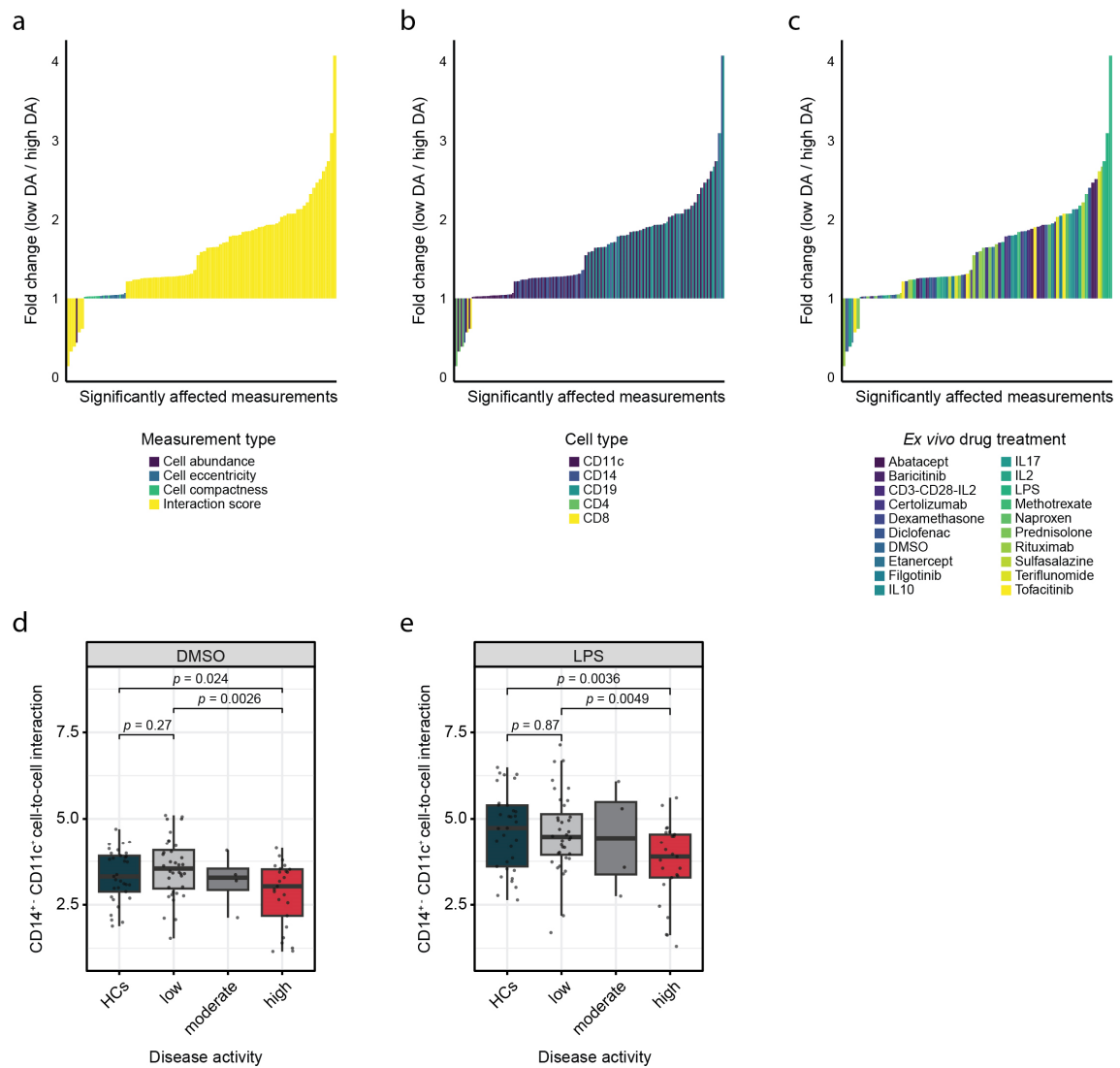
Supplementary Figure 8. *Ex vivo* quantification of PBMC characteristics in patients with high disease activity RA and HCs. (a-c) Significantly differing PBMC characteristics of HCs (n=33) as compared to patients with high disease activity RA (n=25), bars representing individual experimental conditions colour coded for **(a)** measurement type, **(b)** cell type and **(c)** *ex vivo* treatment (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). **(d)** Cell morphological characteristics of CD8⁺ cells of HCs (n=33) and patients with low (n=36), moderate (n=4) and high (n=25) RA disease activity upon CD3-CD28 T cell activator treatment (Welch's t-test). **(e)** CD4⁺ cell morphology upon CD3-CD28 T cell activator treatment in HCs (n=33) and patients with high RA disease activity according to *in vivo* conventional synthetic disease modifying drug treatment (untreated (n=10), methotrexate (n=9), leflunomide (n=3)), (Welch's t-test). px, pixels; HCs, healthy controls; UT, untreated; MTX, methotrexate; LEF, leflunomide.

