Diplomarbeit

Analysis of the JAK-STAT signaling pathway in leucocytes

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Graz, am 13.12.2019

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Graz, am 13.12.2019 *Nicholas Himmer eh*

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Glossary and abbreviations

ACCP-antibodies *(anti cyclic citrullinated peptides antibodies)*

ACPA *(anti-citrullinated protein antibodies)*

ACR *(American College of Rheumatology)*

bDMARD *(biological DMARD)*

boDMARDs *(biological originator DMARDs)*

bsDMARDs *(biosimilar DMARDs)*

CD *(cluster of differentiation)*

cDAI *(Mean clinical disease activity index)*

CMC *(carpometacarpal)* ***joints***

CNTF *(ciliary neurotrophic factor)*

CRP *(C-reactive protein)*

csDMARD *(conventional synthetic DMARD)*

CT *(computed tomography)*

CT-1 *(cardiotropin-1)*

DAS28 *(Mean disease activity score 28)*

DC *(dendritic cell)*

DIP *(distal interphalangeal)* ***joints***

DMARD (disease-modifying antirheumatic drug)

- ***sDMARD*** *(Synthetic DMARDs)*
    - ***csDMARD*** *(Conventional synthetic DMARDs)*
    - ***tsDMARD*** *(Targeted synthetic DMARDs)*
- ***bDMARDs*** *(Biological DMARDs)*
    - ***boDMARDs*** *(Biological originator DMARDs)*
    - ***bsDMARDs*** *(Biosimilar DMARDs)*

