Diploma thesis

Differences in JAK- STAT signaling in leukocytes of the peripheral blood in psoriatic arthritis

Submitted by

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Graz, 05.09.2022

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 05.09.2022 *Daniel Ruben Pietsch eh.*

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Abbreviations

bDMARD	Biological DMARD
CASPAR	Classification criteria for Psoriatic Arthritis
CD	Cluster of differentiation
CRP	C- reactive protein
csDMARD	Conventional synthetic DMARD
DAPSA	Disease Activity Index in Psoriatic Arthritis
DC	Dendritic cell
DMARD	Disease modifying antirheumatic drug
DRE	DNA regulatory element
EULAR	European league against rheumatism
G- CSF	Granulocyte-Colony Stimulating Factor
GAF	Gamma-interferon activation factor
GAS	Gamma interferon activation site
gMFI	Geometric mean fluorescence intensity
GRAPPA	Group for Research and Assessment of Psoriasis and Psoriatic Arthritis
GWAS	Genome- wide association study
HDA	High disease activity
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IFNγ	Interferon γ
IL	Interleukin
ILC	Innate lymphoid cell
ISGF3	Interferon stimulated Gene Factor 3
ISRE	IFN-stimulated response element
JAK	Janus kinase
JH domain	JAK homology domain
LDA	Low disease activity
MAIT	Mucosal- associated T cell
MHC	Major histocompatibility complex
MoDA	Moderate disease activity

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKp44	Natural killer protein 44
NSAID	Non- steroidal anti-inflammatory drug
PDE 4 inhibitor	Phosphodiesterase 4 inhibitor
PGA	Patient global assessment
PsA	Psoriatic arthritis
PsD	Psoriatic disease
RA	Rheumatoid arthritis
REM	Remission
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor gamma
SH domain	Src-homology 2
SJC	Swollen joint count
SOCS	Suppressor of cytokine signalling
SpA	Spondyloarthritis
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tfh	T follicular helper cell
TGF- β	Transforming growth factor β
TH	T helper
TJC	Tender joint count
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNIP	TNFAIP3-interacting protein
TRAF	Tumor necrosis factor receptor associated factor
tsDMARD	Targeted synthetic DMARD
TYK	Tyrosine kinase
VEGF	Vascular endothelial growth factor

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Zusammenfassung

Ziel und Hintergrund der Arbeit

Die immunpathologischen Prozesse bei der Psoriasis- Arthritis (PsA) werden durch Zellen des angeborenen & adaptiven Immunsystems mittels verschiedener Zytokine vermittelt. Diese Zytokine nutzen zu einem großen Teil intrazelluläre Moleküle des JAK / STAT – Signalweges, um die Genexpression von verschiedenen Zellen zu beeinflussen. Ein wichtiges Zeichen von Aktivität in diesem Signalweg ist die Phosphorylierung von STAT – Molekülen. Wir untersuchen in dieser Arbeit die Hypothese, dass sich Unterschiede zwischen dem Ausmaß der Phosphorylierungen in Patientinnen und Patienten mit unterschiedlicher Krankheitsaktivität feststellen lassen und sich diese anhand ihres Phosphorylierungs- Profils in Subgruppen einteilen lassen.

Methoden

Prospektive Querschnittstudie an 49 Patientinnen und Patienten mit PsA [medianes Alter 57 (50.0, 62.0), 42.9% weiblich, Disease Activity Index in Psoriatic Arthritis (DAPSA) 5.0 (1.0, 11.4), 18,4% Moderate / Hohe Krankheitsaktivität (MoDA/ HDA), 81,6% Niedrige Krankheitsaktivität / Remission (LDA/ REM)]. Es wurde die Durchflusszytometrie verwendet, um die Phosphorylierungs- Levels von STATs (pSTATs) verschiedener Immunzellen aus dem peripheren Blut zu bestimmen. Zum Detektieren von Unterschieden wurde das geometrische Mittel der durchschnittlichen Fluoreszenzintensität (gMFI) des jeweiligen pSTAT – Moleküls in den Immunzellen herangezogen und verschiedener statistischer Tests & Verfahren unterzogen.

Resultate

Es zeigten sich statistisch signifikante Unterschiede zwischen den Gruppen LDA/ REM und MoDA/ HDA in den Leveln von pSTAT3 in naiven & Gedächtnis- CD4+ und CD4- T – Zellen sowie pSTAT1 in Granulozyten & Monozyten. Weiter konnten die Patientinnen und Patienten anhand ihrer STAT- Phosphorylierungen in 4 Cluster eingeteilt werden, welche sich signifikant im DAPSA, C- reaktiven Protein (CRP), der CD19+ - Zellfrequenz sowie pSTAT3 in Monozyten, B- Zellen, naiven & Gedächtnis- CD4+ und CD4- T- Zellen, pSTAT5 in naiven und Gedächtnis- CD4+

T- Zellen, pSTAT1 in T- Zellen und pSTAT4 in Granulozyten, Monozyten, B-Zellen sowie naiven CD4+ and CD4- T- Zellen unterschieden.

Schlussfolgerung

In dieser Arbeit konnte gezeigt werden, dass sich PsA - Patientinnen und PsA - Patienten mit unterschiedlicher Krankheitsaktivität in den STAT-Phosphorylierungen von Immunzellen des peripheren Blutes unterscheiden und sich anhand dieser Phosphorylierungen in Subgruppen mit Unterschieden in klinischen als auch biologischen Parametern einteilen lassen.

Abstract

Background and purpose

The immunopathological processes in psoriatic arthritis (PsA) are orchestrated through cells of the innate and adaptive immune system and different cytokines. These cytokines, in large parts, use intracellular molecules that belong to the JAK/ STAT signaling pathway and that can alter gene expression in different cells. An important sign of activation in this pathway is the phosphorylation of STAT molecules. In this work, we investigate whether there are differences in phosphorylation levels between PsA patients with different disease activity and furthermore, if the patients can be divided into subgroups according to their STAT phosphorylation profile.

Methods

Prospective cross-sectional study on 49 patients with PsA [median age 57 (50.0,62.0), 42,9% female, Disease Activity Index in Psoriatic Arthritis (DAPSA) 5.0 (1.0, 11.4), 18.4% Moderate / High disease activity (MoDA/ HDA), 81.6% Low disease activity / remission (LDA/ REM)]. Flow Cytometry was used to determine the phosphorylation levels of STAT molecules (pSTATs) in different immune cells of the peripheral blood. The geometric mean fluorescence intensity (gMFI) of the given STAT molecule in the immune cell was used to detect differences with several statistical tests & procedures.

Results

We found statistically significant differences between the groups LDA/ REM and MoDA/ HDA in the levels of pSTAT3 in naïve / memory CD4+ and CD4- T cells as well as in pSTAT1 in Granulocytes & Monocytes. We further could divide the PsA patients into 4 clusters based on the STAT phosphorylation levels. These clusters differed significantly in DAPSA, CRP, CD19+ cell frequency, pSTAT3 levels in Monocytes, B cells, naïve & memory CD4+ and CD4- T cells, pSTAT5 in naïve & memory CD4+ T cells, pSTAT1 in T Cells and pSTAT4 in Granulocytes, Monocytes, B cells, and naïve CD4+ and CD4- T cells.

Conclusion

This work shows that PsA patients with differences in disease activity also differed in the STAT phosphorylation levels of immune cells of the peripheral blood. Furthermore, the patients could be divided into subgroups based on the phosphorylation patterns and the clusters differed in clinical as well as biological parameters.

1. Introduction

1.1. Psoriatic Arthritis

1.1.1. Definition

Psoriatic Arthritis (PsA) is a chronic, immune- mediated inflammation of the joints and entheses of the peripheral and axial skeleton that is associated with psoriasis (Ps) (1). Due to its interrelation with other diseases like cutaneous psoriasis and further inflammatory entities like inflammatory bowel diseases (IBDs), PsA could be described as part of a heterogeneous spectrum of diseases which share some common genetic and pathophysiological background. The umbrella term for these conditions has been given the name Psoriatic disease (PsD) (2).

1.1.2. Epidemiology

The global prevalence of PsA ranges from 0.01% in the Middle East to 0.13% in North America and 0.19% in Europe. A general estimate of prevalence is not possible since there are several methodologic challenges that make it difficult to narrow the wide variation of these estimates, which reflects the heterogeneity of the disease. For example, the variation in case definitions, the lack of validated classification criteria and different general study designs never allowed for this until now (3). PsA affects both genders equally (4, 5).

Around 30% of psoriasis patients develop psoriatic arthritis (6). Skin psoriasis often precedes PsA, but it seems that PsA is largely underdiagnosed in patients with skin psoriasis. A meta- analysis concluded that around 10.1- 15.5% of patients with psoriasis that are seen by a dermatologist may have undiagnosed PsA (7).

1.1.3. Etiology & Pathogenesis

The pathogenesis of PsA is a complicated process that is not fully understood yet. As of now, the disease model we have is considered to be a multi-facet process that involves genetic susceptibility loci, environmental triggers and different parts of our innate & adaptive immune system as well as local tissue cells that facilitate the inflammation (8).

1.1.3.1. Familial & Genetic Risk Factors

Psoriatic Arthritis has a strong familial component, with first-degree relatives and siblings showing a risk ratio of up to 39 and 31, respectively (9, 10). Interestingly, the risk ratio for skin psoriasis is much lower in these patients with 7.6 and 8.8 for first-degree relatives and siblings, respectively (10). This leads to the assumption, that there might be PsA – specific gene loci.

PsA has a strong association with MHC class I alleles, for example *HLA-A*0201, HLA-B*08, B*27, B*38 & HLA-C*0602* (11-13) . Certain HLA types are not only found to be overrepresented in these patients, but they also seem to correlate with certain disease presentations. For example, *B*27:05:02-C*01:02:01* has a strong association with enthesitis & dactylitis, while *B*08:01:01 / C*07:01:01* is more associated with joint fusion, deformities and asymmetrical sacroiliitis (12). Interestingly and in contrast to rheumatoid arthritis (RA), there are no strong associations with MHC Class II loci in PsA (14).

There are many other loci of interest besides MHC I. For example, the *IL23R* gene showed a very strong association with PsA in a large genome- wide association study (GWAS) (15). IL-23 has been implicated in the pathogenesis of enthesitis by acting on enthesal resident T cells that expressed the IL-23 receptor in a mouse model (16). Other significant loci for Ps and PsA are *TRAF3IP2, IL12B, IL23A-STAT2, TNIP1, TYK2.* A PsA- specific loci has been found in the long arm of chromosome 5 (5q31) and more specifically at the SLC22A5 gene, that encodes a transmembrane cation transporter with important functions in intestinal absorption. Given the association of psoriatic disease and IBDs, this might be a hint to a role of the gut microbiome in the pathophysiologic process *(11)*.

1.1.3.2. Environmental Triggers

Recently, the association between the microbiome and immune- mediated inflammatory diseases has drawn more and more attention. There is evidence that a disturbed microbiome is involved in the pathogenesis of PsA, since dysbiosis might involve overgrowth of certain bacteria that, in genetically susceptible individuals, lead to aberrant activation of the immune system, mostly through molecular mimicry (8).

Another important concept is the so called “mechanoinflammation”, which describes the activation of the immune system as consequence of recurrent (micro-) trauma at the enthesis or other joint structures (1, 8). Other environmental factors are infections, stress, obesity, and smoking (17).

1.3.3.3. Innate Immune Cells

There are several cells of the innate and adaptive immune system that play a role in the pathogenesis of PsA.

Dendritic cells (DCs) are an important link between innate sensing of pathogens and activation of the adaptive immune system. A study of synovial fluid and peripheral blood of PsA patients has shown that DCs in the synovial fluid of PsA patients exhibit an immature phenotype that might contribute to inflammation by sampling of the synovial environment and by presentation of arthritogenic antigen to cells of the adaptive immune system (18). In PsA patients, there is an increase in immature DCs that show an upregulated expression of the Toll-like receptor 2 (TLR-2), which in turn leads to a TH1- type inflammatory pattern with increased production of TNFα, IFNγ and IL-2 (19).

Macrophages are important secretors of proinflammatory cytokines & matrix metalloproteinases and present antigens to the cells of the adaptive immune system. They also play an important role in bone resorption and therefore contribute to the structural damage at the site of inflammation in inflammatory arthritis (1, 14).

Type 3 Innate Lymphoid Cells (ILC3) have an important role in psoriatic skin lesions and are increased in the synovial fluid of patients with psoriatic arthritis, where they show increased expression of the chemokine receptor 6 (CCR6) and the activating receptor NKp44 and moreover, increased production of IL-17A (20). ILC3s are an important source of IL-17 and IL-22 and have been found to be increased in active PsA in comparison to ILC2, which in contrast tend to produce pro-resolving cytokines like IL-9 and IL-13. Interestingly, the ILC3/ ILC2 - ratio was reversed during remission (21, 22).

Mucosal- associated invariant T (MAIT) cells are a T cell subset that belongs to the innate immune system and seems to be mainly derived from the gut, since MAIT cells are not present in germ-free mice (23). They are part of the innate

immune response against bacteria or yeast and are distributed throughout the body, where they are mainly found on mucosal sites, but can also be detected in synovial fluid of PsA patients. In one study, MAIT cells were mainly CD8+ and showed a functionally active IL-23 receptor, were significant producers of IL-17A, which is produced upon IL-23 activation and furthermore produced IFNγ and TNFα (24). Therefore, they might play an important role in the pathophysiology of PsA due to their contribution to the IL-23 / IL-17 – axis, which will be described later.

Mast cells that express high amounts of IL-17 have been found in the synovia of patients with RA and peripheral SpA, where they promote the inflammatory process in the joints (25).

NK cells are a type of lymphocyte that seems to be involved in arthritis by increased production of IFNγ and TNFα at the synovial site (26).

1.3.3.4. Adaptive Immune Cells

T Lymphocytes are the main adaptive immune cell type in the inflamed joints of PsA patients which is emphasized by the cytokines that are found in the synovial fluid, like IL-2, IFNγ, TNFα and IL-17A (27). CD4+ as well as CD8+ T cells play a role in driving the disease, but CD8+ T cells seem to be the major culprit, which is underpinned by the strong genetic association with the MHC I loci as described above.

PsA has long been considered a Th1- driven disease with higher amounts of IFNγ and IL-12 in the synovial fluid (28). Since more T cell subtypes have been discovered, the focus now heavily lies on TH17 cells.

TH17 Cells, which are mostly CD4+ T cells, are increased in the circulation as well as in the synovial fluid of PsA patients (29). They are considered a major contributor to disease process, since they produce large amounts of IL-17, which induces inflammation and angiogenesis in the joint. IL-17 furthermore upregulates matrix metalloproteinases and activates osteoclasts, which consequently leads to structural damage of the inflamed joints (1).

Another involved T cell subtype is the **TH9 cell**, which is an effector cell that belongs to the CD4+ population and is linked to several autoimmune diseases.

A significant increase in Th9 cells has been found in the blood, synovial tissue, and gut of PsA patients (30). These cells, as well as Th17 cells, can produce IL-9, which has pro- inflammatory properties by induction of TH17 cells from naïve CD4+ T cells together with TGF-β and furthermore protective functions by enhancement of suppressive functions of Treg cells, while signaling mainly through STAT3 and STAT5 (31).

Th22 cells are another CD4+ subpopulation that is characterized by production of IL-22, which can, again, be furthermore produced by TH17 cells. IL-22 plays a role in pathological bone formation and osteoclastogenesis in PsA (14).

Subclinical gut inflammation has been detected in PsA patients and has been further characterized by a strong TH17 and TH22 cell involvement, as well as TH9 cells with IL-9 overexpression. Thereby providing another link between the IBDs and PsA (30).

B Lymphocytes on the other hand haven't been shown to contribute majorly to pathogenesis in PsA, although they might be involved through antigen presentation, co- stimulation of T Lymphocytes and production of cytokines (1).

1.3.3.5. Angiogenesis, structural pathology and its mediators

The pathology of PsA is characterized by aberrant angiogenesis, bone & cartilage destruction and furthermore, osteoproliferation (14). These processes are mediated by synovial fibroblasts, osteoclasts, osteocytes, and osteoblasts and are orchestrated by many different cytokines, hormones and mechanical stress.