Supplementary Figure 9



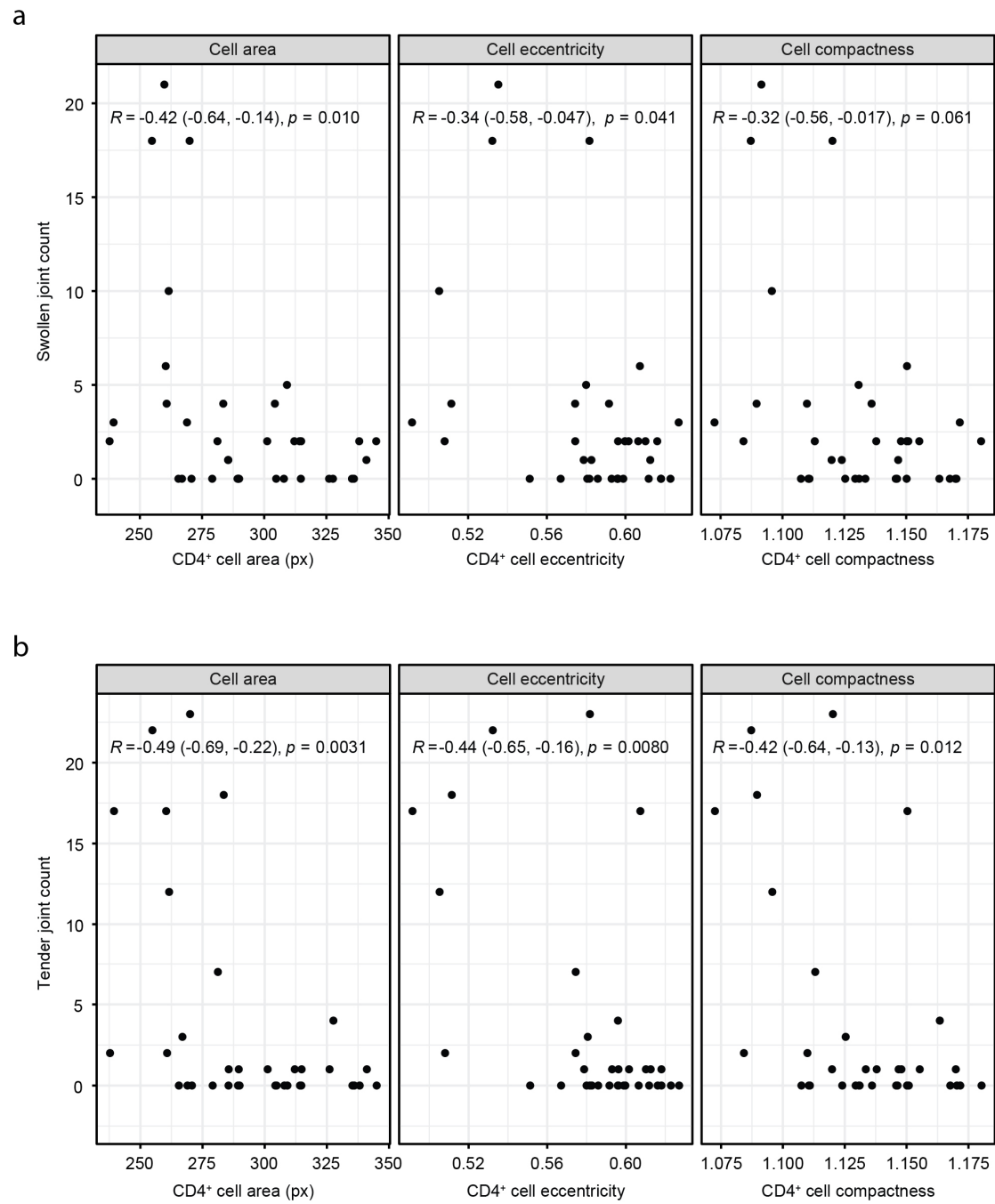
Supplementary Figure 9. *Ex vivo* quantification of PBMC characteristics in patients with low disease activity RA and HCs. (a-c) Significantly differing PBMC characteristics of HCs (n=33) as compared to patients with low disease activity RA (n=36), bars representing individual experimental conditions colour coded for (a) measurement type, (b) cell type and (c) *ex vivo* treatment (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). (d) CD8⁺ T cell abundance in HCs (n=33) and patients with low (n=36) moderate (n=4) and high (n=25) RA disease activity, point colour indicating the *in vivo* csDMARD treatment (Welch's t-test). (e) Venn diagram comprising individual measurements significantly differentiating between HCs, and patients with low and high RA disease activity. DA, disease activity.