DRB1 *(DR beta chain 1)*

EDTA *(Ethylenediaminetetraacetic acid)*

EPO *(erythropoietin)*

EULAR *(European league against rheumatism)*

FSC-A *(forward scatter parameter)*

GH *(growth hormone)*

GM-CSF *(granulocyte macrophage colony-stimulating factor)*

HLA *(human leucocyte antigen)*

IFN *(interferon)*

IgA/IgE/IgG/IgM *(Immune globulin A/E/G/M)*

IL *(interleukin)*

JAKs *(Janus kinases)*

LIF *(leukaemia inhibitory factor)*

MAPK pathway *(mitogen-activated protein kinase pathway)*

MCP *(metacarpophalangeal)* ***joint***

MHC *(major histocompatibility complex)*

MRI *(Magnetic resonance imaging)*

mRNA *(messenger ribonucleic acid)*

MTP *(metatarsophalangeal)* ***joint***

MTX *(methotrexate)*

NNT-1/BSF-3 *(novel neurotrophin-1/B cell-stimulating factor-3)*

NSAIDs *(nonsteroidal anti-inflammatory drugs)*

NK-cells *(natural killer cells)*

OSM *(Oncostatin M)*

PIP *(proximal interphalangeal)* ***joints***

pSTAT *(phosphorylated STAT)*

PRL *(prolactin)*

RA *(rheumatoid arthritis)*

RF *(rhesus factor)*

SAPK pathway *(stress-activated protein kinase pathway)*

SE *(shared epitope)*

SSC-A *(side scatter parameter)*

STATs *(Signal transducers and activators of transcription)*

TF *(transcription factor)*

Th cells *(T helper cells)*

TNF *(tumor necrosis factor)*

TNFi *(tumor-necrosis factor inhibitor)*

TPO *(thrombopoietin)*

tsDMARD *(targeted synthetic DMARD)*

TYK *(tyrosine kinase)*

US *(ultrasound)*

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1 Zusammenfassung

1.1 Einleitung

Rheumatoide Arthritis (RA) bezeichnet eine chronisch-entzündliche Erkrankung und die häufigste rheumatische Autoimmunkrankheit. Bei diesem klinischen Syndrom führend verschiedene Arten von Entzündungskaskaden zu einer gemeinsamen Erkrankung mit anhaltender synovialer Entzündung und Schädigung von Gelenksknorpeln sowie anliegender Knochenstrukturen. Als Hauptsymptom zeigt sich eine fortschreitende Polyarthritis, Nebensymptome können unterschiedlich ausfallen, abhängig von Lage und Stadium der RA. Unbehandelt führt das Fortschreiten der Erkrankung zu schweren Gelenksschäden mit körperlicher Beeinträchtigung sowie zu systemischer Manifestation der Erkrankung.
In der Pathogenese der RA spielen Zytokine und der JAK-STAT Signalweg eine wichtige Rolle. Daher war das Ziel dieser Arbeit, ein Laborprotokoll sowie weiterführendes Prozedere zu etablieren, um die Phosphorylierung von STAT-Molekülen in Leukozyten zu analysieren. Dabei wollten wir potenzielle Unterschiede zwischen Patientinnen und Patienten mit RA und gesunden Personen identifizieren. Außerdem wurde das Kollektiv der Patientinnen und Patienten auf das Vorhandensein bestimmter Endotypen untersucht.

1.2 Material und Methoden

In einer prospektiven Studie wurde peripheres Blut von 6 gesunden Personen und 17 Personen mit RA analysiert. Die Phosphorylierung von STAT-Molekülen in Leukozyten wurde anhand der Messung ihrer MFI (mittleren Fluoreszenzintensität) untersucht. Mittels Durchflusszytometrie wurde sowohl Zytokin-stimuliertes Blut als auch unstimuliertes Blut ausgewertet. Der MFI von Zelluntergruppen innerhalb der Leukozytenpopulation wurde mittels hierarchischem Clustering verglichen und ursächliche Parameter für signifikante Unterschiede identifiziert. Ergebnisse wurden anschließend mit den Zytokinwerten der jeweiligen Personen verglichen.

1.3 Resultate und Diskussion

Die Phosphorylierung von STAT3-Molekülen unterschiedlichen Zelllinien innerhalb des RA-Kollektivs im Vergleich zum gesunden Kollektiv zeigte signifikante Erhöhungen, bei einer geringeren Anzahl von Zelluntergruppen war außerdem die Phosphorylierung von STAT4 und STAT5 signifikant erhöht. Eine statistisch signifikante Unterscheidung zwischen krankem und gesundem Kollektiv anhand der gesamten STAT-Phosphorylierung war allerdings nicht möglich, das gesamte Kollektiv konnte aber durch unterschiedlich starke Ausprägung der STAT-Phosphorylierung in Untergruppen eingeteilt werden. Diese Ergebnisse zeigen zwar

eine unterschiedliche Ausprägung der STAT-Expression im Kollektiv, Endotypen innerhalb des RA-Kollektivs konnte aber nicht signifikant nachgewiesen werden.
Diese Resultate könnten von weiterem Interesse für das Verständnis der Pathogenese der RA sowie ihrer Behandlung. Da unser untersuchtes Kollektiv im Vergleich zur Anzahl der analysierten Variablen sehr klein war, wäre die Weiterführung des Laborprotokolls und damit eine Vergrößerung des Kollektivs sinnvoll.

2 Abstract

2.1 Introduction

Rheumatoid arthritis (RA), is a chronic inflammatory disorder and the most common autoimmune-rheumatic disease. As a clinical syndrome, several types of inflammatory cascades lead to a common pathway with persistent synovial inflammation and defect to articular cartilage as well as underlying bone. Main clinical symptom is a progredient polyarthritis, other symptoms may differ, depending on location and stage of RA. Untreated, disease progression leads to joint destruction, disability and systemic manifestation.
While the pathogenesis of RA is not jet fully understood, cytokines and the JAK-STAT signaling pathway play an important role in RA. The intention of the study was, to establish a laboratory protocol and procedure in order to analyse the phosphorylation of STAT-molecules in leucocytes. Therein, we aimed to identify potential variations between patients with rheumatoid arthritis and healthy subjects. We also planned to analyse whether certain endotypes exist within the subjects collective.