The dysfunctional angiogenesis happens at an early disease stage and is characterized by increased, immature blood vessels in the psoriatic synovium induced by an increase in growth factors like Vascular endothelial growth factor (VEGF) and mainly T cell - derived cytokines like TNFα and TGF-β (32, 33). These immature vessels harbor dysfunctional endothelial cells that allow for an influx of immune cells into the synovial tissue, which is probably mediated by hypoxia due to the high metabolic activity of the synovium in inflammation (34).

The following infiltration and inflammation are on the one hand mediated by Dendritic cells, that are a key player to attract & activate further immune cells. These are especially T cells, that in consequence secrete pro- inflammatory

cytokines as mentioned above. On the other hand, additional immune cells like macrophages, NK cells & MAIT cells further contribute to inflammation with distinct cytokines as described above.

A key resident cell is the fibroblast- like synoviocyte, that in turn produces pro-inflammatory cytokines, Matrix- degrading enzymes, growth factors, chemokines and promotes osteoclastogenesis (35). Consequently, cartilage and bone are broken down, which on the macroscopic level emerges as narrowing of the joint space. But not only the breakdown of bone, also the generation of new bone tissue in form of osteophytes is a feature of PsA that seems mainly be mediated by IL-22- activated osteoblasts (36).

An important feature of PsA is the inflammation of the enthesis, which is the term for the insertion of the muscle tendons and ligaments into the bone and that is composed of fibrous, cartilaginous and calcified tissue (37). The entheses of the lower limbs, especially of the plantar fascia and Achilles tendon, are more often affected than those of the upper extremities, probably due to the higher mechanical forces they are exposed to (38). Mechanical stress leads to local prostaglandin E2 (PGE2) production, which is considered an early mediator of enthesitis that causes vasodilation and influx of immune cells, which facilitate the following inflammation in susceptible people (14). The inflammatory process is considered to be driven in large parts by T cells that are activated by PGE2 & IL-23 from resident CD14+ myeloid cells and consequently produce IL-17, a sequence that is often referred to as the IL-23/ IL-17 axis, which finally leads to the amplification of local inflammation (14, 39). Besides IL-17, the accumulation of IL-23 leads to IL-22 expression by entheseal cells, which in turn promotes bone formation in the enthesis by osteoblasts (16). The concept of stress- induced inflammation is often referred to as mechanoinflammation.

Taken all together, the most important cytokines in PsA pathophysiology seem to be TNFα, IL-17, IL-23 and IL-22 which in turn activate resident cells in the synovium and the entheses (1). Excellent reviews of this complicated topic have been provided by Douglas J Veale et Ursula Fearon and furthermore by Camel Stober (1, 14).

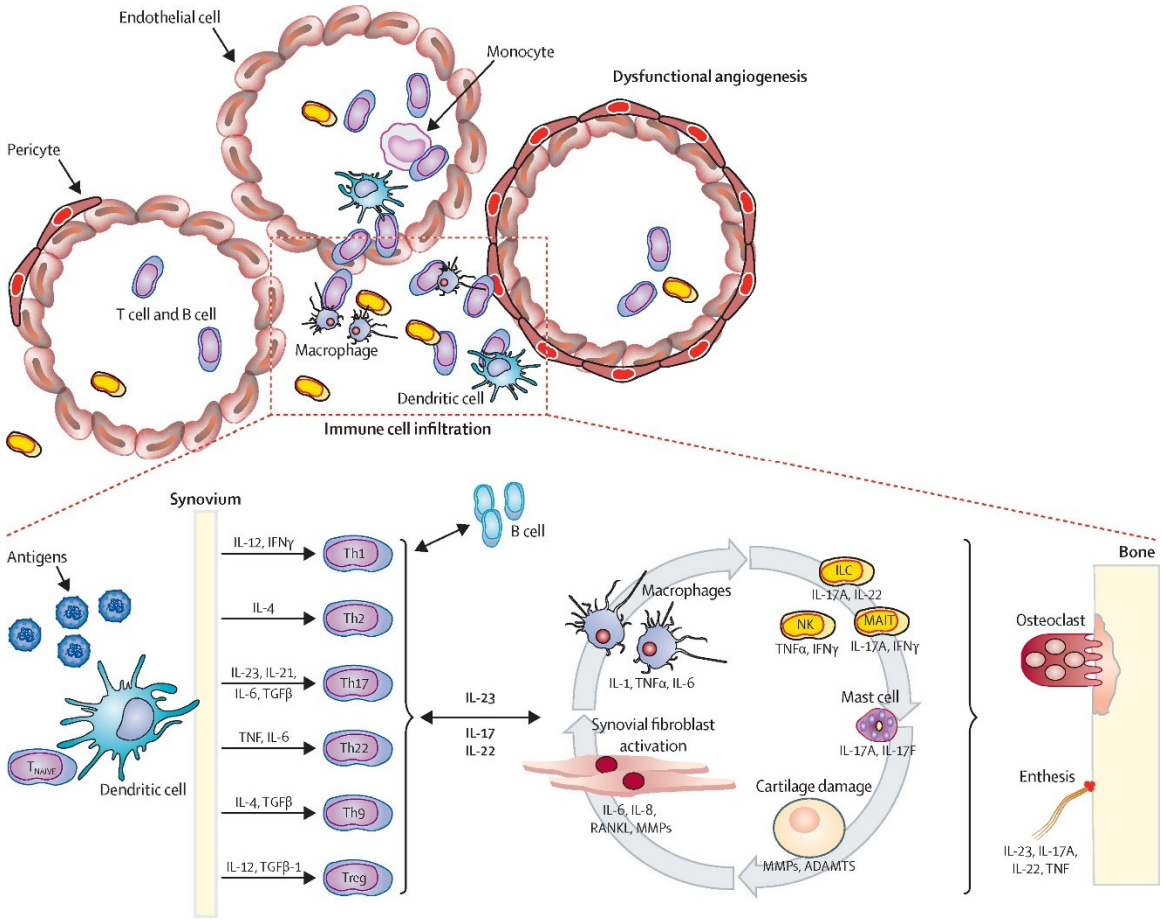


Figure 1: Schematic illustration of the cells and organs involved in the pathophysiology of PsA. Reproduced with friendly permission of Elsevier ® (1)

1.1.4. Diagnosis

Psoriatic Arthritis is a heterogeneous disease which must be differentiated from other rheumatologic diseases. The main differential diagnoses for these patients are other types of inflammatory arthritis like rheumatoid arthritis or crystal arthropathies (40).

1.1.4.1. Classification Criteria

Since 2006, the most common criteria used are the CASPAR criteria (Classification criteria for Psoriatic Arthritis) which were established in a large collective of PsA patients that were compared with patients who had other types of inflammatory arthritis. The criteria have a sensitivity of 91% and a specificity of 99% for the established disease (41). For early forms of PsA however, there is a drop in sensitivity to 87%, although the specificity remains as high as in established disease (42).

To fulfill the criteria for the diagnosis of PsA, a patient with inflammatory articular disease (joint, spine, or enthesal) must at least have 3 points of the following score:

CASPAR- Criteria	**Point Value**
Skin Psoriasis	
Current	2
Personal history	1
Family history (1st / 2nd degree relative)	1
Current typical psoriatic nail lesions (oncholysis, pitting, hyperkeratosis)	1
Dactylitis (current or history of, if documented by a rheumatologist)	1
Negative rheumatoid factor	1

Table 1: The CASPAR Criteria for the diagnosis of PsA, adapted after Taylor et al. (41)

1.1.4.2. Disease Activity Index

To assess the disease activity in PsA, the DAPSA score (Disease Activity Index in Psoriatic Arthritis) has been implemented in 2010. It includes the number of tender and swollen joints, tender joint count (TJC) and swollen joint count (SJC), respectively. Moreover, the disease activity according to the patient, called patient global assessment (PGA), and the overall level of joint pain during the preceding week, each on a scale of 0- 10. Lastly, the CRP level in mg/dl is added. All these parameters are added up and result in a linear scaled number. Based on this score, the patient can be classified as being in remission or having low, moderate or high disease activity (43).

DAPSA score	**Disease activity**
< 4	Remission (REM)
> 4 to ≤ 14	Low (LDA)
> 14 to ≤ 28	Moderate (MoDA)
> 28	High (HDA)

Table 2: DAPSA score and disease activity categories.

1.1.5. Therapy

Therapy according to current GRAPPA (Group for Research and Assessment of Psoriasis and Psoriatic Arthritis) recommendations can differ depending on the health- care setting. The decision for a given drug largely depends on the site of disease, the time of the disease course and lastly on a shared decision made by the physician and the patient.

For symptomatic therapy, it is advised to treat PsA with oral or intra-articular steroids and non- steroidal anti-inflammatory drugs (NSAIDs).

To beneficially influence the course of disease, so called disease modifying antirheumatic drugs (DMARDs) are used in a wide array of rheumatic disease and also in PsA. As of now, we have synthetic and biological DMARDS (bDMARDs) in the repertoire of drugs. The latter are antibodies against cytokines or their receptors, like TNF inhibitors, IL-12/ IL-23 inhibitors, IL-17 inhibitors & IL-23 inhibitors, to name the few that are approved for PsA. Synthetic DMARDs on the other hand can be further divided into conventional synthetic DMARDs (csDMARDs), that include Methotrexate, Leflunomide and Sulfasalazine and into targeted synthetic DMARDs (tsDMARD), that include JAK inhibitors and phosphodiesterase 4 inhibitors (PDE4 inhibitors).

For treatment- naïve patients, csDMARDs can be used as first- line therapy. A follow- up assessment of effectiveness and safety should occur 12 to 24 weeks after initiation of therapy and early escalation should be considered in this time frame if treatment response is not sufficient. There is also good evidence to use a TNFα inhibitor or any other tsDMARD or bDMARD in treatment- naïve patients with early disease (44). For the patients that started on a csDMARD and show insufficient treatment response, therapy escalation to bDMARDs or tsDMARDs is recommended (45).

The EULAR recommends starting with a csDMARD in patients with peripheral arthritis. In case of treatment failure, a bDMARD should be commenced. Moreover, if treatment in arthritis and/ or enthesitis with at least one csDMARD and one bDMARD fails, it is recommended to treat with a JAK inhibitor, switch to another bDMARD or PDE4 inhibitor (46).

1.2. JAK – STAT Pathway

The Janus Kinase ("Jak") and signal transducer and activator of transcription ("STAT") pathway represents a fast and direct membrane-to-nucleus signaling pathway that is used by more than 50 cytokines and growth factors (47). The pathway was detected in the early 1990's based on the finding that Type I and II Interferons alter transcriptional activity in immune cells (48).

1.2.1. Janus Kinases

Janus Kinases are protein- tyrosine kinases (PTKs) that are associated with the intracellular domain of the transmembrane receptor that binds the cytokine or growth factor (49). As of now, we know four different tyrosine kinases that play a role in the JAK- STAT Pathway: JAK1, JAK2, JAK3 and Tyrosine Kinase 2 (TYK2) (50-53). These Kinases structurally consist of 7 domains, the so called JAK homology (JH) domains, which are highly conserved among all JAK molecules (54).

The JH1 domain possesses tyrosine kinase activity, while the JH2 domain negatively regulates the kinase activity of the JH1 domain and therefore might inhibit auto- activation in absence of ligand binding (54-56). The other domains are mainly concerned with structural stability and the attachment of the JAK to its receptor (57).

Following cytokine interaction with the predominantly dimeric receptor, the signaling cascade is induced by a conformational change of the transmembrane helices that allows approximation and transphosphorylation of the two dimeric JAKs at the activation loop site of the kinase/ JH1 domain (58, 59). The JAKs are therefore activated and phosphorylate tyrosine residues at the intracellular proportion of the receptor chains, which enables them to interact with cytosolic STATs subsequently (60).

1.2.2. Signal Transducers and Activators of Transcription (STATs)

The STATs are proteins that, as indicated by their names, have a dual function. First, they transduce the signal of the cytokine from the cell membrane into the nucleus and second, they activate or enhance the transcription of certain genes (48). As of now, we know of 7 distinct mammalian STATs: STAT1- 4, STAT5a, STAT5b and STAT6 (57).

The STATs consist of the following structure: The N- terminal region, which is followed by the so called coiled- coil domain, which consists of four long helices and has an important function in dimerization. Next comes the DNA- binding domain, which is followed by the linker domain that connects the DNA- binding domain to the SH2 domain, which is lastly followed by the C-terminal transcriptional activation domain. Between the last two domains, there lies a single phosphorylation site which is required for ligand- induced STAT activity (61, 62).

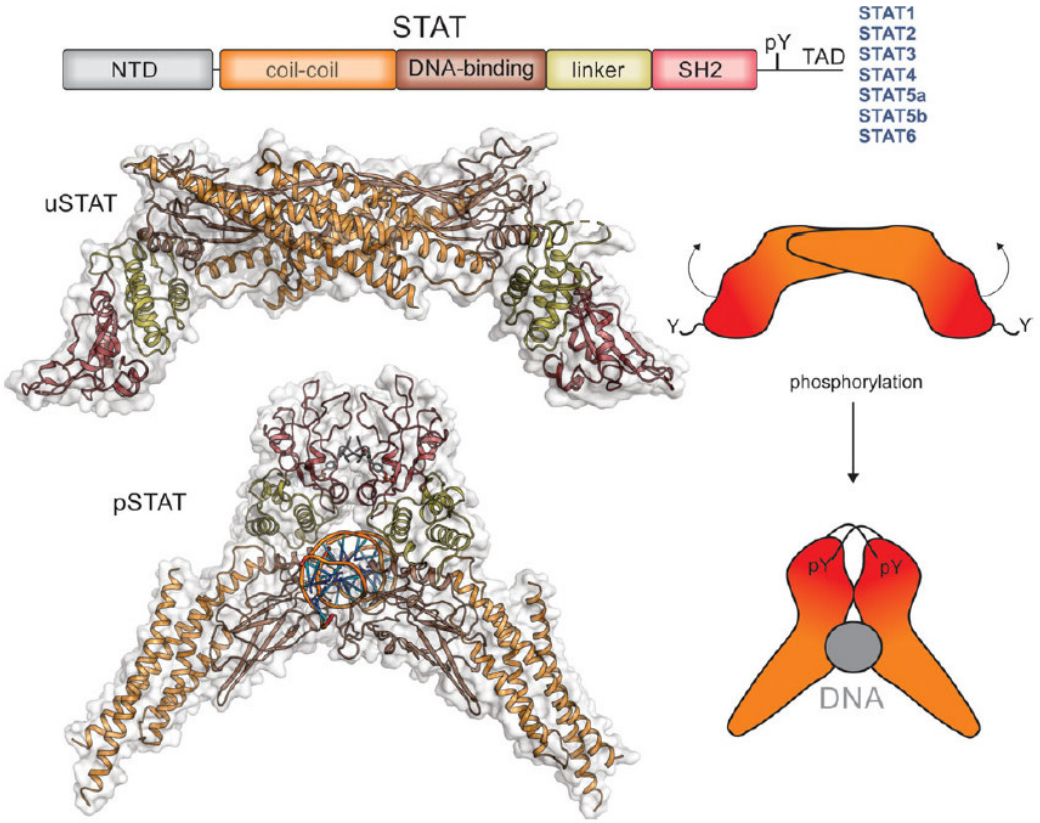


Figure 2: Structure of unphosphorylated (uSTAT) and phosphorylated (pSTAT) STAT molecules. All STATs share the same architecture and phosphorylation of tyrosine residues leads to a change in conformation which allows for interaction with the DNA. Reproduced with friendly permission of John Wiley and Sons ® (57)

Before ligand- induced activation, STATs exist as inactive monomers or dimers in the cytoplasm (63). Following the previously described phosphorylation of the tyrosine residues at the intracellular portion of the given receptor, the STATs are then able to bind to these residues with their SH2 domain (57). The SH2 domain is the part of the STAT that decides which cytokine receptor the STAT will bind to

(64). The activation of a specific STAT is furthermore dependent on the type of receptor that is engaged. Therefore, different cytokines can activate the same STAT, since their receptors share similar chains and/ or distinct motifs (65).

After binding to the receptor, the STATs are activated by JAK- mediated phosphorylation of a tyrosine residue between the SH2 and transactivation domain. The SH2 domain of the STATs now binds to this newly created residue of the other monomer instead of the phosphorylated receptor, which leads to dissociation of the STAT dimer from the receptor (57). The phosphorylated STAT molecule (pSTAT) is subject to the investigations that are conducted in the study.