Supplementary Figure 10



Supplementary Figure 10. *Ex vivo* quantification PBMC characteristics in patients with high and low RA disease activity. (a-c) Significantly differing PBMC characteristics of patients with high RA disease activity (n=25) as compared to patients with low RA disease activity (n=36), bars representing individual experimental conditions colour coded for (a) measurement type, (b) cell type and (c) *ex vivo* treatment (adj. $p < 0.05$, Welch's t-test adjusted for multiple testing according to Benjamini and Hochberg). (d-e) CD14⁺ to CD11c⁺ cell-to-cell interaction in HCs (n=33), and patients with low (n=36) moderate (n=4) and high (n=25) RA disease activity upon 24h of *ex vivo* treatment with DMSO (d) and LPS (e) (Welch's t-test). DA, disease activity.

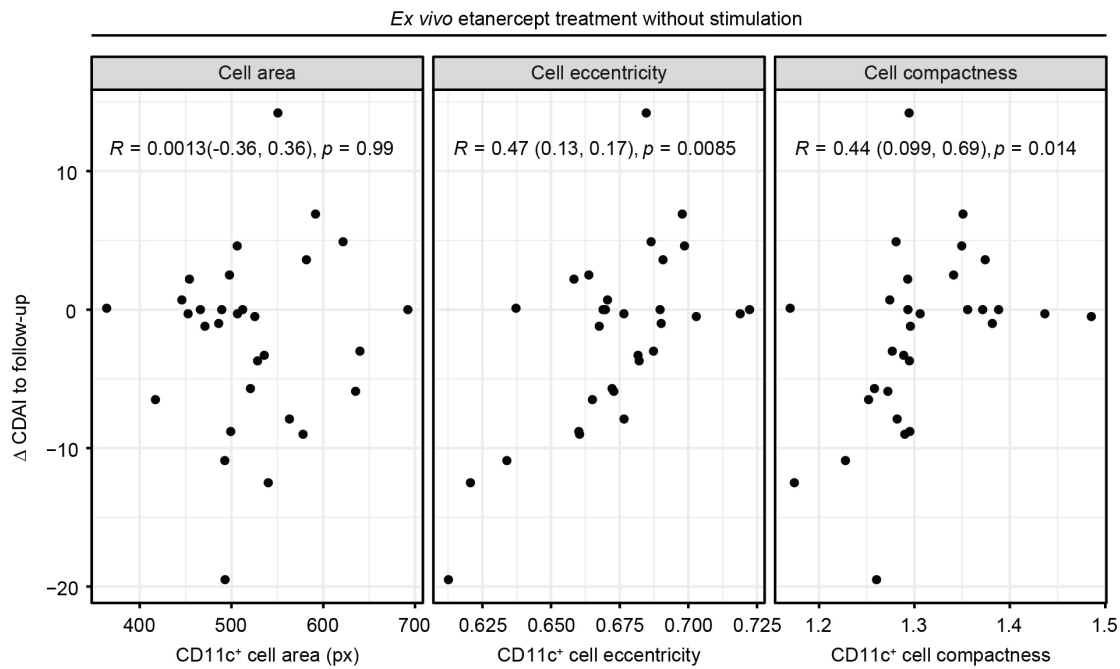
Supplementary Figure 11



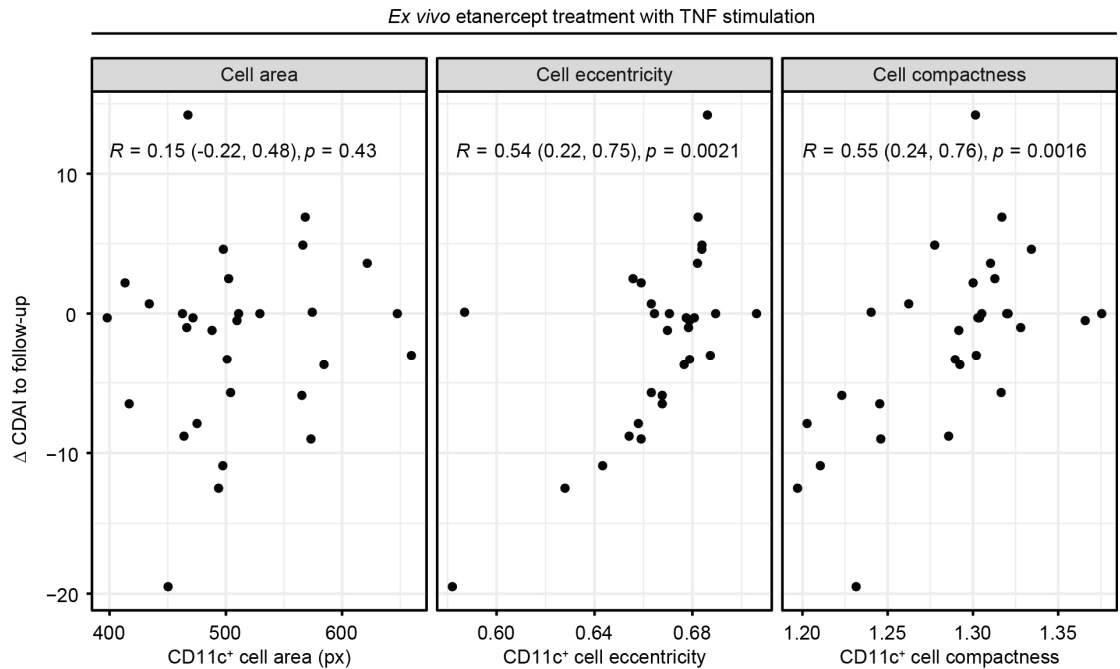
Supplementary Figure 11. Correlation of cell morphological characteristics of CD4⁺ cells with clinical outcomes (a) Correlation of CD4⁺ cell area (left), cell eccentricity (middle) and cell compactness upon 24h *ex vivo* CD3-CD28 T cell activator treatment with swollen joint count of 28 joints (n=43). (b) Correlation of CD4⁺ cell area (left), cell eccentricity (middle) and cell compactness upon 24h *ex vivo* CD3-CD28 T cell activator treatment with tender joint count of 28 joints (n=43). (a, b) Spearman's rank correlation coefficient *R* is indicated with its 95% confidence interval (in parentheses) and its statistical significance (p value). px, pixels.