2.2 Material and Methods:

In a prospective study peripheral blood from 17 patients with RA and 6 healthy controls was analysed. Phosphorylation of STAT-proteins was examined via measuring the mean fluorescent intensity (MFI) in leucocyte populations with flow cytometry. Subjects blood was analysed both unstimulated and stimulated with cytokines prior to flow cytometric analysis. We compared the MFI of lymphocyte subsets with hierarchical clustering and identified parameters responsible for clustering. Additionally, findings were compared to subject's cytokine levels.

2.3 Results and Discussion

We found significantly increased phosphorylation of STAT3 in different cell types in RA subjects, compared to healthy subjects. Increase also concerned phosphorylation of STAT4 and STAT5 to a lesser extent. Overall STAT-phosphorylation did not allow for a clear separation of healthy subjects and subjects with RA in hierarchical cluster analysis.
Yet, distinct distribution in the expression of STAT-phosphorylation allowed for the overall classification of all subjects into subgroups. While these findings show a heterogenous expression of STAT-molecules, endotypes within the RA-collective were not found significantly. Still, results might be of interest in the understanding of RAs and its treatment. As our subjects' collective was relatively small compared to the number of analysed variables, further expansion of the collective is reasonable.

3 Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease which is common to affect joints, where it leads to synovialitis, arthritis, bursitis and tendovaginitis ultimately resulting in joint damage and disability. Although there is not yet a cure, patients may reach a temporarily state of remission, which, with optimal early treatment, is an achievable goal. That is why it is of grave importance to diagnose RA early and correctly in order to ensure optimal treatment. [1,2,3,4,5]

While cytokines play a crucial part in the pathogenesis of RA, a direct measurement of cytokines is quite difficult, due to their local distribution and limited half-live. Cytokines induce the activation of the JAK-STAT signaling pathway, which seems to play an important role in the pathogenesis of RA, further resulting in the phosphorylation of STAT-molecules, leading to gen-transcriptions inside the nucleus. [6,7,8]

Therefore, we assumed that the phosphorylation of STAT-molecules could provide better information on the distribution of cytokines, which would ease the process of differentiation between healthy patients and patients with rheumatoid arthritis. We also assumed that there would be different subgroups – so called endotypes – within patients with rheumatoid arthritis.

The characterisation of such endotypes could be of great benefit to making prognostic statements according medical treatment and its effectiveness.

That is why the leading question of this thesis was defined as follows:

“Is there a difference between patients with rheumatoid arthritis and healthy patients regarding the phosphorylation of STAT-molecules in leucocytes? Are there subgroups within RA (so-called endotypes)?”

In order to answer this question, we examined the phosphorylation of STAT molecules in leucocytic populations of healthy subjects as well as those of subjects with rheumatoid arthritis.

3.1 Rheumatoid arthritis

Rheumatoid arthritis (RA), also called chronic polyarthritis, is an autoimmune disorder that leads to chronic inflammation of the synovial membrane in joints, tendon sheaths and bursae. Untreated, the progression of the disease leads to joint destruction and disability.[1(p792),2(p660)]

3.1.1 Epidemiology and Aetiology

RA concerns between 0,5 and 1% of the population under the age of 55, patients older than 55 have an incidence of about 2% of being affected by RA, with a peak between the age of 55 and 75. Women are 2-4 times more likely to be affected than men. Therefore, RA is the most common inflammatory-rheumatic disease. [1(p792),2(p660) 4]

3.1.2 Pathogenesis

The pathogenesis of the RA is quite intricate and seems to be multifactorial, which makes it even more difficult to understand. A way to understand its pathogenesis, is to see RA as a clinical syndrome with several subsets. Each subset results in its own inflammatory cascade which leads to one common pathway with persistent synovial inflammation and defect to articular cartilage as well as underlying bone.[3(p1094)]