The STAT dimers then find their way into the nucleus, where they alter gene transcription. STAT1 for example, binds to importin-α5, which is a part of the nucleocytoplasmatic transport machinery, and thereby gets transported into the nucleus of the cell (66, 67).

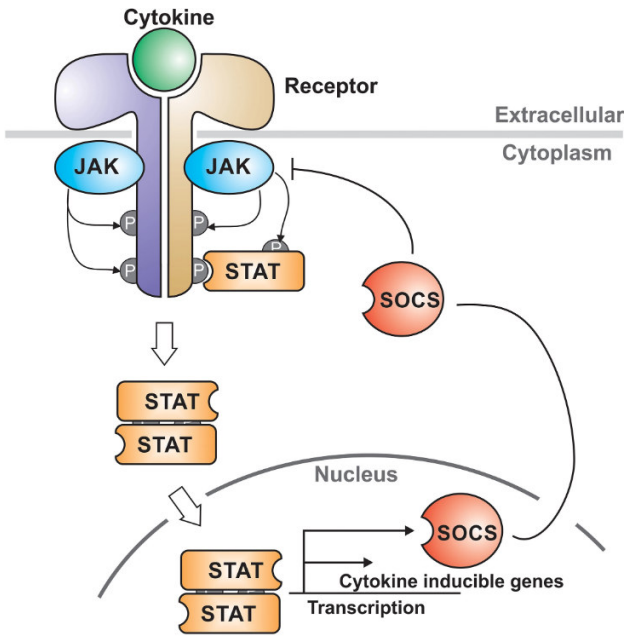


Figure 3: Schema of the JAK / STAT pathway. Reproduced with friendly permission of John Wiley and Sons ® (57)

After that, the STAT dimers bind to gene promoters with a fitting binding site and further co- activators of transcription are recruited, resulting in enhancement of transcription of these genes (57).

DNA binding of STATs occurs at DNA regulatory elements (DREs) that are either proximal or distal to the target genes. Many of these proximal binding sites are not directly linked to increased gene expression and this interaction is therefore called “neutral binding” (68). Distal binding sites can be either enhancers, epigenetic hotspots, or non-coding loci like microRNA. Interestingly, STATs interact with enhancers of lineage- defining genes, like *Ifng, IL-4 / IL-13* and *IL-17* and are therefore important parts of lineage determination (69). They often co- localize with other lineage- defining transcription factors like T-bet or RORγt in T cells (47). Furthermore, the STATs aggregate with other transcription factors to form complexes such as the Interferon Stimulated Gene Factor 3 (ISGF3), which is induced upon IFNα stimulation and that consists of STAT1, STAT2 and IRF9 (70).

Additionally, we know that STATs can regulate the epigenetic status of genes in an either permissive or repressive way (71, 72). This function might be mediated either by physical interaction with or by transcriptional control of certain proteins that mediate epigenetic changes, like p300 or EZH2 (47, 73, 74).

One of the mysteries in JAK- STAT signaling is how specificity of only 7 STATs is achieved, since each STAT has unique functions on cellular and organismal level but is utilized in the downstream pathway of lots of different cytokines, which sometimes have even contrary effects. The answer to this question probably lies in multiple facets of the pathway, which I will describe in the following.

All STATs can bind, although not exclusively, to a certain DNA sequence, called the GAS motif. GAS stands for Gamma interferon activation site and has initially been described in IFN signaling but has now been established as a terminal end of JAK- STAT signaling in general (75). Specificity could be achieved through small variations in the GAS motif that could lead to different affinities of a certain STAT to a certain DRE. Epigenetics may also play an important role in differentiating the function of STATs as described above and are probably the main reason why STAT activity is lineage- dependent, since they control the access of the transcription factors to the genes (47).

Another factor is posttranslational modification. For example, STAT1 is involved in type I and type II IFN signaling, which both results in transcription of particular genes and therefore has distinct effects on the cell. Phosphorylation on tyrosine residue Y701 is necessary for initial activation. But for maximum transcriptional activity, STAT1 (and STAT3) need to be further phosphorylated on a single serine residue (residue S727) (76). In the case of STAT1, the post- transcriptional modification differentiates the downstream pathway of type I and type II IFN. For conduction of the type I IFN signal, an additional phosphorylation of yet another serine residue (residue S708) is required for the formation of the ISGF3 complex and for inhibition of the homodimerization of STAT1 (GAF complex), since the latter would lead to type II IFN gene transcription (77).

Another factor is called “priming”, a term that describes the phenomenon that prior exposure to cytokines affects following responses to cytokines and has been investigated mostly in IFN signaling. For example, priming with IFNγ increased the concentrations of the transcription factor complex ISGF3 and thereby lead to an increased responsiveness to IFNα in IFN-resistant melanoma cells (78). Many other examples of priming have been found and summarized in an excellent review by van Boxel- Dezaire et al. (79).

Furthermore, qualitative differences in JAK/ STAT signaling contribute to the differences. IL-6 & IL-10 for example, both rely on STAT3 to induce their inflammatory (IL-6) or anti- inflammatory (IL-10) effects on macrophages. SOCS3, a part of the regulatory machinery in this pathway, is responsible for the opposite effects of these cytokines, since in absence of SOCS3, even IL-6 induces anti-inflammatory effects. However, in the presence of SOCS3, IL-6 doesn’t upregulate the genes of the anti- inflammatory response anymore which in turn, leads to increased inflammatory effects. Therefore, differences in the cytokine receptors are considered to play a major role in differentiating the type of response that is conducted via different STATs, since SOCS3 exerts its inhibitory effects on the gp130 subunit of the IL-6 receptor but doesn’t interfere with the IL-10 receptor (80, 81).

1.2.2.1. STAT1

STAT1 was discovered alongside STAT2 as they both, combined as a heterodimer, are part of the ISGF3 complex that is activated following IFNα. Therefore, STAT1 acts as an intermediary in the antiviral response induced by Type I Interferons (70, 82). Additionally, STAT1, but not STAT2, can be activated by IFNγ and therefore facilitate its key role in macrophage activation & defense against intracellular pathogens, TH1 cell responses, production of antiviral proteins & phagocytic receptors, just to name a few (83, 84). STAT1 has shown paradoxical effects in T cells, since STAT1 induction upon type I IFNs in vitro lead to antiproliferative effects, while in vivo leading to CD8+ T cell expansion upon viral infection. This might reflect the complexity of the signaling cascade, since in vivo, many other cytokines besides type I IFNs participate in the anti-viral response, while in vivo stimulation of cells is usually performed with a few or just a single cytokine (85). In an animal model, pSTAT1 was necessary to protect T cells from NK cell- mediated cytotoxicity which consequently lead to maintenance of T-cell-mediated inflammation (86). To sum it up, STAT1 in T cells is considered to be pro- inflammatory, anti- proliferative and pro-apoptotic (87).

Furthermore, STAT1 has an important role in NK cell cytolytic and anti- tumor activity (88). Moreover, it is important for regulation of lymphocyte survival and proliferation (89). Additionally, STAT1 might be required for the development and functionality of regulatory T cells (Treg) and might therefore be important for maintenance of immunological self- tolerance (90).

Levels of unphosphorylated STAT1 (uSTAT1) show delayed upregulation following STAT1 phosphorylation, since pSTAT1 increases the expression of the STAT1 gene (91, 92). Consequently, many genes that are initially expressed in an increased fashion, like the ones encoding for antiviral proteins, are further expressed at a constant rate to clear the virus in the infected cell completely (93).

Regarding Psoriatic Disease, an increase of pSTAT1 in keratinocytes of psoriatic skin lesions has been found, but not in the synovial tissue (94, 95). In PsA, pSTAT1 levels in circulating immune cells did not differ in patients with active or inactive disease in one study (96).

1.2.2.2. STAT2

The most important role of STAT2, known yet, is its participation in the previously described ISGF3 complex with STAT1 and the transcription factor IRF9. Therefore, it mediates antiviral and antiproliferative effects of Type I IFNs. Unphosphorylated STAT2 can even bind IRF9 in absence of STAT1 and in combination with NF-κB bind to the ISRE in the *il6* promoter, which leads to increased expression of IL-6 (97).

Besides that, STAT2 might have a role in myogenic differentiation (98).

1.2.2.3. STAT3

STAT3 was initially detected as a factor that could bind to the enhancer element in the promoter region of genes that encode for acute phase proteins following IL-6 stimulation in hepatocytes (99, 100). Activation of STAT3 occurs following stimulation by cytokines of the gp130 family like IL-6 & IL-11, furthermore by cytokines with homodimeric receptors like G- CSF and moreover by different growth factors like the epidermal growth factor (101-103).

Unphosphorylated STAT3 (uSTAT3) on the other hand increases following pSTAT3 induced gene transcription and serves as part of a transcription factor complex including NF-kB, which regulates genes that are usually not affected by pSTAT3 signaling (104).

STAT3 underlies an autoregulatory amplification loop since it induces its own gene expression (105). Therefore, gene regulation by pSTAT3 and uSTAT3 might be induced & multiplied following initial STAT3- induced gene expression.

The most important transcriptional functions of pSTAT3 in immunity are TH17 cell differentiation through IL-23 signaling, steady state & emergency granulopoesis, which is important in fighting bacterial and fungal infections, and development & normal function of dendritic cells (106-109).

In phagocytes, STAT3 is part of anti- inflammatory pathways like the one induced by IL-10. This has been proven to be of upmost importance for the maintenance of intestinal homeostasis, since absence of STAT3 in different types of phagocytic cells lead to chronic bowel inflammation in multiple mouse models (110-112).

In adaptive immunity, STAT3 plays a role in B cell development and terminal B cell differentiation (113, 114). Moreover, it is required for IL-6 mediated T cell survival, induction of T follicular helper (Tfh) cells but is also able to inhibit generation of regulatory T cells. (115-117). Furthermore, it is necessary for functional maturation of memory CD8+ T cells (118). Mutations in STAT3 are also an underlying cause for Hyper-immunoglobulin E syndrome (HIES), which is a primary immunodeficiency that is, amongst other symptoms, characterized by recurrent staphylococcal skin abscesses, pneumonia, and high serum IgE levels (119).

Besides its important role in immunity, STAT3 is crucial for normal development of different tissues in the body (120). Other than its role in gene transcription, STAT3 has important functions in cellular respiration, metabolism, autophagy, and the potential to induce cancer (108).

One important role of STAT3 in the pathophysiology of PsA is the involvement in the induction of CD4+ TH17 T cells, which are a key player in the IL-17 / IL-23 axis by production of IL-17 (121).

1.2.2.4. STAT4

STAT4 has been shown to have significant functions in IL-12 and type I IFN signaling (122-124). It is therefore of upmost importance for TH1 differentiation (125, 126). Moreover, STAT4 is involved in the signaling of IL-23, since the IL-12 and IL-23 receptor share a similar subunit (127). Additionally, STAT4 plays a role in IL-2, IL-27 and IL-35 signaling, just to name a few (128).

In experimental models auf autoimmunity, the knockout of STAT4 led to a significant reduction in arthritis incidence (129).

1.2.2.5. STAT5

STAT5 consists of two isoforms, namely STAT5α and STAT5β that are highly homologous proteins (130-132). In the following, I will summarize the functions of both isoforms and refer to them only as STAT5.

STAT5 is involved in NK cell proliferation and cytolytic activity through IL-2 & IL-15 signaling (133, 134). Furthermore, it plays a role in differentiation of naïve CD4+ T cells into the TH1 & TH2 lineages via IL-2 dependent upregulation of the IL-12Rβ2-chain (TH1) and IL-4Rα- chain (TH2) but is also involved in inhibition of

TH17 differentiation via IL-2 signaling (135-138). Moreover, STAT5- induced transcription of *foxp3* via IL-2Rβ is crucial for the induction of regulatory T cells (139). Another role for STAT5 is the involvement in CD8+ T cell proliferation and survival, probably through IL-2, IL-7 and IL-15 signaling (140).

Besides its immunological functions, STAT5 acts downstream of Prolactin and is therefore important for adult mammary gland development and lactation (141). STAT5 also plays a role in malignancy, amongst other things through its role in induction of Tregs, which can suppress antitumor immunity (142).

1.2.2.6. STAT6

STAT6 is a major part of TH2 type inflammation, which is mainly induced by IL-4 and IL-13 and is characteristic for asthma and anti-parasitic immune responses (143, 144) .

In B cells, STAT6 plays an important role in development, activation, function, and tolerance, mainly through IL-4 signaling (145).

In RA patients, pSTAT6 levels in circulating immune cells was associated with treatment response to DMARDs (146). For PsA, no such investigation has been undertaken yet.

1.2.3. Negative regulation of JAK / STAT signaling

The JAK/ STAT pathway has a few negative- feedback mechanisms to prevent overly excessive activation. The primary negative- feedback regulators are the so-called SOCS proteins, which stands for suppressor of cytokine signaling. These proteins are induced by many cytokines and lead to degradation of cytokine receptors and potentially other substrates, therefore mitigating activation of the JAK/ STAT pathway (57).

On the other hand, we know of phosphatases that are associated with the cytokine receptor, like CD45, or that are located in the cytoplasm, like PTP1b (147, 148). These molecules are constantly expressed and could therefore not be considered a negative feed- back mechanism, but much more of a regulative component in this pathway (57).

Cytokines	Tyrosine Kinase	Associated STAT
IL-2, IL-7, IL-9, IL-15, IL-21	JAK 1, JAK 3	STAT3, STAT5
IL-4		STAT6
IL-12, IL-23	JAK 2, TYK 2	STAT3 (IL-23), STAT 4 (IL-12)
IFNα / β	JAK 1, TYK 2	STAT1, STAT2, STAT4, STAT3
IFNγ	JAK 1, TYK 2	STAT1
IL-10, IL-22	JAK 1 / TYK 2	STAT3
TNFα	JAK 1	STAT3, STAT5

Table 3: List of cytokines that have a prominent role in PsA, listed with their associated tyrosine kinases and STAT molecules. (57, 149, 150)

2. Aims of the study

The aim of this work is to investigate differences in JAK/ STAT – signaling in patients with PsA. We therefore focus on the phosphorylation status of STAT molecules, which is considered a sign of activation of this pathway, in different types of immune cells.

This study is mainly concerned with differences in JAK/ STAT – signaling of PsA patients, especially in the context of clinical implications but also regarding the underlying pathophysiology. We have the hypothesis, that there are different phosphorylation patterns in patients with a higher disease activity (MoDA/HDA) and lower disease activity or remission (LDA/REM). Moreover, we postulated that disease activity might correlate with pSTAT levels, and that disease duration influences the phosphorylation level. Furthermore, we hypothesize that these types of differences could be observed between genders. A specific observation from unpublished data of our lab was that female RA patients had higher pSTAT5 levels in T cells than male RA patients.

Finally, we postulate that PsA patients could be divided into subgroups according to their molecular signature in terms of pSTAT levels in immune cells. A similar approach has already been used for RA patients (151). The purpose of stratifying patients with rheumatic disease lies in the idea that therapy regimens could be guided more effectively, since approximately 40% of patients with PsA don’t respond to therapy with csDMARDs, TNFi or other biologic treatments, leaving

physicians with a trial- and- error approach in pharmacological therapy (152). Therefore, the EULAR put the determination of biomarkers for the treatment response, which might help in stratification of patients, on their research agenda for PsA (46).

This work is part of a longitudinal study.

3. Methods

3.1. Study design

This was a prospective cross- sectional study on patients with PsA.

3.2. Patients

50 patients with the diagnosis of PsA, based on the 2006 CASPAR criteria, were prospectively enrolled. Exclusion criteria were an ongoing infection and other autoimmune diseases besides that of PsA & Psoriasis. Clinical data including disease duration, comorbidities, diagnosis of skin psoriasis or general skin involvement as well as medication was collected. Furthermore, the disease activity score (DAPSA) was calculated for each patient if possible. This study was approved by the Institutional Review board of the Medical University Graz and written informed consent was obtained from each patient.