Supplementary Figure 12

a



b



Supplementary Figure 12. Correlation of cell morphological characteristics of CD11c⁺ cells with change as measured by clinical disease activity index (CDAI) (a) Correlation of CD11c⁺ cell area (left), cell eccentricity (middle) and cell compactness upon 24h *ex vivo* etanercept treatment with change in CDAI to the follow-up visit (n=30). (b) Correlation of CD11c⁺ cell area (left), cell eccentricity (middle) and cell compactness upon 24h *ex vivo* etanercept treatment plus artificial TNF stimulation with change in CDAI to the follow-up visit (n=30). (a, b) Spearman's rank correlation coefficient *R* is indicated with its 95% confidence interval (in parentheses) and its statistical significance (p value). px, pixels, CDAI, clinical disease activity index.

3 Discussion

3.1 General discussion

3.1.1 Disease-specific aspects

Evidence-based research has transformed our understanding of RA from a chronic progressive relative to gout, that mainly affects the poor and women (Entezami, Fox, Clapham, & Chung, 2011), into a mechanistically well characterized autoimmune disease amenable to treatment with highly specific targeted therapy regimens (Smolen et al., 2018).

In autoimmune diseases as well as malignancies, the phenotypic presentation and hallmarks of pathology are often far more complex than the integrated consequence of individual mutations observed in cancer (Hanahan, 2022) or dysregulated immune components for autoimmunity (Song, Li, & Wu, 2024). In RA specifically, the identification of key serological factors like RF, but also ACPAs, which demonstrate a higher sensitivity and specificity than RF (Vander Cruyssen et al., 2005), indeed led to some amount of disease stratification (Ajeganova & Huizinga, 2015). The same is true for clinical (Smolen et al., 2020) and socioeconomic factors (Y. Xu & Wu, 2021) of an individual as well as the identification of different patient sub populations based on their synovial cellular composition (Bykerk, 2023). However, except for the autoantibody status (yielding a maximum of 3 points if RF and ACPAs are positive) and APRS (1 point), current disease classification criteria primarily rely on clinical features (Aletaha et al., 2010). And neither of the previously described static PM or FPM approaches have been validated or are significant enough for their integration into the ACR/EULAR treatment recommendations (Smolen et al., 2023) and have therefore found their way into wide application in routine clinical use. Not even the growing mechanistic understanding of RA pathophysiology and large-scale clinical studies have resulted in precise measures of disease stratification, prediction of preferential treatment MOA or at least recommendation for a preferential sequence of bDMARDs (Smolen et al., 2023). This results in an unavoidable trial-and-error medicine until the matching bDMARD is found in patients that do not reach remission with a csDMARD monotherapy, an already poor prognostic factor (Smolen et al., 2023), highlighting the need for the development of PM tools for the identification of the preferential bDMARD treatment. In addition, monitoring of disease primarily relies on clinical scoring, with only a few serological markers like CRP being reflected in some of the disease activity scores (G. Wells et al., 2009), due to the inexistence of definite biomarkers for disease activity and early treatment response (Lequerré et al., 2019). Ultimately this lack of PM for RA and consequentially the trial-and-error-based, often suboptimal treatment regimen, leads to

significant individual, but also socioeconomic burden (Uhlig et al., 2014), highlighting the importance of studies like the one presented within this thesis.