Yet unknown triggers (viral and/or bacterial antigens) may induce an autoimmune disease with infiltration of the synovial membrane with autoreactive T-helper-cells, B-lymphocytes, plasma cells and dendritic cells. Those cells lead to a progressive inflammation of the synovial membrane which presents itself microscopically with an intensified vascularisation and hyperplastic processes regarding the synovial membrane. This inflammatory process results in the development of a hyperplastic degenerated synovial membrane, the so-called “pannus”. While the term “pannus” is used in several medical contexts, in rheumatology it refers to the aggressively hypertrophic part of the synovium, consisting of different inflammatory cells, producing collagenolytic enzymes. As the pannus subsequently develops, it leads to the erosion of cartilage as well as bone, resulting in joint destruction.[4(p37),5(p3)]

Until today, the cause of the RA is not yet fully known, but it seems that the disease appears familiar accumulated, with about 70% of RA-patients having the HLA-complex variation (human leukocyte antigen complex) DRBI 104, a gene complex encoding the HLA-proteins. These cell-surface proteins are responsible for the presentation of antigens to CD4+ T-helper cells making them an important part of our immune system. The DRBI 104 complex variation appears to be causally connected to the rheumatoid arthritis. [1(p792),2(p660) 4]

Further indicators for the presence of RA are rheumatic factor (RF) -antibodies and antibodies against cyclic citrullinated peptides (aCCP-antibodies or ACPA). When examining blood serum of patients, it was shown, that a part of these patients had detectable amounts of

rheumatic factors or ACPA, years before developing RA. While this might be quite interesting, it is yet unclear what number of RF-and ACPA-positive objects might develop RA.[4(pp22-23)]

3.1.3 Clinical stages of rheumatoid arthritis

In recent years, research on RA has focused strongly on the early and earliest stages of RA. That is hardly surprising, considering that nowadays the main goal is, to prevent late stages of RA and their impact to the patient by treating early stages of RA. In order to do so, it became necessary to introduce a preclinical stage of RA, thereby addressing risk factors, isolated symptoms without the whole syndrome of RA and also the preclinical presence of autoimmune activity. This stage, the "Pre-rheumatic arthritis" will be discussed in the following chapters.

3.1.3.1 *Pre-rheumatic arthritis*

Research's focus on early RA has revealed, that circulating antibodies, especially the immunoglobulin RF and ACPA and also acute phase proteins precede the clinical onset of RA. These autoantibodies can be present a median of 5 years before RA becomes clinically symptomatic. Those data strongly suggested a preclinical phase to RA which is present for several years and may allow the opportunity of an important therapeutic window. In order to facilitate research to understand the mechanisms responsible for the immunological abnormalities and the stimuli, resulting in the manifested disease of RA, the European league against rheumatism (EULAR) formed a study group in 2011, whose goal was to better describe specific stages leading to the development of RA. [9(p1)]

As a result, it was recommended to describe the preclinical period of RA that precedes the onset of clinically detectable RA as "pre-rheumatic arthritis". This pre-RA includes the interaction of the two main risk factors (genetic and environmental risk factors) as well as the development of disease related autoantibodies and symptoms and signs, considered unspecific or unclassified for RA.[10,11]

The following phases were described[9(p2)] as parts of the pre-rheumatic arthritis on its way to developing into RA:

- Genetic risk factors for RA
- Environmental risk factors for RA
- Systemic autoimmunity associated with RA
- Symptoms without clinical arthritis
- Unclassified arthritis [9(p2)]

Importantly, not every patient has to experience each phase, also those stages don't necessarily have to occur in the above order, they are able to occur in a combinatorial manner.[4(p21)]