3.3. Whole blood processing

Peripheral venous blood was drawn from each individual using EDTA tubes and immediately put on ice to prevent auto- activation of leucocytes and to minimize changes in phosphorylation status of intracellular molecules. 200 µl of whole blood were utilized for each of the two panels in the subsequent analyses. In a first step, Panel 1 was stimulated with viability dye for 10 minutes. Panel 2 didn't allow for staining with a viability dye due to limitation in the number of fluorochromes. After that, both tubes were fixed by addition of 2 ml pre-warmed Lyse/Fix Buffer to the samples, followed by a vortexing step and a 10-minute incubation period in a 37°C water bath. Next, the tubes were centrifuged at 600g for 6 minutes and after that, the supernatant was removed, and the remaining cell pellet was disrupted with another vortexing step. Then, a washing step was conducted by addition of 2 ml PBS and centrifugation. After removing the supernatant, the cells were permeabilized by addition of 1 ml pre- chilled Perm Buffer III. The tubes were then

vortexed again and incubated for 30 minutes on ice. Following the incubation, 3 ml of PBS were added to each tube and a centrifugation step was conducted again. After removing the supernatant, the washing step was repeated 2 times with 3 ml of PBS each. Following the washing steps, the supernatant was removed, and the cells were stained with the antibodies. After an incubation period of 60 minutes, protected from light, another washing step was conducted by addition of 3 ml PBS and centrifugation. Lastly, the supernatant was removed, the cells were disrupted by vortexing and 50 µl of staining buffer were added to each tube. After that, the flow cytometry was performed.

Reagent	Name	Company	Cat. No.
BD Phosflow ™	Lyse/ Fix Buffer 5x	BD Biosciences ®	558049
BD Phosflow™	Perm Buffer III	BD Biosciences ®	558050
eBioscience ™ Flow Cytometry	Staining Buffer	Invitrogen by Thermo Fisher Scientific ®	00-4222-26

Table 4: List of reagents.

3.4. Antibodies & flow cytometry

Antibodies for cell surface, as well as for intracellular staining were used. Surface antibodies, such as CD3, CD19, CD16, CD45RA and Tbet, were used to differentiate cell types and intracellular antibodies against pSTAT1-6 were used to measure phosphorylation levels. In panel 1, we added a viability dye to investigate the number of dead cells in the sample.

Panel 1 antigen	Dye	Company	Cat. Nr.	Volume per 50 µl test
pSTAT1	AF647 (APC)	Cell Signaling Technology ®	8009	1µl
pSTAT2	PE	Cell Signaling Technology ®	77366	1 µl
pSTAT6	AF488 (FITC)	Cell Signaling Technology ®	68600	2.5 µl
CD3	PerCP	BD Biosciences ®	345766	10 µl
CD19	V450 (PacificBlue)	BD Biosciences ®	560353	2.5 µl
CD16 APC-Cy7	APC- Cy7	BioLegend	302018	2.5 µl

Table 5: List of antibodies for surface and intracellular staining for Panel 1. Cat. Nr., Catalog number

Panel 2 antigen	Dye	Company	Cat. Nr.	Volume per 50 µl test
pSTAT3	AF647 (APC)	Cell Signaling Technology ®	4324	1 µl
pSTAT4	PE	Cell Signaling Technology ®	13223	1 µl
pSTAT5	AF488 (FITC)	Cell Signaling Technology ®	3939	1 µl
CD3	APC-eF780 (APC- Cy7)	eBioscience ™	47-0036-42	2.5 µl
CD4	eF506 (AmCyan)	eBioscience ™	69-0049-42	2.5 µl
CD45RA	PE-Cy7	BD Biosciences ®	337186	2.5 µl
Tbet	PerCP-Cy5.5	eBioscience ™	45-5825-80	2.5 µl

Table 6: List of antibodies for surface and intracellular staining for Panel 2. Cat. Nr., Catalog number

All stained cells were analyzed on a BD FACSlyric™ Flow Cytometry System. The analysis of the FACS data was performed on FlowJo™ version 10.8.1.

3.5. Statistical analysis

All statistical analyses were performed using the IBM ® SPSS ® software, version 26.0 (Armonk, NY, USA). Results were described as median and range or mean and standard deviation as appropriate. The Shapiro- Wilk- Test was performed to explore the distribution of the data. In case of a normal distribution of the variable, the two- sided Student's t-test was performed. In case of non- parametric distribution, the Mann- Whitney U test was applied. For correlation analysis, the Spearman's rank correlation coefficient was used. The cluster analysis was performed with Ward's method, using the squared Euclidean distance. Comparisons between clusters were conducted with the Kruskal-Wallis test. Significance level was set as $p < 0.05$. For analyses that included the DAPSA, 8 patients were excluded because of missing values that were necessary to calculate the score correctly. In the cluster analysis, an additional 2 patients were excluded because of missing gMFI values.

4. Results

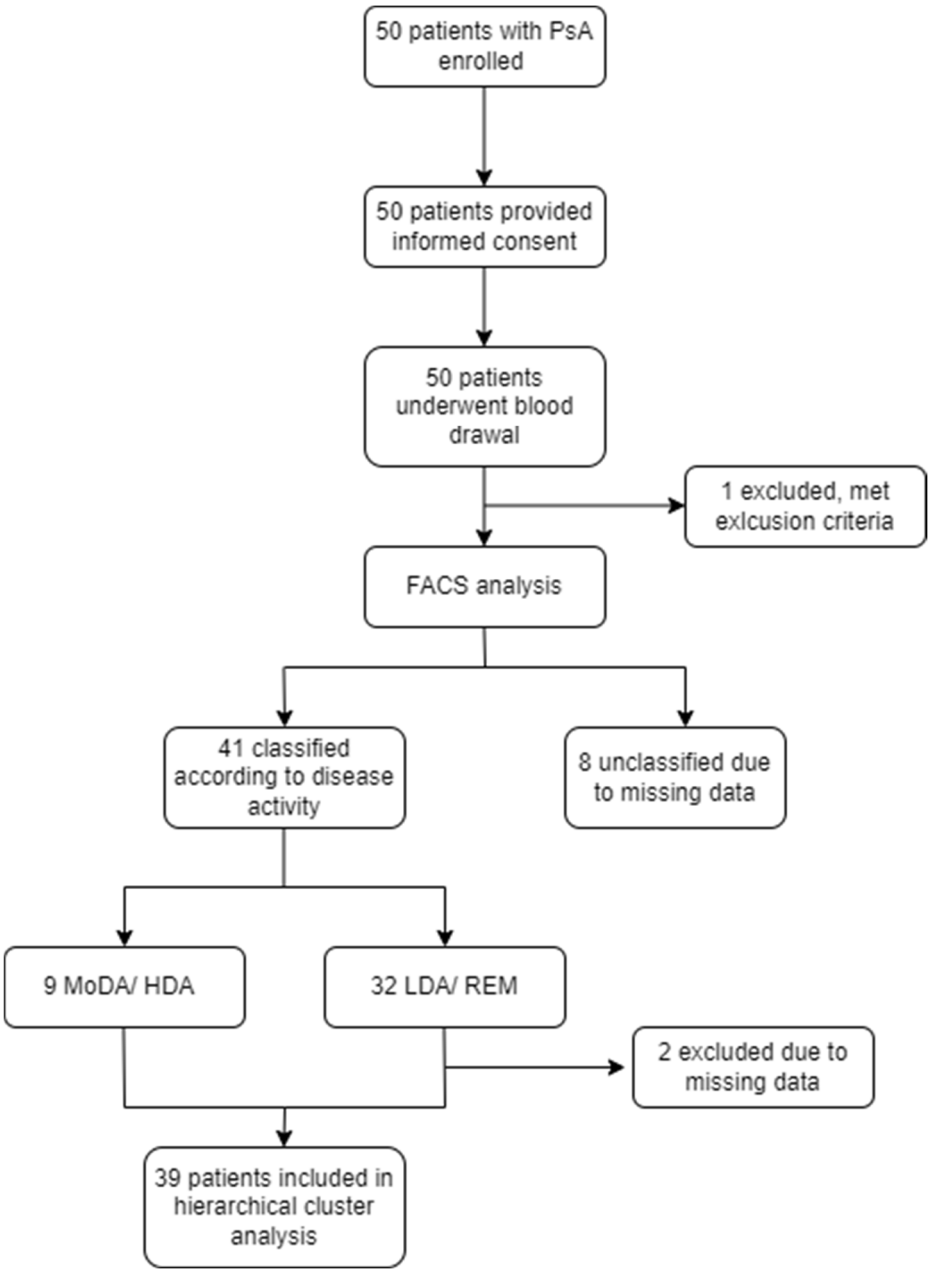


Figure 4: Trial profile.

4.1. Patient's Characteristics

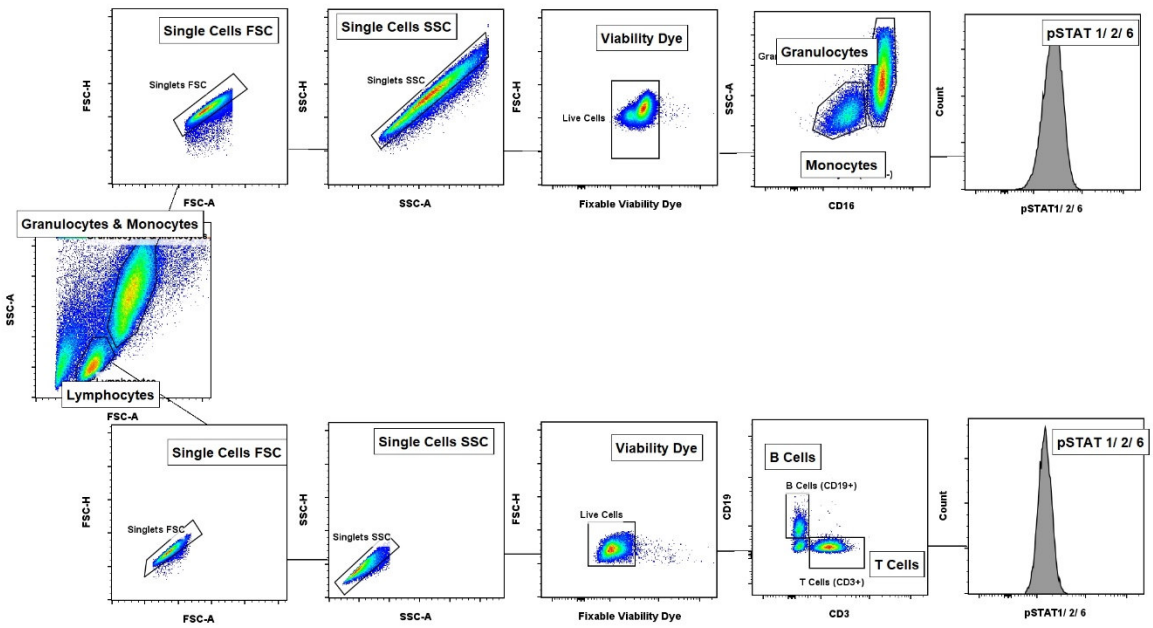
	Overall (n= 49)	**LDA / REM (n= 32)**	**MoDA/HDA (n=9)**	**p- value****
Age (years)*	57 (50.0, 62.0)	57 (44.0, 63.0)	56 (53.0, 56.0)	0.508
Sex (n)				
Female	21 (42.9 %)	14 (43.8%)	5 (55.5%)	0.709
Male	28 (57.1%)	18 (56.2%)	4 (44.5%)	0.709
disease duration (years)*	7.0 (1.0, 15.0)	10.0 (2.0, 15.0)	7.0 (3.0, 16.0)	0.849
Clinical parameters (n)				
TJC*	0.0 (0.0, 2.0)	0.0 (0.0, 1.0)	6.0 (2.0, 12.0)	0.001
SJC*	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	4.0 (0.0, 5.0)	0.000
CRP (mg/dl) *	1.9 (0.7, 4.0)	1.2 (0.5, 4.0)	4.0 (1.3, 6.0)	0.097
BSG (mm/h) *	7.0 (5.0, 13.0)	6.0 (5.0, 13.0)	10.0 (3.0, 19.0)	0.521
PGA*	5.0 (3.0, 7.0)	2.0 (0.0, 3.0)	5.0 (4.0, 8.0)	0.000
PhGA*	3.0 (2.0, 6.0)	1.0 (0.0, 2.0)	5.0 (3.0, 6.0)	0.000
Pain (NRS)*	2.0 (2.0, 6.0)	1.0 (0.0, 3.0)	6.0 (3.0, 7.0)	0.000
DAPSA*	5.0 (1.0, 11.4)	2.7 (1.0, 7.1)	23.4 (16.1, 27.9)	0.000
Treatment (n)				
Methotrexate	18 (36.7 %)	11 (34.3 %)	3 (33.3 %)	1.0
Prednisone	3 (6.1 %)	1 (3.1%)	1 (11.1 %)	0.395
Leflunomid	1 (2.0 %)	0	0	NA
Sulfasalazin	1 (2.0 %)	0	1 (11.1 %)	0.220
JAK- Inhibitor	2 (4.0 %)	2 (6.3 %)	0	1.0
TNFa- Inhibitor	20 (40.8 %)	12 (37.5 %)	4 (44.4 %)	0.717
IL-17A- Inhibitor	12 (24.5 %)	8 (25.0 %)	2 (22.2 %)	1.0
PDE- Inhibitor	4 (8.1 %)	2 (6.3 %)	1 (11.1 %)	0.535
Other Conditions (n)				
Skin Psoriasis	26 (53.0%)	19 (59.4%)	3 (33.3 %)	0.260
Hyperuricemia	8 (16.3%)	4 (12.5 %)	3 (33.3 %)	0.165
Hypertension	6 (12.2%)	5 (15.6%)	1 (11.1%)	1.0
Osteoarthritis of the knee	12 (24.5%)	7 (21.9 %)	2 (22.2 %)	1.0

**median (25th, 75th percentile)*
***p value was calculated with the Mann- Whitney- U- Test for continuous variables and with fisher's exact test for categorical variables for comparison between groups*

Table 7: Baseline characteristics of patients, stratified by group (REM/LDA, MoDA/HDA).