3.3.2 Discussion in the context of previous FPM approaches

Current FPM approaches were mainly developed in the field of oncology and hematology, where cell type-specific drug effects on malignant cells can easily be quantified by the assessment of cell viability (Malani et al., 2022). The simplest approach facilitates ATP-based assays as a surrogate for cell viability, however, with the caveat that these techniques only yield information on the level of whole cell populations per condition, not discriminating between malignant and healthy cells. This limitation is overcome by single cell techniques, like automated flow cytometry or imaging-based approaches, that allow for the cancer cell population-specific assessment of drug action (Letai et al., 2022; Napoli et al., 2022; Snijder et al., 2017). Assays for FPM beyond the application in malignant diseases are however scarce, especially in the domain of rheumatic diseases.

To address this, we designed a proof-of-concept study, representing the first application of a high-content high-throughput microscopy-based assay in combination with ex vivo drug treatment and immune stimulation as an approach to FPM in autoimmune disease.

Based on a previously established 2D monolayer culture, that allows for the systematic assessment of ex vivo drug treatment effects on immune cell-to-cell interaction (Vladimer et al., 2017), and that was already successfully applied as an FPM tool in hematology (Kornauth et al., 2022; Kropivsek et al., 2023; Snijder et al., 2017), we specifically aimed to demonstrate the feasibility of this methodology to identify immune-related PBMC phenotypes that are specific to RA treatment and disease.

3.3.3 Limitations of the PBMC model culture

In detail, we set out to combine ex vivo drug and immune stimulus treatment of RA patients' and HCs' PBMCs with high-content high-throughput imaging and computational image analysis for the identification of in vivo and ex vivo drug effects as well as disease and disease state specific PBMC phenotypes. However, this focus on cells from the peripheral blood limits the informative value of the model culture for disease pathophysiology and transforms the readouts of the assay into a surrogate rather than a reflection of the process in the immunological niche of the synovium (Orr et al., 2017) and possibly also missing unknown factors contributing to diseases. The lack of stromal cells for example disregards the complex inflammation-dependent interplay of FLS and immune cells (Kugler et al., 2023) and the cellular components of peripheral blood might not reflect the cell types involved in the synovial process. B-cells in the blood are different from the ones found in B-cell-rich

synovium (Weyand, Seyler, & Goronzy, 2005; Yeo et al., 2015) and circulating monocytes in the blood differ significantly from joint-invading and resident macrophages (Udalova, Mantovani, & Feldmann, 2016). In addition, the 2D culture does not resemble the highly dynamic 3D architecture of a synovial membrane, where steady mechanic stress induces pro inflammatory cytokines and constant synovial remodeling (Piluso et al., 2025; Shimomura et al., 2014). In addition, the model does not take into account the fact, that disease stratification based on synovial subtypes demonstrates differential potential to form erosions (Triaille & Lauwerys, 2019) and omits the contribution of the highly complex immune and stromal cell interplay of the pannus tissue completely.

Microfluidic devices, that aim to model the synovium functionally (e.g. by simulating mechanical or sheer stress) and in addition allow for the real time assessment of immune function, definitely resemble this immunological niche more precisely and would in addition allow for the temporally resolved assessment of cellular interactions (Thompson et al., 2023). However, these highly complex culture systems come with relevant limitations like labor intensiveness, the need for specialized equipment for live cell culture and screening as well as the significantly longer culture duration restricting their application as tools for FPM, where turnover times are critical for the initiation of a preferential treatment.

3.3.4 Aspects of novelty

The current study represents a novel approach for FPM in autoimmune disease, as previous PM profiling approaches mainly focused on the assessment of static features like PBMC composition (Kubo et al., 2025) or integration of genetic with clinical components at baseline (Gosselt et al., 2021). To gain insight into ex vivo drug response of PBMCs, we designed a comprehensive ex vivo drug treatment panel covering all currently approved cs, ts and bDMARD MOAs as well as GSs and NSAIDs (Smolen et al., 2023). This panel was supplemented with a set of pro- and anti-inflammatory reference immune stimuli, and to mimic the systemic inflammation the complete set of drugs and reference stimuli was cultivated in unstimulated and TNF pro-inflammatory conditions. The incubation of PBMCs in 384-well plates allows for the formation of a mono layer of adherent and non-adherent cells, enabling the morphologic assessment and the formation of cell-to-cell interactions like immunological synapses (Dustin, 2014). However, the short ex vivo treatment period of 24h does neither reflect typical treatment durations nor in vivo pharmacokinetics and pharmacodynamics. These vary significantly between fast and short acting drugs like Diclofenac (Davies & Anderson, 1997), GCs that are usually tapered (Frey & Frey, 1990) and csDMARDs like MTX that can take up to months to reach their full therapeutic potential (Rezaei et al., 2012). In addition, the designed drug plate does not address possible

synergistic effects of treatment combinations, a limitation also known for FPM in malignant diseases, where complex combination therapies are more common (Letai et al., 2022) and the limited number of reference stimuli indeed does not reflect the complex network of pro- and anti-inflammatory components driving RA. Finally, the procedure of fixing the cells for consecutive immunostaining prohibits any kind of longitudinal PBMC phenotype assessment, turning the data from our methodology into a snapshot of immune cell behavior rather than a time resolved functional assessment of immune cells.

3.3.5 Technical limitations

During the setup phase of this study, we have made great effort to optimize the seeding, fixing, staining and imaging protocols to acquire the most robust data possible. This included the meticulous optimization of seeding and fixing procedures to limit perturbation of the monolayer to minimize noise in the cell-to-cell interaction data that would have been introduced by washing artifacts. Marker-specific cell surface staining was performed in 4 overlapping panels, however the applied immune cell markers are only a coarse representation of the complex cellular immune system. A more refined PBMC subset staining panel, however, would indeed come with a significantly increased number of cell surface marker combinations, which is also technically limited by the number of available fluorescence channels. Flow cytometry, indeed allows for far more complex staining panels (Preglej et al., 2023), however misses spatial resolution. Experimental approaches that try to overcome this limitation, for example combine high-content microscopy with single cell mass spectrometry (Rosenberger et al., 2023), but are limited by throughput, as is the acquisition of spectral images with consecutive linear (Zimmermann, Marrison, Hogg, & O'Toole, 2014) or ML-based (McRae, Oleksyn, Miller, & Gao, 2019) unmixing. To increase the number of available immune cell markers per well, we aimed to establish a 5th fluorescent channel using transfer dyes, however, failed due to significant bleed-through as a technical limitation.

Advancements of automated image analysis aim to overcome the limitations of cell surface marker staining by the application of ML-based models that allow for the identification of malignant cells solely based on DAPI+ BF images (Heinemann et al., 2022). However, these trained ML approaches are in danger of misclassification of novel, previously unknown phenotypes not included in the training data. In addition, this approach might be limited in the context of identification of immune cells from the peripheral blood with physiological features, rather than identifying a malignant cell clone in a population of healthy cells.

The acquired data comprised of 484,224 image sets with a total of 1,936,896 individual images in 42 ex vivo treatment conditions for all individual donors (33 HCs and 65 RA

patients). To extract information about immune cell frequencies, morphology and intercellular interaction patterns from these images, a tailored CellProfiler-based (McQuin et al., 2018) computational image analysis pipeline was developed. In brief, the DAPI staining allowed for the identification of individual nuclei from local maxima and cell type-specific fluorescent surface staining was used to identify cellular morphology and quantify intensity in the membrane compartment. This led to the phenotypic characterization of 6.9×10^8 individual cells on a single cell level, a number comparable to other studies in the field of imaging-based FPM (Kropivsek et al., 2023). To overcome limitations of manual gating for cell type classification a bi-Gaussian fitting was established using log-transformed median cell membrane intensity values. In contrast to previous applications of this model culture for immune cell interactions profiling, interactions were not calculated based on cellular proximity (Vladimer et al., 2017) but rather by extracting the cell-to-cell interaction information from touching individual objects. Indeed, the set of cellular features extracted for the purpose of this study was limited to identity, area, elongation, eccentricity and interaction propensity. However, the quantification of additional features per individual cell would have led further towards the curse of dimensionality, a challenge in the analysis of high-dimensional data where the number of measurements vastly outnumbers the number of individual observations, bearing the risk of overfitting (Altman & Krzywinski, 2018; Gygi, Kleinstein, & Guan, 2023). Indeed, to avoid these statistical pitfalls, we conducted our statistical analysis as accurately as possible with extensive use of testing data integrity and visual data inspection. And even though the most meticulous approach to overcome this limitation would be the validation of the findings in a separate validation cohort, this would not fully protect against systematic and technical bias.

Microscopy-based high-content and high-throughput assays are widely applied as versatile tool in phenotypic drug discovery studies e.g. by the application of cell painting (Seal et al., 2025). We therefore first mapped the RA-specific drug space for its effect on RA and HCs PBMCs and indeed found clustering according to MOA, as well as known immunologic phenomena such as lymphoblast formation upon T cell activation (Smith-Garvin, Koretzky, & Jordan, 2009) and B-cell depletion in RTX condition (Pierpont et al., 2018) as proof of concept for the technique. Cell type-specific activation can of course also be quantified based on induced expression of cell surface markers and clonal T-cell expansion via flow cytometric assays (Cossarizza et al., 2021). The applied 2D monolayer culture in combination with the advanced single cell contact-specific readout was however, sensitive enough to detect cell contact-specific morphological changes as a measure for cell activation. In addition to known phenomena, the ex vivo treatment revealed previously

undescribed effects on PBMCs such as cell morphologic changes upon Etanercept treatment that were accompanied by more pronounced cell-to-cell clustering.