4.2. Gating strategy

A



B

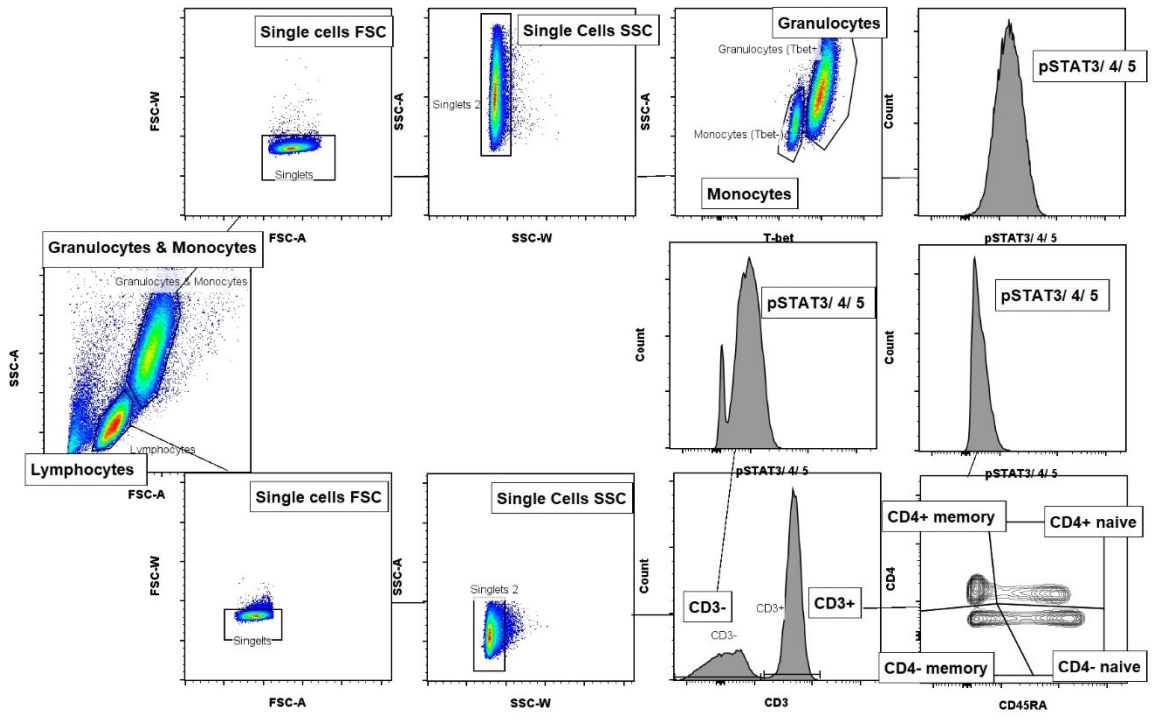


Figure 5: Gating strategy. **A:** Panel 1; **B:** Panel 2

4.3. Cell frequencies

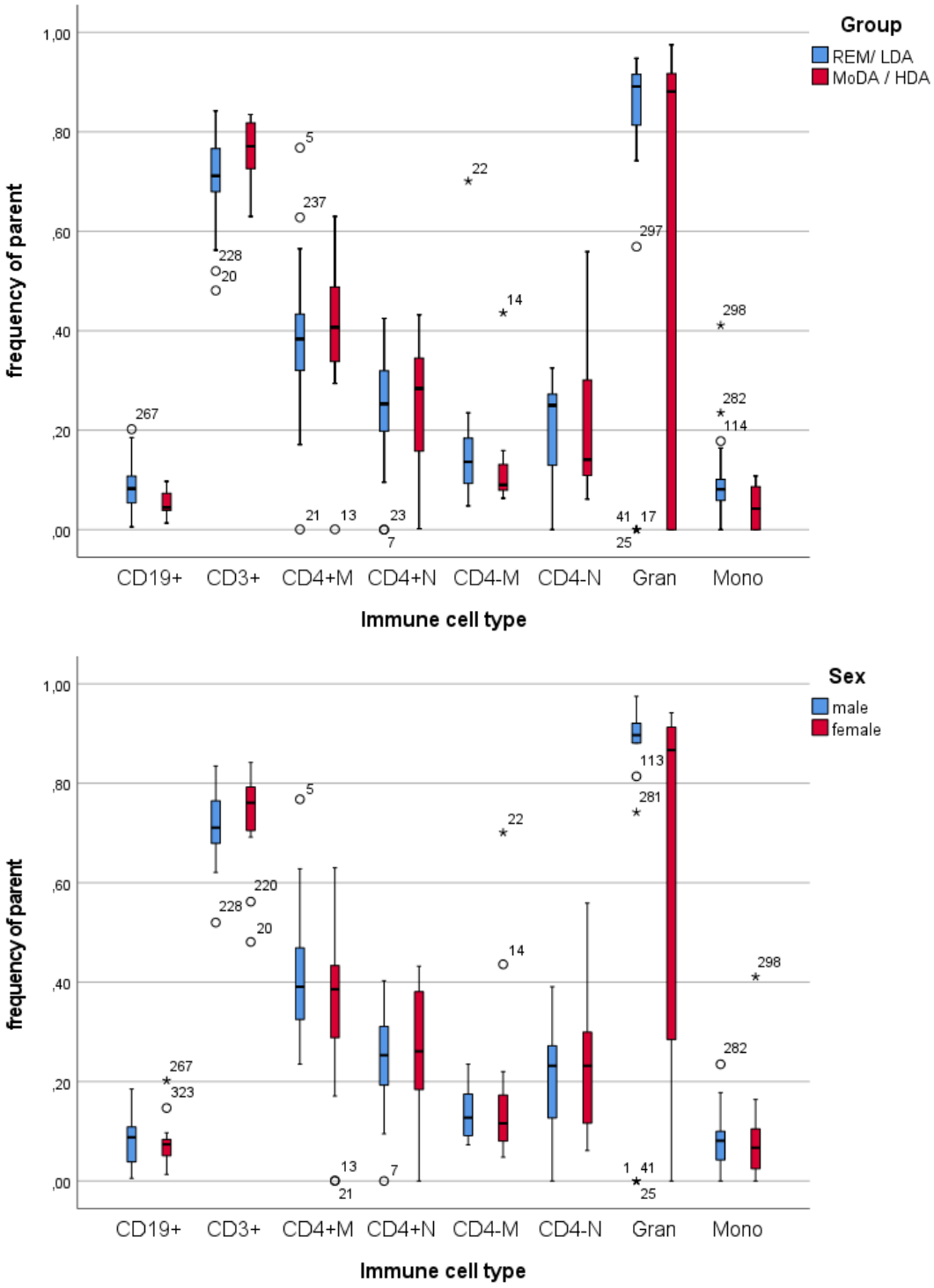


Figure 6: Frequency of immune cells, grouped according to disease activity and gender.

There were no significant differences in cell frequencies between the LDA/ REM & MoDA/HDA group nor between genders.

4.4. Differences between REM/LDA & MoDA/ HDA in STAT phosphorylation

To test whether there are significant differences in the STAT phosphorylation levels of PsA patients in the REM/ LDA and MoDA/ HDA group, we observed the gMFI of the given STATs in different cell types and subpopulations with FACS analysis as described above and performed the Mann- Whitney U test.

We found significant differences for pSTAT3 in CD4+ naïve & memory cells and in CD4- naïve & memory cells. Patients with MoDA/ HDA showed significantly higher pSTAT3 levels in CD4+ & CD4- naïve and memory T cells (see table 8).

For pSTAT1, we found significant differences between groups in Granulocytes & Monocytes. Patients in REM/ LDA showed significantly higher pSTAT1 levels in Granulocytes and Monocytes (see table 9).

	Naive CD4+	Memory CD4+	Naive CD4-	Memory CD4-
REM/ LDA	238.0 (99.0)	190.0 (58.0)	233.0 (104.0)	161.5 (58.0)
MoDA/ HDA	284.5 (125.8)	227.0 (109.0)	297.5 (106.0)	209.0 (80.5)
U	44.5	42.0	42.5	70.5
Z	-2.766	-2.852	-2.329	-2.315
p- value	0.006	0.004	0.020	0.021
r (effect size)	-0.43	-0.45	-0.36	-0.36

Table 8: Significant differences in pSTAT3 levels between groups [median and interquartile range].

	Granulocytes	Monocytes
REM/LDA	2509.0 (2850.0)	255.0 (167.25)
MoDA/HDA	1330.5 (1679.0)	144.0 (108.25)
U	38.0	40.0
Z	-2.08	-1.99
p- value	0.038	0.047
r (effect size)	-0.32	-0.31

Table 9: Significant differences in pSTAT1 levels between groups [median and interquartile range].

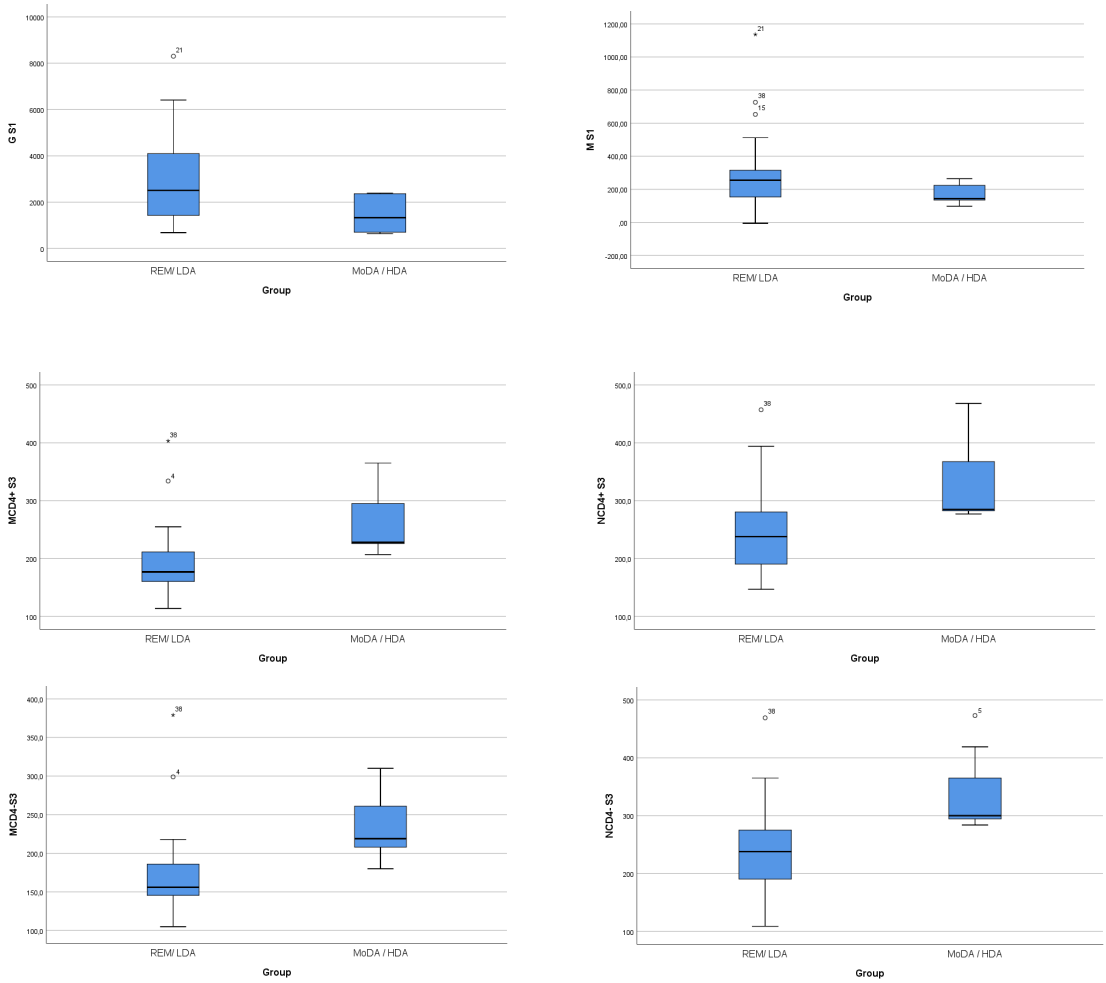
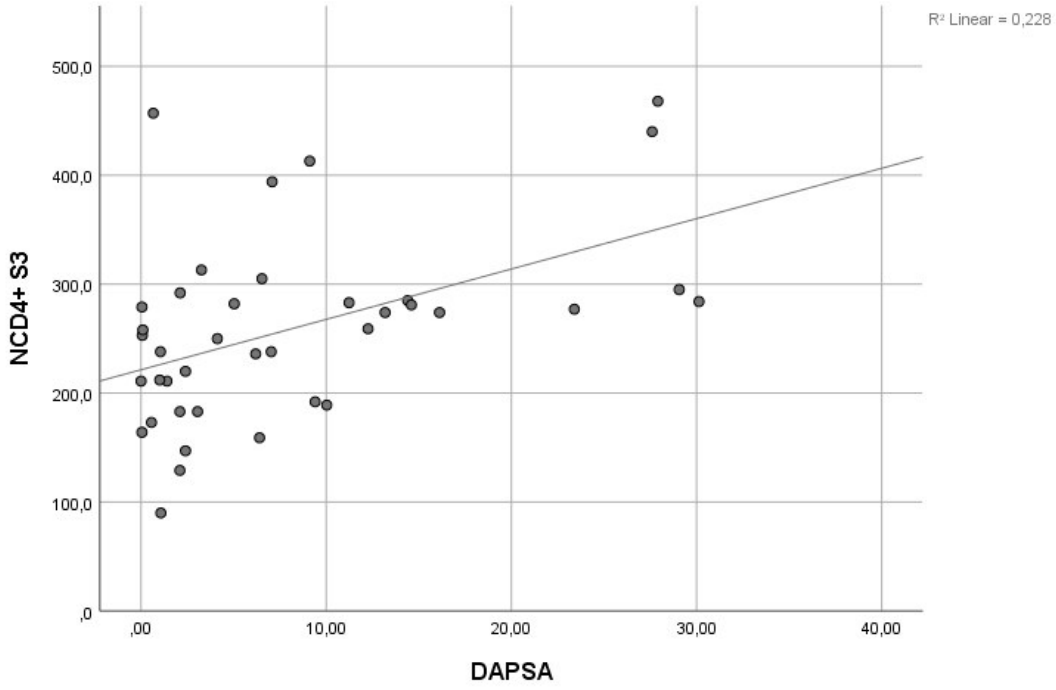


Figure 7: Box plots of pSTAT levels (gMFI) in different cells show significant differences between the REM/LDA and MoDA/HDA group. G, Granulocytes; M, Monocytes; MCD4+/ NCD4+, memory / naïve CD4+ T cells; MCD4-/ NCD4-, memory / naïve CD4- T cells; S1, pSTAT1; S3, pSTAT3

4.5. Correlation of pSTAT levels and clinical disease activity

To investigate whether the STAT phosphorylation levels correlate with clinical disease activity, Spearman's correlation coefficient ρ (rho) was calculated. Significant positive correlations were found between the DAPSA score and pSTAT3 in CD4+ naïve (ρ (rho) = 0.48, p = 0.002) and furthermore CD4+ memory T cells (ρ (rho) = 0.45, p = 0.004).

Significant negative correlations were found between the DAPSA score and pSTAT1 in Granulocytes (ρ = -0.45, p = 0.007) and Monocytes (ρ = -0.34, p = 0.05).



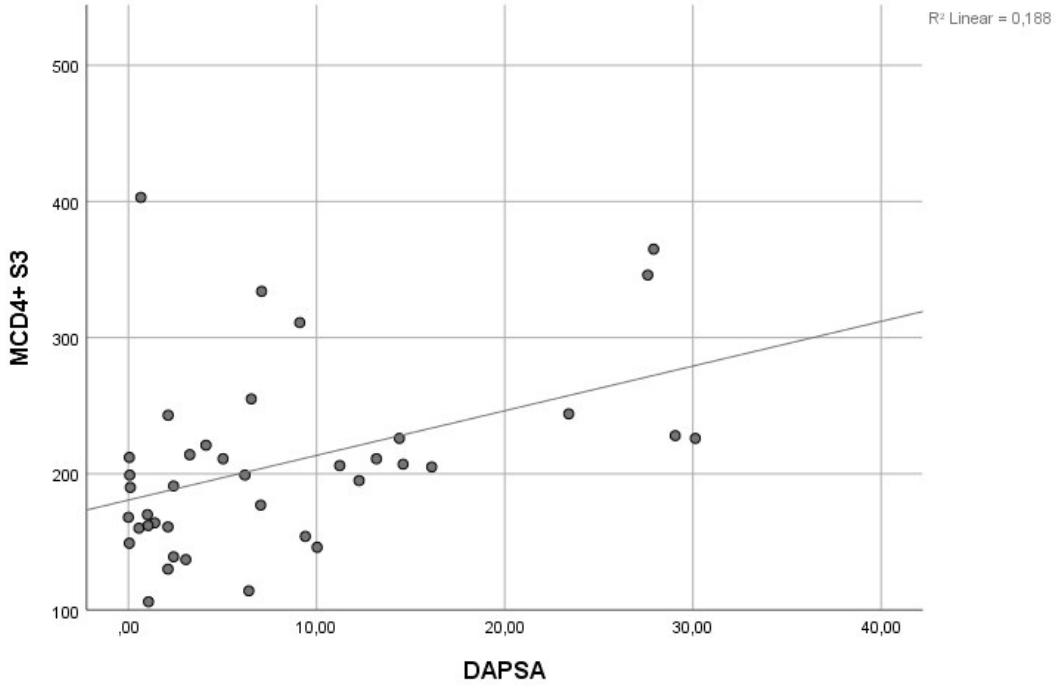


Figure 8: Scatterplots of significant positive correlations between pSTAT3 levels (gMFI) in naive & memory CD4+ cells and the DAPSA score. NCD4+ S3, naïve CD4+ pSTAT3; MCD4+ S3, memory CD4+ pSTAT3

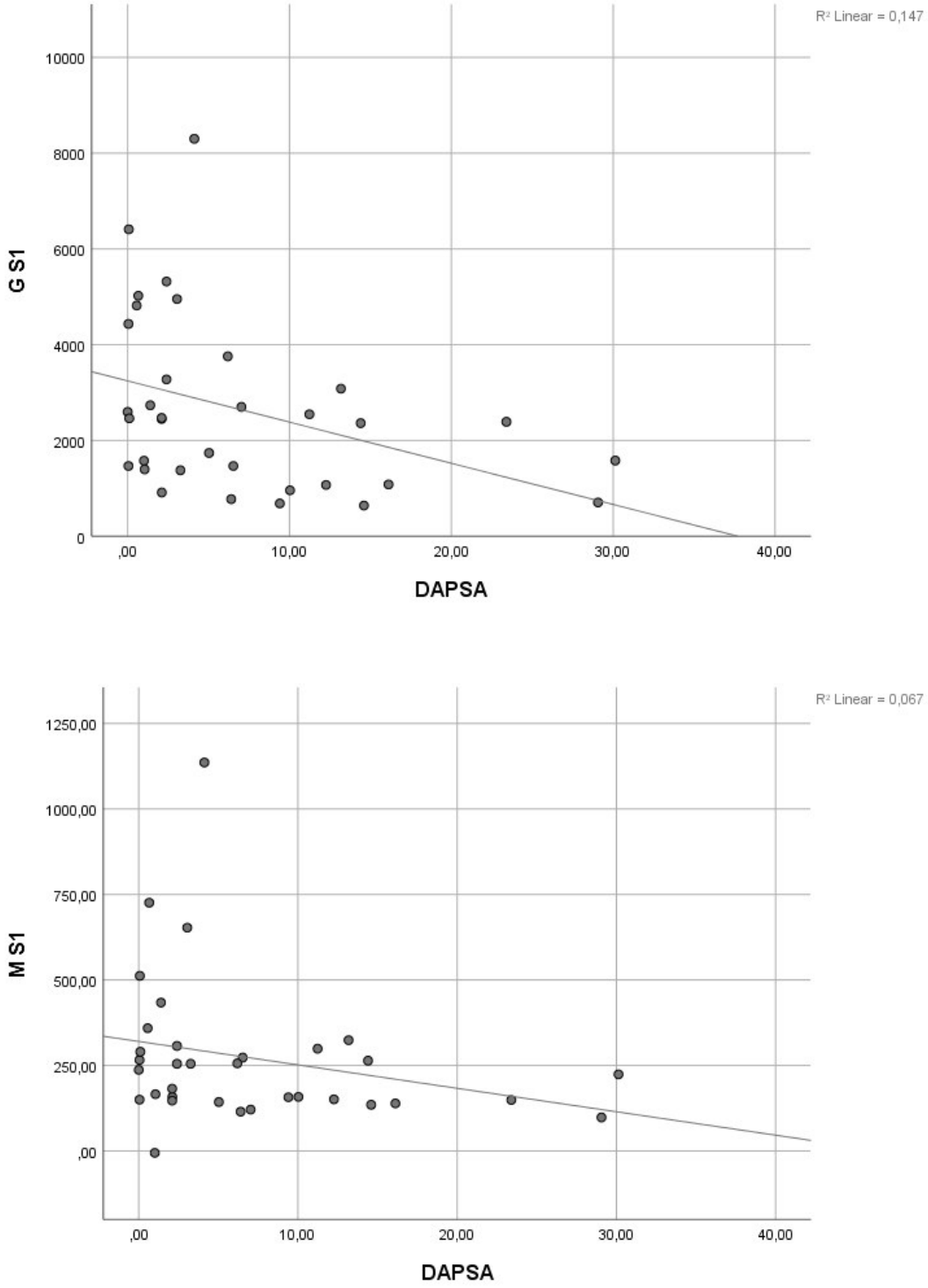


Figure 9: Scatterplots of the significant negative correlations between pSTAT1 levels (gMFI) in Granulocytes & Monocytes and the DAPSA score. G S1, Granulocyte pSTAT1; M S1, Monocytes pSTAT1

4.6. Correlation between pSTAT levels and disease duration

To test whether pSTAT levels correlate with disease duration, Spearman's correlation coefficient ρ (rho) was calculated.

Significant negative correlations were found between disease duration and pSTAT6 levels in Monocytes and B cells (ρ (rho) = -0.37, p = 0.017 and ρ (rho) = - 0.43, p = 0.002). Furthermore, we found significant negative correlations between disease duration and pSTAT5 levels in memory CD4- T cells and B cells (ρ (rho) = - 0.29, p = 0.050 and ρ (rho) = - 0.39, p = 0.005). Additionally, pSTAT4 levels in Monocytes showed a negative correlation with disease duration (ρ (rho) =- 0.30, p = 0.036).

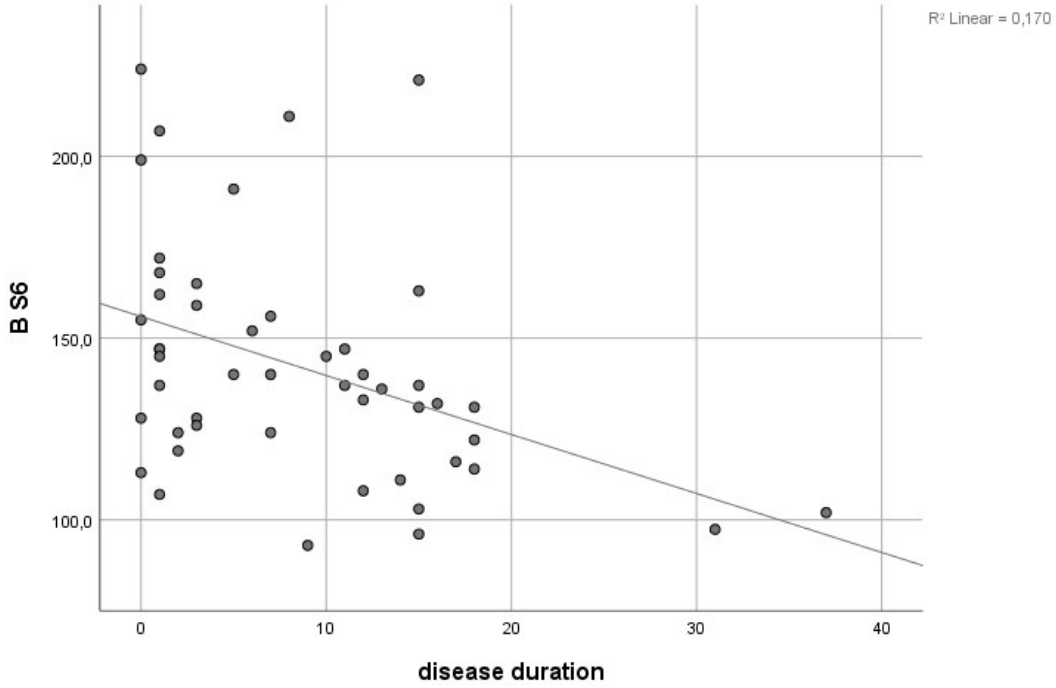


Figure 10: Scatterplot of significant negative correlation between pSTAT6 levels (gMFI) in B cells and disease duration. B, B cell; S6, pSTAT6

4.7. Gender- related differences between pSTAT5 levels in T cells

To test whether female patients have different pSTAT5 levels in T cells than male patients, we used a non- parametrical test that showed significant gender-related differences for pSTAT5 in CD4+ naïve & memory cells and for CD4- naïve cells. Female patients had significantly higher pSTAT5 levels in several T cell subsets as shown in table 10.

	MCD4+	*NCD4+*	*NCD4-*
female	234.0 (60.0)	209.0 (40.0)	183.0 (61.0)
male	165.0 (67.0)	150.0 (58.0)	150.0 (40.0)
U	68.5	61.0	65.5
Z	-3.357	-3.569	-2.299
p- value	0.001	0.000	0.022
r (effect size)	-0.52	-0.56	-0.36

Table 10: Significant differences in pSTAT5 levels between genders [Median gMFI and IQR].

4.8. Correlation of pSTAT5 in T cells and DAPSA in female patients

In the next step, Spearman's correlation coefficient ρ (rho) was calculated to test whether there is a correlation between pSTAT5 in T cells and the DAPSA score in female patients. The results showed no significant correlation between the DAPSA score and pSTAT5 levels in the three T cell subsets that were found to be different between genders.

4.9. PCA & Cluster analysis

To find patients with similarities in STAT phosphorylation, a principal component analysis (PCA) was performed to find the STATs that contribute most to variability in the data set. The idea of performing a PCA before a cluster analysis arose from the consideration that unsupervised dimension reduction is closely related to unsupervised learning techniques like clustering (153). The decisions for the inclusion of variables were made based on achieving a maximum value on the Kaiser- Meyer- Olkin measure of sampling adequacy, which was 0.75 and therefore well above the minimum of 0.5 (154). Included variables are shown in the SPSS output that is depicted in table 11.

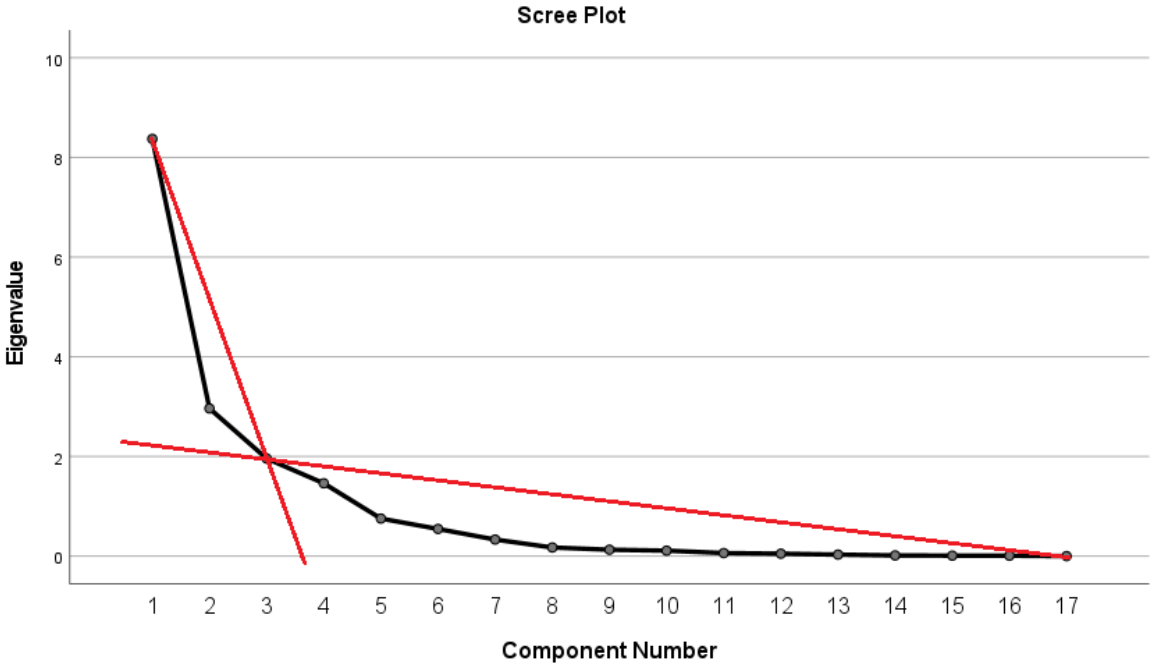


Figure 11: Scree plot of the conducted PCA. Red lines represent the “elbow”, which is used to decide between the number of components.

The decision for a 2- component solution was made based on the elbow method on the scree plot, shown in figure 11, although the Kaiser’s rule to simply retain components whose eigenvalues are greater than 1 suggested a 4- component solution. However, since the 3rd and 4th component equated only for a small portion of variance, this suggestion was rejected.

Pattern Matrix[a]		
	Component	
	1	2
NCD4+ S3	,914	
MCD4+ S3	,890	
MCD4- S3	,877	
B S3	,866	
B S4	,807	
NCD4+ S4	,775	
M S3	,756	
G S3	,687	
M S4	,636	,438
G S4	,581	,314
G S5		
MCD4- S5		,924
MCD4+ S5		,906
NCD4+ S5		,897
B S5		,826
MCD4+ S4	,459	,587
MCD4- S4	,509	,557

Extraction Method: Principal Component Analysis

Rotation Method: Oblimin with Kaiser Normalization.[a]

a. Rotation converged in 5 iterations

Table 11: Pattern Matrix of the Principal Component Analysis from SPSS®.

The results showed a high loading of pSTAT5 on component 2, while especially pSTAT3 in T cells, B cells, Granulocytes & Monocytes was high on component 1.

Following the PCA, the hierarchical clustering analysis was conducted. For variable selection, the variables of the PCA that showed a high component loading and therefore proved to account the most for variability in the data set were chosen. An arbitrary cut- off was set at 0.8 component loading and all variables above 0.8 were included in the hierarchical cluster analysis.

Consequently, pSTAT3 in naïve & memory CD4+ T cells, memory CD4- T cells and B cells, pSTAT4 in B cells, pSTAT5 in B cells, memory and naïve CD4+ and memory CD4- T cells were incorporated. We chose Ward's method with the squared Euclidean distance. Z- standardized gMFI values were used in the calculation.
This time, we decided to set the cutoff at a 4- cluster solution based on the scree plot and the consideration that this approach might provide more information about potential subgroups.

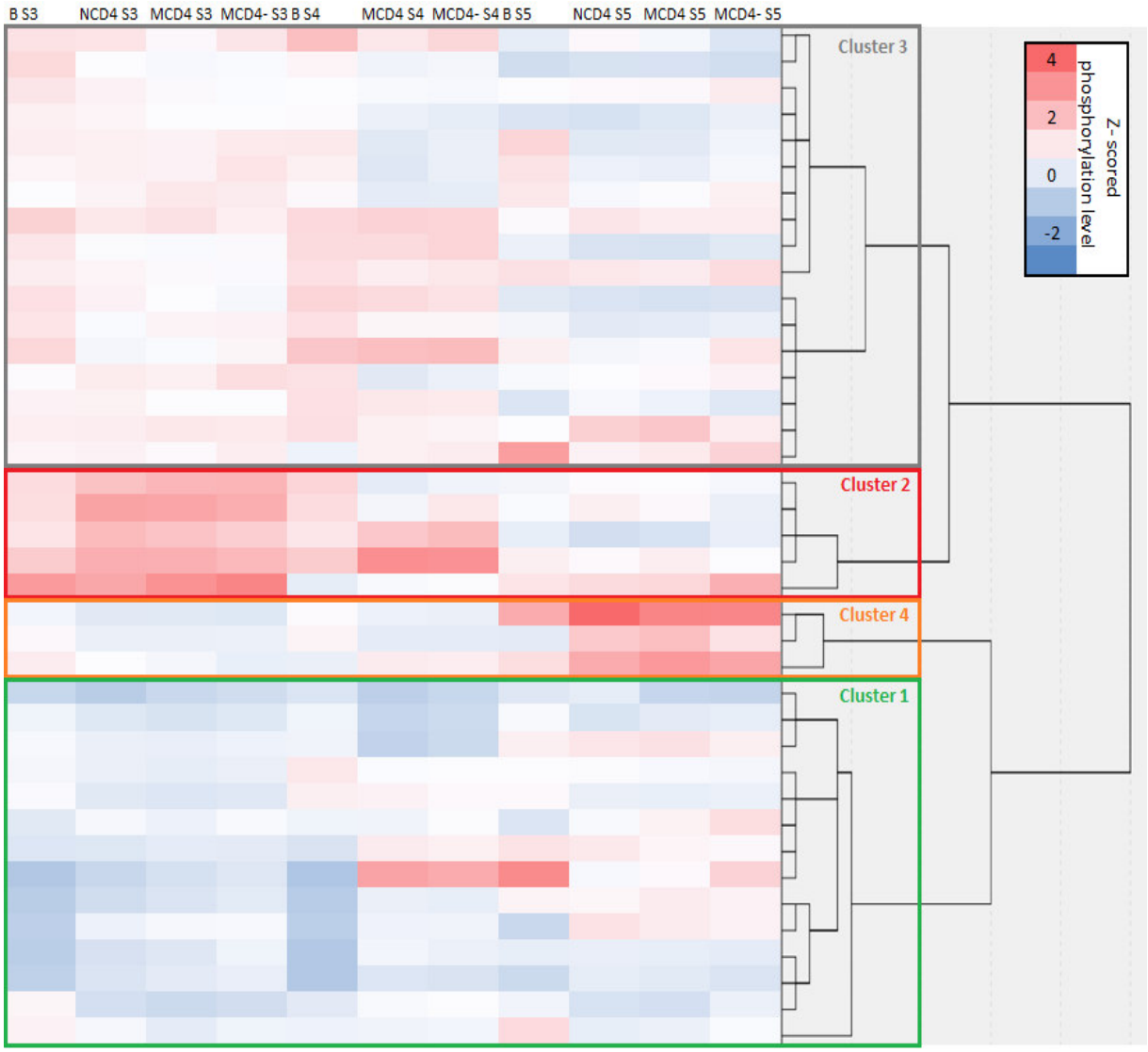


Figure 12: Heatmap & dendrogram of the cluster analysis.