Due to the exploratory nature and the previously unknown effect sizes of disease and treatment on PBMC phenotypes, this study was conducted in 65 RA patients of varying disease activity according to CDAI (low: n=36; moderate: n=4; high: n=25) and HCs in accordance with the ethical committee of the Medical University of Vienna. On the one hand, the number of patients and their heterogeneity in treatment might have limited the full potential of detecting disease state-specific PBMC phenotypes or patterns specific to early disease. On the other hand, this diversity allowed us to detect alterations that were specific for in vivo csDMARD and bDMARD treatment. These included the clustering of in vivo bDMARD based on MOA as well as the csDMARD-specific changes in CD8⁺ T-cell abundance.

As described, the RA cohort comprised of patients with varying disease activity, which allowed for the identification of ex vivo PBMC phenotypes that were associated with high disease activity as compared to HCs. One of these was the reduced reactogenicity to activation by T-cells from patients with high RA disease activity, most likely because of immunologic reprogramming due to constant activation (Lotz et al., 1986). However, CDAI was the only clinical measure of disease activity used throughout the study, leaving the question open, that other measures of disease activity (e.g. DAS-28 CRP) could have been more accurate as they include biological biomarkers of systemic inflammation. In addition, the study design did not include resampling of individual patients. A longitudinal approach, including resampling, would have allowed for the assessment of disease and treatment effects on PBMC phenotypes over time, whilst a longer observational phase could have possibly detected PBMC patterns associated with radiographic findings. Yet we were able to identify T-cell responsiveness to activation to correlate with clinical disease activity at baseline and change of CDAI to the follow-up visit. This might however reflect the fact that patients with higher initial disease activity can reach higher deltas as a measure of improvement.

One technical aspect that hinders wide application in clinical routine application, is the need of specialized infrastructure for the presented methodology. In addition to sterile conditions for sample handling and cultivation, a specialized 384-well pipetting robot and an automated confocal microscope are needed in combination with matching computational resources to generate comparable data sets. In an optimal scenario, the individual key parameters identified by our pipeline could be reduced to a simplified laboratory test, probably in the form of a microfluidics device, minimizing the need for labor and equipment. The analysis,

however, would have to be translated into a standardized analytical tool, that comes with regulatory complications.

3.2 Conclusion & prospects

This work represents the first proof of concept study for FPM in autoimmune diseases. Fundamental technical advancements were key to reaching the necessary analytical precision for the robust detection of ex vivo and in vivo treatment-specific as well as RA disease-specific PBMC phenotypes. This work therefore forms the foundation for future applications of high-content, high-throughput microscopy in combination with ex vivo perturbation in translational research and FPM.

These planned future applications include the systematic investigation of ex vivo PBMC phenotypes of various rheumatic and musculoskeletal diseases. Given the clear results from the current RA cohort, an obvious starting point would be the characterization of seronegative arthritides such as PsoA, AxSpA and peripheral SpA, that not only present clinically different to RA, but also appear to have overlapping and differing pathophysiology as reflected by the clinical response to partially other bDMARD MOAs. Indeed, assessing these diseases in our ex vivo phenotyping pipeline would require the adaptation of the current drug library, including bDMARDs targeting IL-17 as well as IL-12 and IL-23. The same is true for the used reference stimuli, requiring adaptation to reflect disease pathophysiology. Additional disease entities in the rheumatic field beyond arthritides that might demonstrate with specific ex vivo PBMC phenotypes include forms of connective tissue diseases and various types of vasculitis.

Another approach to strengthening the significance of our technology would be adding stainings for intracellular signaling molecules in the form of phosphorylation site-specific antibodies against signaling proteins and other surrogates for intracellular signaling such as nuclear translocation of relevant transcription factors. Such targets could comprise components of the JAK-STAT signaling cascade like individual phospho-STATs, or nuclear translocation of p65 for NF- κ B signaling. For the quantification of NF- κ B and IRF5 activation, that was demonstrate to be relevant in SLE, we e.g. already applied a tailored, microscopy-based assay for the cell type-specific quantification of transcription factor nuclear translocation in cell lines and primary material of SLE patients and HCs (Boeszoermyeni et al., 2023; Heinz et al., 2020). Therefore, these two studies provide evidence for the value of high-content microscopy in combination with computational image analysis as a valuable tool for the mechanistic investigation of disease mechanisms and screening for novel compounds selectively targeting the IRF5 pathway. PBMC profiling, especially in combination with pathway-specific stainings, might represent a promising avenue to extend our approach to SLE, for which clinical measures of disease activity are well established,

but biomarkers for treatment prediction are missing (Capecchi, Puxeddu, Pratesi, & Migliorini, 2020).