		Cluster 1	Cluster 2	Cluster 3	Cluster 4	p-value*
pSTAT1	T cells	22.8 [38.6]	155.0 [169.0]	46.5 [15.7]	46.7	0.009
pSTAT3	Mono	271.0 [170]	580.0 [174]	469.0 [324.0]	335.0	0.037
	B cells	62.2 [100.5]	166.0 [77.5]	138.0 [28.5]	121.0	0.000
	MCD4+	154.5 [30]	346.0 [62]	212.0 [22]	170.0	0.000
	MCD4-	141.5 [28.3]	299.0 [71.0]	196.0 [31]	146.0	0.000
	NCD4+	183.0 [57.3]	440.0 [59]	281.0 [22]	212.0	0.000
	NCD4-	176.0 [91]	444.0 [94]	291.5 [25]	228.0	0.000
pSTAT4	Gran	404.0 [106]	509.0 [74]	467.0 [102]	395.0	0.025
	Mono	345.5 [98]	469.0 [156]	414.0 [111]	359.0	0.015
	B cells	155.5 [715]	919.0 [529.5]	861.0 [335.0]	551.0	0.000
	NCD4+	152.0 [307.1]	409.0 [320]	362.0 [119]	246.0	0.001
	NCD4-	170.0 [1028]	936.0 [620.5]	688.0 [238]	496.0	0.011
pSTAT5	MCD4+	181.5 [72]	212.0 [83.5]	188.0 [73]	389.0	0.032
	NCD4+	162.5 [60]	193.0 [80]	174.0 [72]	335.0	0.031

Table 12: Median gMFI and IQR of pSTATs that differed significantly between clusters. *p- value of comparison between all groups in the Kruskal-Wallis test; Mono, Monocytes; MCD4+, memory CD4+ T cells; MCD4-, memory CD4- T cells; NCD4+, naïve CD4+ T cells; NCD4-, naïve CD4- T cells; Gran, Granulocytes

		Cluster 1	Cluster 2	Cluster 3	Cluster 4
No. of patients		14	5	17	3
Age*		52.00 [21]	57.00 [22]	56.00 [13]	65
Group	REM/ LDA	14 (100%)	3 (60%)	11 (64.7%)	3 (100%)
	MoDA/ HDA	0	2 (40%)	6 (35.3%)	0
Sex	Male	8 (57.1%)	2 (40%)	11 (64.7%)	1 (33.3%)
	Female	6 (42.9%)	3 (60%)	6 (35.3%)	2 (66.6%)
Med.	Cortisol	0	1 (20%)	1 (5.9%)	0
	MTX	2 (14.3%)	1 (20%)	10 (58.8 %)	1 (33.3%)
	Sulfasalazine	0	1 (20%)	0	0
	JAK-i	1 (7.1%)	0	1 (5.9%)	0
	TNFα-i	9 (64.3%)	3 (60%)	3 (17.6%)	0
	IL-17A-i	3 (21.4%)	1 (20%)	4 (23.5%)	1 (33.3%)
	PDE-i	0	0	2 (11.8%)	1 (33.3%)
No drug therapy		0	1 (20%)	2 (11.8%)	0
Monotherapy		13 (92.9%)	1 (20%)	10 (58.8%)	3 (100%)
Combination therapy		1 (7.1%)	3 (60%)	5 (29.4%)	0
DAPSA*		2.10 [2.96]	9.11 [23.88]	11.23 [11.67]	1.01
CRP*		0.85 [3.6]	6.60 [6.4]	1.80 [3.1]	1.00
Disease duration*		9.00 [13]	1.00 [13]	7.00 [14]	8.00
Psoriasis		8 (57.1%)	2 (40%)	11 (64.7%)	1 (33%)
Hyperuricemia		1 (7.1%)	0	6 (35.3%)	0
Art. Hypertension		3 (21.4%)	1 (20%)	2 (11.8%)	0
Osteoarthritis of the knee		3 (21.4%)	1 (20%)	3 (17.6%)	2 (66.7%)

Table 13: Clinical characteristics of patients in each cluster. JAK-i, Jak inhibitor; TNFα-i, TNFα inhibitor; IL-17A-i, IL-17A inhibitor; PDE-i, phosphodiesterase 4 inhibitor

*median [IQR]

The Kruskal- Wallis- Test was performed to detect differences between the 4 clusters regarding several variables and to describe the characteristics of the different clusters.

DAPSA scores were significantly different between groups (H(3)= 10.39, p= 0.016). Pairwise comparisons with adjusted p- values showed that there were significant differences between cluster 1 & 3 (p= 0.03, r= 0.50). No significant differences were observed in the other pairwise comparisons for the DAPSA score, although it could be observed that cluster 1 & 4 and cluster 3 & 4 have similarities.

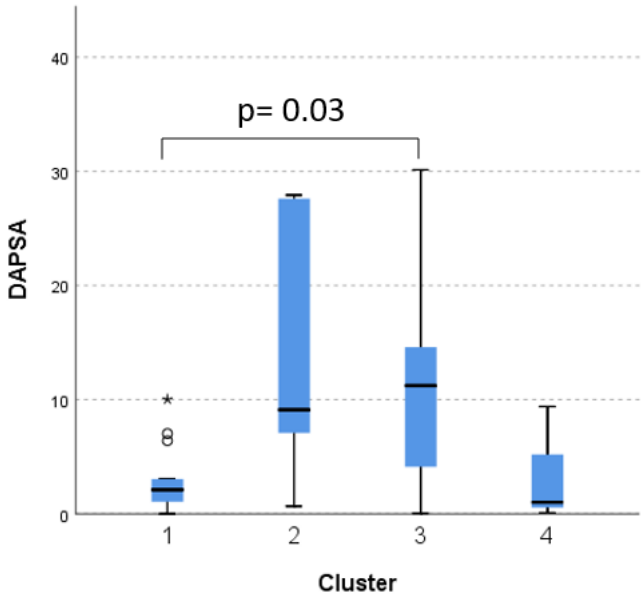


Figure 13: Comparison of DAPSA scores between the clusters. Significant differences were found between clusters 1 & 3.

CRP levels showed significant differences in the Kruskal-Wallis test (H(3) = 9.39, p = 0.024). Significant differences in pairwise comparisons were found between Cluster 1 & 2 (p = 0.02, r = 0.67), but not between the other clusters.

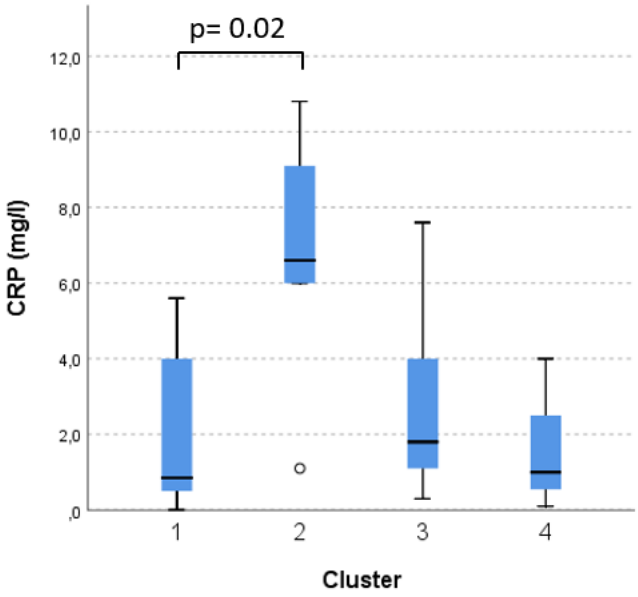


Figure 14: Comparison of CRP levels between clusters. Significant differences were found between clusters 1 & 2.

Age and disease duration did not differ significantly, although the median age was the lowest in Cluster 1 and the highest in Cluster 4. Disease duration was the lowest in Cluster 2. Cell frequencies didn't differ significantly, except for CD19+ cells (H(3)= 10.84, p= 0.013) and pairwise comparison showed significant differences between cluster 1 & 3 (p= 0.027, r= 0.51). Overall, clusters 1 & 4 showed the highest CD19+ cell frequency.

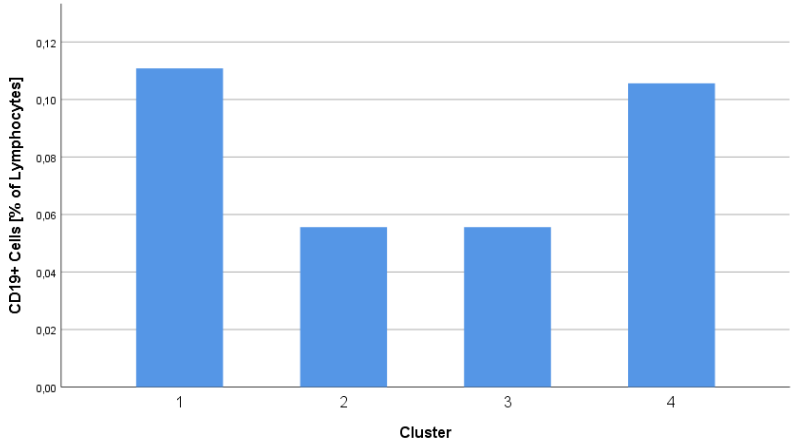


Figure 15: Significant differences in the frequency of CD19+ cells between clusters.

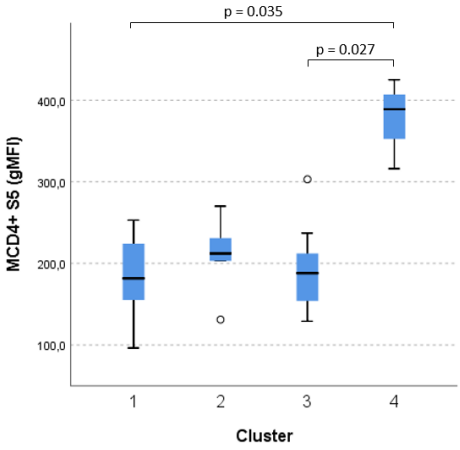
Moreover, there were differences in the drug therapy regimens. Clusters 1 & 4 had a significant higher percentage of patients on monotherapy (p= 0.008 on the fisher's exact test). Clusters 2 & 3 had a higher percentage of patients on a combination therapy with different drugs, although not significantly.

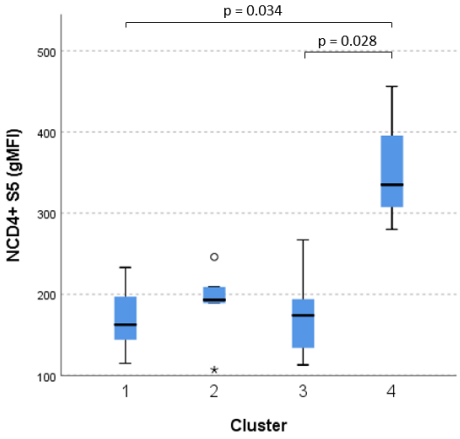
Cluster 1 consisted of 14 patients that were all in the REM/ LDA group, with approximately even distribution of gender. This cluster was characterized by a low disease activity and long disease duration. This group, together with cluster 2, had a significantly higher percentage of patients on TNFα- inhibitor therapy (p= 0.017 on fisher's exact test). The overall pSTAT levels were decreased, although Cluster 1 had the highest pSTAT1 levels in Granulocytes and pSTAT6 levels in Granulocytes & Monocytes. However, these differences were not significant. (Data not shown)

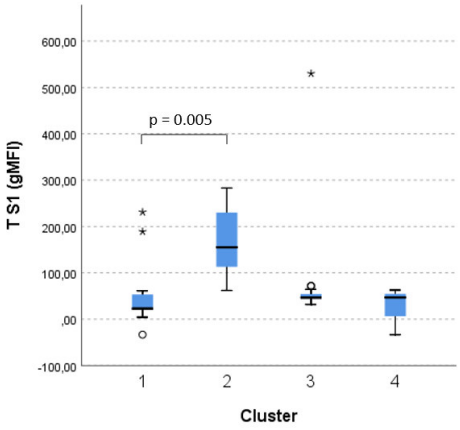
Cluster 2 consisted of 5 patients of whom 2 were in the MoDA/ HDA group. This cluster emerged due to very high phosphorylation levels in several STATs, especially pSTAT1 in T cells, pSTAT4 in Granulocytes, Monocytes, B cells and naïve CD4+ & CD4- T cells. Furthermore, high levels of pSTAT3 in Monocytes, B cells, naïve & memory CD4+ as well as CD4- T cells were observed. This cluster had the second highest DAPSA scores and the CRP was much higher compared to all other groups. The two patients that were in the MoDA/ HDA group had a combination therapy, with a TNFα inhibitor & Cortisone or IL-17A inhibitor and Sulfasalazine. Two patients in the REM/ LDA group had a TNFα inhibitor, one in combination with Methotrexate, while the 3rd patient had no drug therapy.

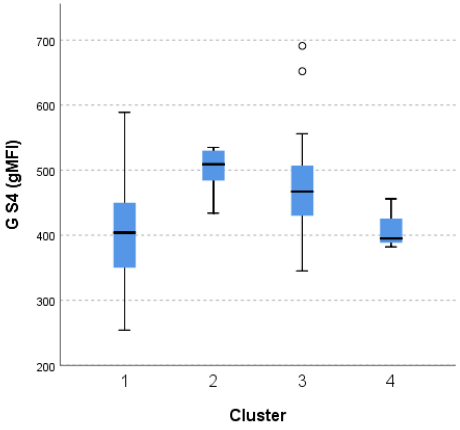
Cluster 3 consisted of 17 patients, of whom 11 were in the REM/ LDA group and the other 6 were in the MoDA/ HDA group. This cluster had almost 2 times as much male as female patients. Cluster 3, like Cluster 2, had high DAPSA scores, but lower CRP levels. It also had the highest percentage of patients on MTX therapy and the second highest percentage of patients with combination drug therapy. On the molecular and partially clinical level, Cluster 3 was most like cluster 2, although overall expression levels of pSTATs were lower.

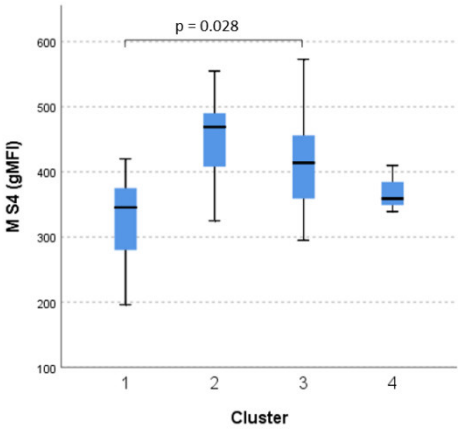
Cluster 4 consisted of 3 patients, which were all in the REM/ LDA group. Interestingly, pSTAT5 levels in naïve and memory CD4+ T cells were the highest among this cluster. All patients were on a different monotherapy drug regimen.

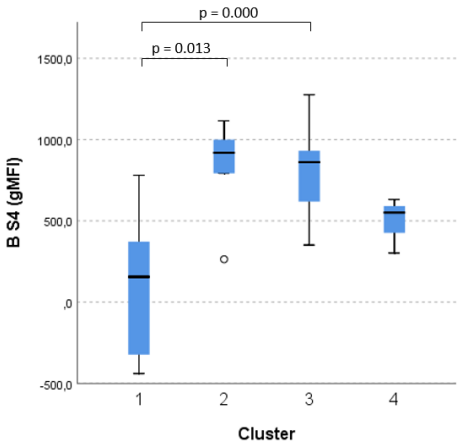












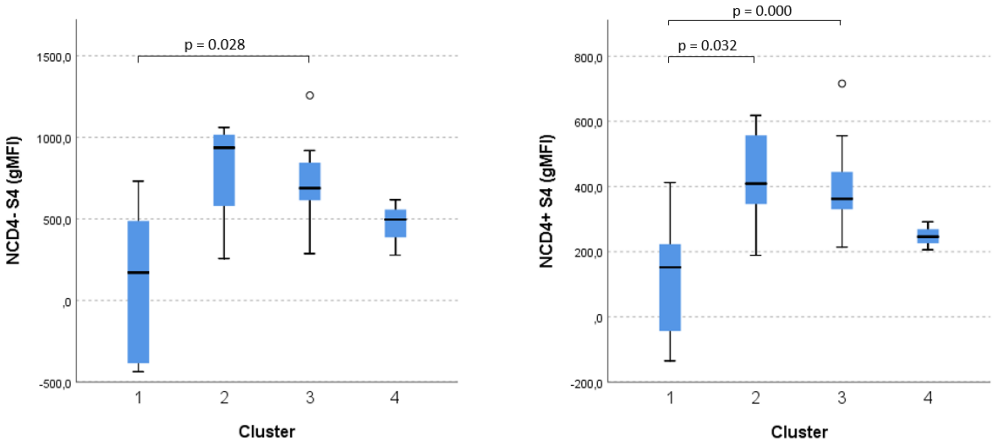


Figure 16: Significant differences in pSTAT1/ 4/ 5 between clusters. NCD4+, naïve CD4+ T cells; MCD4+, memory CD4+ T cells; T, T cells; G, Granulocytes; B, B cells; M, Monocytes; NCD4-, naïve CD4- T cells; S1, pSTAT1 (gMFI); S4, pSTAT4 (gMFI); S5, pSTAT5 (gMFI)

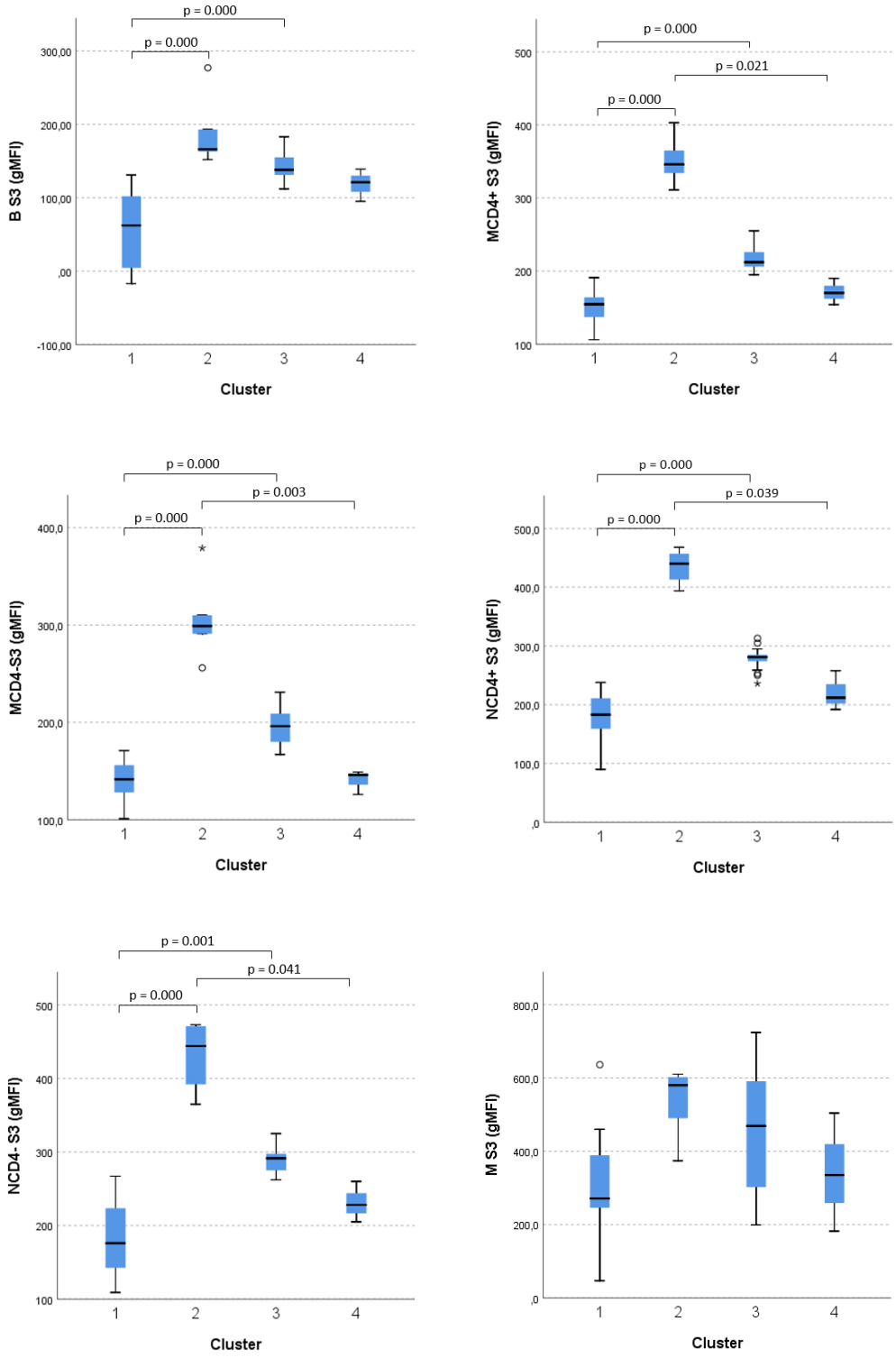


Figure 17: Significant differences in pSTAT3 between clusters. B, B cells; MCD4+/ NCD4+, memory/ naive CD4+ T cells; MCD4-/ NCD4- , memory / naïve CD4- T cells; M, Monocytes; S3, pSTAT3 (gMFI)

5. Discussion

Finding biomarkers for stratified therapy is of upmost importance in patients with rheumatic diseases since therapeutic success is often achieved based on trial-and- error principle. This is mostly because we lack guidance in which treatment to choose for a given patient. More easily accessible biomarkers like cytokines haven't shown to be sufficient to serve this purpose. Therefore, investigation of the downstream intracellular pathways might be a more promising approach.

In this work we describe the phosphorylation levels of pSTAT molecules in different immune cells of patients with established PsA. We found several differences between patients with moderate/ high disease activity and remission / low disease activity.

We found significantly higher pSTAT3 levels in CD4+ & CD4- T cells of patients in the MoDA/ HDA group and found a significant positive correlation of pSTAT3 in CD4+ T cells and disease activity. Given the results of our study, we conclude that higher pSTAT3 levels in several T cell subsets are associated with more severe disease. This observation could be explained due to increased activation of the IL-23/ IL-17 axis on the one hand with involvement of CD4+ TH17 Cells and due to increased activation of CD4- T Cells, especially CD8+ T Cells that are a major contributor to pathology in PsA as described above.

A limitation of our study is the lack of a healthy control group, since it was designed for intragroup comparisons of PsA patients. We can't therefore tell whether high pSTAT3 levels in T cells are a hallmark of PsA or immune mediated diseases in general, although it is well known that constitutive hyperexpression of pSTAT3 in CD4+ T cells under certain circumstances is sufficient to promote psoriasis- like arthritis and enthesopathy in a mouse model (121).

Moreover, positive correlations between pSTAT3 in immune cells and disease activity in PsA has been observed by other groups (96).

Phosphorylated STAT1 on the other hand, which plays a prominent role in IFN signaling, shows a negative correlation with DAPSA in Granulocytes and Monocytes. A similar observation has been made for patients with RA, where high disease activity correlated with lower pSTAT1 levels following IL-10 stimulation in

several cell populations, although not specifically monocytes or granulocytes. However, in this particular study, the RA patients showed significantly lower pSTAT1 levels following IFNα stimulation, with the lowest being in monocytes (155).

An explanation for this observation might be that, during active disease, certain cytokines are highly increased in comparison to others. Therefore, the highly abundant cytokines activate their respective STAT molecules excessively in the downstream pathway, leading to high phosphorylation levels of those STATs, while other cytokines and their STATs are downregulated, probably below their baseline levels, due to compensatory shift of protein synthesis and consequently cytokine and STAT abundancy.

Furthermore, an upregulation of STAT1 in exhausted monocytes has been observed in sepsis models (156). However, in our study cohort the pSTAT1 levels of Granulocytes & Monocytes did not correlate with disease duration, although exhaustion of cells doesn't necessarily have to interrelate with long term duration of disease but could instead be characterized by duration & intensity of disease flares.

STAT1- activating cytokines, like IFNγ, have the capability to suppress TH17 responses. Thus, the higher pSTAT1 levels, although in monocytes and granulocytes and not in T cells, might reflect an anti- inflammatory counter-regulation in the patients with low disease activity or remission (157).

The negative correlation between pSTAT6 and disease duration in Monocytes and B cells might reflect a negative feedback mechanism involved in JAK/ STAT signaling, mostly through upregulation of SOCS proteins that inhibit phosphorylation of JAKs and STATs as described above. This feedback mechanism could be upregulated long term through constant activation of the pathway. The same could apply for pSTAT5 in CD4- T cells, since pSTAT5 is involved in proliferation and survival of CD8+ T cells. Overall, this could reflect an exhaustion of the immune response in long standing disease that might be, at least partially, mediated by the negative feedback mechanisms involved in JAK/ STAT signaling.

The gender- related differences in pSTAT5 levels in several T cell subsets are hard to interpret, since no differences between females and males regarding prevalence, disease manifestation or severity in PsA have been observed yet and neither have been in this study. An explanation for the higher pSTAT5 in T cells might be the higher prolactin levels in females that lead to a higher baseline pSTAT5, since prolactin is able to act on T cells, for example by inhibiting the suppressive function of Treg cells (158-160).

The results of the cluster analysis are discussed in the following.

The conducted hierarchical cluster analysis allowed to decide mainly between 2 or 4 clusters. The decision for 4 clusters was made to account for the complexity and heterogenicity of this disease regarding the underlying pathophysiology. However, this led to clusters of uneven size and especially the conclusions drawn from the small clusters should be interpreted with caution.

The selection of variables for cluster analysis is a complicated subject, since the goal is to find variables that are “important” and to sort out variables that mainly add “noise” to the data, since even a few noisy variables can interfere with a clear cluster structure (161, 162).

In summary, we can state that we found 4 clusters, which could further be pooled together into 2 clusters based on disease activity. Cluster 4, with its high pSTAT5 levels in T cells, could be viewed as a subgroup within a low disease activity cluster that is characterized by increased anti- inflammatory counter regulation on the molecular level. On the other hand, cluster 2 could be regarded as a more aggressive subgroup of a high disease activity cluster, at least on the molecular level which is depicted by its higher CRP and higher pSTAT3 levels.

Clusters with higher disease activity (2 & 3) had a lower B cell frequency. However, this could partially be explained by the gating strategy, since CD19 was plotted against CD3. Consequently, the CD3 frequency was higher in these clusters, although not significantly. Since the disease process in PsA is driven more by T cells rather than B cells, in regard to the lymphocyte compartment, a higher T cell frequency would be well in line with this assumption.

No differences in cell frequencies have been observed between disease activity groups or gender. Clonal expansion of different cell subsets has been observed mostly in synovial fluid and not necessarily in peripheral blood of PsA patients (163). Additionally, only a limited number of cell subsets have been analyzed in this study.

It isn't much of a surprise that the two clusters with the highest disease activity scores were also the ones with the highest pSTAT3 levels, since pSTAT3 levels had a positive correlation with disease activity and was significantly higher in the MoDA/ HDA group as shown above.

The levels of pSTAT1 in T cells differed significantly between groups with the highest being in Cluster 2. As mentioned before, STAT1 in T cells has many, even contrary effects depending on the cell environment and the T cell subtype. Considering the high disease activity that is present in this cluster, the high pSTAT1 levels could be interpreted as a sign of maintenance of a pro-inflammatory state, since for example IFN- independent pSTAT1 upregulation can promote T cell survival by upregulation of MHC I, thereby protecting them from NK-mediated cytotoxicity. The investigators that discovered this mechanism proposed IL-7 as a potential cytokine, which has been shown to signal through STAT1. Since IL-7 is also considered to mainly signal through STAT3 and STAT5, the higher pSTAT3 levels in cluster 2 would support this hypothesis (86).

Moreover, the finding that pSTAT4 in Granulocytes, B cells, naïve CD4+ and CD4- T cells was significantly different between clusters and the highest among cluster 2 & 3 was interesting since pSTAT4 wasn't significantly associated with disease activity in the analyses that were conducted before. An explanation for this might be an increase in IL-23 signaling in the cellular environment of these patients, which is partially conducted by STAT4 and STAT3 (127).

Interestingly, cluster 4 had the lowest DAPSA score, but the highest pSTAT5 levels in naïve & memory CD4+ T cells. Since STAT5 is crucial for development of Treg cells, which can be found in the CD4+ repertoire, this could be a sign of increased induction of these anti- inflammatory T cells and therefore account for the lower clinical disease activity.

Cluster 1 had a significantly higher number of patients on TNFα inhibition. This could account for the lower pSTAT3 & pSTAT5 levels in several cell types of these patients, since TNFα, besides its canonical pathway, has been shown to conduct its signal by usage of these STATs (150, 164, 165) .

The observation that clusters with lower disease activity had more patients on drug monotherapy while clusters with higher disease activity had more patients on a combination of different drugs is interesting and could reflect the increased need for therapy escalation in these patients.

Although mechanistical reasoning seems to be able to explain most of the differences between these clusters, the conclusions drawn from these explanations need to be interpreted with caution. The redundancy of JAK/ STAT signaling in almost every cytokine pathway can lead to contrary conclusions when a mechanistical approach is used. Moreover, the JAK/ STAT pathway is more complicated than it seems at first sight. The role and implication of phosphorylated STATs has been well studied, but the role of unphosphorylated STATs, epigenetic modifications, priming and crosstalk to other pathways, just to name a few, are additional factors that are generally not included. The reason is that it seems nearly impossible to do so because of the complexity and knowledge gaps we still have about these subjects. Additionally, to gather a more inclusive overview of the pathophysiological process it is necessary to include cytokine measurements & mRNA expression levels of target genes of the investigated STATs.

To investigate whether phosphorylation levels or patterns can be useful in therapy selection, follow- up investigations are necessary.

6. Limitations of the study

The first limitation of our study is the number of patients enrolled. Due to randomly missing data points this number has been further reduced for several analyses that were conducted. Another limitation is the lack of a healthy control group, although the study has been designed for intragroup comparison of PsA patients.

A further limitation of the study lies in the cell types that have been investigated due to the limited number fluorochromes that can be used at once with the FACS technology. For example, it would have been interesting to investigate pSTAT levels in TH17 cells, a cell type that is well known for its role in pathogenesis of PsA as described above.

Moreover, the number of patients was small considering the clustering approach. The cluster analysis resulted in the choice between a 2- or a 4- cluster solution. In general, it is common practice to choose a solution with nearly even distribution of clusters, although this approach wouldn't had given us much more information about the cohort. Therefore, the 4- cluster approach seemed to be appropriate, although the lower number of patients reduced the power of the results. A larger number of patients would be necessary to confirm the results of the analysis on the one hand and to draw serious conclusions from it on the other hand. A large proportion of clustering algorithms generate clusters even if the underlying data has no actual, inherent cluster structure. That is why validation tools are usually required to confirm the results, which is not applicated in this work (162).

As stated above, cytokine levels and mRNA expression analysis are needed to depict a more complete picture of the processes involved in the JAK/STAT pathway on the one hand and to confirm the accuracy of the pSTAT measurements on the other hand. Both techniques are planned to be used in the related study.

Furthermore, the fact that this analysis was conducted in peripheral blood and not in synovial fluid might be a limitation, since most studies about immunopathology of PsA have been conducted at the site of inflammation and therefore, observations & mechanistical conclusions from other studies in STAT – signaling of the immune cells in the joint can't be fully applied to peripheral immune cells.

7. Conclusion

In conclusion, in this work we describe differences in phosphorylation levels of STAT molecules of different leukocytes populations in the peripheral blood of PsA patients. We show that pSTAT levels differ across patients with different disease activity and that patients can be stratified into clusters according to their phosphorylation profile. This might provide an important foundation for future analyses. In the next steps, cluster stability over a longer period and correlation with treatment response could be investigated to find out if the phosphorylation of STATs could serve as potential biomarker that helps in the guidance of treatment.

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