Finally, a possible future application of our methodology on the interphase between oncology and rheumatology would be the quantitative assessment of PBMC phenotypes associated with immune-related adverse events occurring after checkpoint inhibitor treatment (Martins et al., 2019). These complications include rheumatological conditions such as seronegative arthritis and tenosynovitis and myositis, however the mechanistic understanding of the underlying processes is poor and systematic evidence for current treatment approaches is mainly based on retrospective cohort or case-control studies (Haanen et al., 2022; Martins et al., 2019). Our technique might be valuable for the immunological profiling of patients that developed such autoimmune conditions. In addition, ex vivo assessment of PBMC could be used to monitor the immune system during checkpoint inhibitor treatment and possibly identify parameters that predict this adverse overshooting immune reactions.

Possibly the biggest shortcoming of the study to overcome in future applications is the lack of prospective data, therefore follow-up studies are needed to identify ex vivo PBMC phenotypes that are directly linked to b- and tsDMARD-specific treatment response. Phenotypic readouts in these trials could comprise measurements already established like cell type quantification, morphological readouts and cell-to-cell interaction. Additionally, these could also be extended to activation markers that are specific to a cell type, drug MOA or disease, thereby additionally supporting FPM.

Indeed, the complementation with orthogonal techniques like high-dimensional immune cell profiling (Preglej et al., 2023) or synovial biopsy data (Myles J. Lewis et al., 2025) are possible and might assist in the stratification of different RA subtypes. Co-cultivation of PBMCs with autologous PBMCs would allow for additional readouts like the quantification of immune cell interaction with FLS from the affected joints and measurement of activation markers on PBMCs that are in direct contact with FLS. In addition, inflamed joints often present with effusion, which can be sampled via joint puncture or during synovial biopsies. This synovial aspirate holds high numbers of immune cells that can be analyzed by flow cytometry (Attrill et al., 2024; Kuryliszyn-Moskal, 1995) but might also be valuable primary material for further analysis in the presented ex vivo phenotyping platform.

One study that is currently conducted with contribution of MUV and in collaboration with CeMM makes use of various primary materials from patients with RA is the BioTest trial (ISRCTN 80852529), a multicentric observational trial, aiming to identify biomarkers for treatment response prediction. In brief, RA patients that did not respond to TNFi are

randomized to bDMARDs of differing MOA and simultaneously characterized in a multi-omics approach, not only looking at peripheral blood but also at synovial tissue from the site of inflammation via histology, molecular profiling and by assessing synovial fibroblast function. The high-content high-throughput imaging arm of the study will assess PBMC phenotypes in an approach similar to this study, however with a more complex immune stimulation panel and additional cell surface markers not only staining for immune cell identity but also for cell-specific activation.

A growing field of medicine is based on the application of ML for the support of diagnosis, prediction of treatment and prognosis, and also optimization of treatment regimens and clinical workflows (Rajkomar, Dean, & Kohane, 2019). These approaches demonstrated especially valuable in the context of image analysis. On the one hand by supporting radiologist interpreting medical images (Chilamkurthy et al., 2018), on the other by assisting histopathology (Steiner et al., 2018). In the context of high-content imaging-based FPM, previous studies already applied ML for cancer cell identification for a broad range of hematologic malignancies in general (Heinemann et al., 2022) and multiple myeloma specifically (Kropivsek et al., 2023). Indeed, the ex vivo PBMC immune cell profiling data from our study provides a formidable basis for the future assessment employing ML tools. Indeed, these might be applied at different steps of the analysis: Firstly, cell identification, secondly cell segmentation, thirdly feature identification and finally on a patient level for unbiased stratification. However, supervised ML approaches need annotated data for training models, which can be done on this data set, not using ML for scoring for example. On the other hand, the multi-omics data set currently produced in the context of the BioTest trial will also be used in another multi-centric consortium (AutoPix), that focuses on the development of ML approaches for precision medicine in rheumatic diseases. Based on the data acquired during the BioTest trial, in combination with additional samples screened for treatment response to bDMARDs of varying MOA we will aim to develop ML tools to support image analysis and ultimately lead to patient stratification.

In conclusion, this thesis provides the foundation for the use of FPM approaches in the domain of rheumatic and related autoimmune diseases. Importantly, we could demonstrate the performance of imaging-based high-content microscopy in combination with ex vivo drug and immune stimulus perturbation as a powerful tool for the identification of relevant disease phenomena. The utility of this method is currently evaluated in multiple clinical follow-up studies extending the discovery space regarding disease entity and drug treatment. Furthermore, the robustness of the underlying imaging data provides a formidable basis for the currently ongoing implementation of ML-assisted analysis

approaches. These efforts hold promise to further advance this technology, ultimately aiming for the clinical applicability as FPM for RA.

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