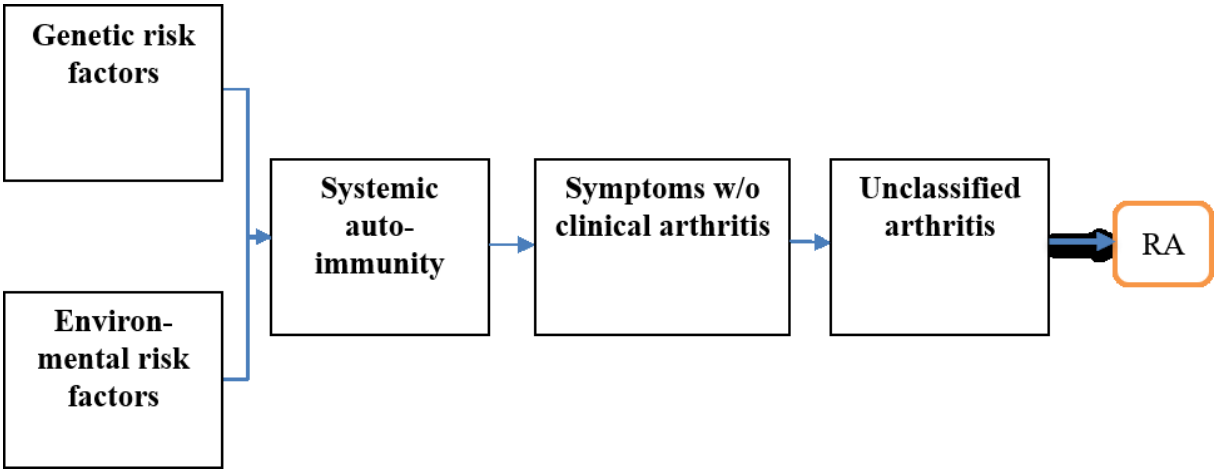

*Figure 1 | **The different stages of pre-RA in the development of rheumatoid arthritis.***

3.1.3.2 *Genetic risk factors for RA*

Genetic factors seem to play a major part in the development of RA. These parameters include the general increased appearance of RA within families, with familiar risk contribution of between 40% and 50% of seropositive RA, whereas the strongest risks are seen in first- degree relatives. Also, genetic factors in RA seem to be suggested by the fact, that certain racial groups exhibit increased prevalence rates of RA, such as North American natives with prevalence rates of 5-7%.[12(pp2-3)]

Currently, more than 100 risk factors for RA have been identified, with many of them being quite common in the population, yet most of them have a relatively small odds-ratio below 2, meaning that carrying a single risk factor does not significantly increase the risk of suffering from RA. More interesting is the fact, that there are some specific genetic loci that seem to be associated with increased risk of RA, while other loci have a decreased risk, with several of the identified genes lying on the same pathway, showing, that there are some physiological processes (T-cell activation, cell-cycle regulation) which are common to contribute in the development of RA.[4(pp21-22),12(p3)]

The gene variations that are known to contribute the most to the genetical risk factors are a set of alleles located in the MHC-complex that encode for HLA-protein complexes. Those alleles are termed in the aggregate "shared epitope" (= SE). It is still a matter of discussion on how much percent the SE as a group contributes to the development of RA, with some studies suggesting up to 40%. Many of these alleles containing the SE are located in the HLA-DRB1 region, explaining the connection of the HLA-DRB1-gene to the development of RA, which,

at the time of writing, is to be set as the genetic risk factor most common to result in RA. [1(p792),2(p660),4,12(p3) 4]

3.1.3.3 *Environmental risk factors*

The following table adapted from "*Atlas of Rheumatoid Arthritis* ", *by P. Emery* shows the environmental risk factors for RA. However, it seems that the only established environmental risk factor for RA is smoking, especially in persons that carry an HLA-DRB1 genetic variation.[4(p22)]

Risk factor	Degree of evidence
Air pollution	Inconclusive • Increased RA risk has been reported
Infections	Inconclusive • Indirect evidence by findings of increased rates of onset of RA in the winter. • Several microorganisms have shown increased titres of antibodies in RA compared to control groups; however, no single microorganism seems responsible for RA-development.
Smoking	Established • This is the only well-established environmental factor for RA • Predisposition in HLA-DRB1 shared epitope-positive persons for development of ACPA-positive RA (anti-citrullinated protein antibodies)
Coffee	Inconclusive • Both positive and negative effects have been observed
Alcohol	Inconclusive • RA patients report lower intake of alcohol, unclear whether alcohol is truly protective
Parity	Inconclusive • Some studies report a lower RA risk in parous women
Hormone replacement therapy/ oral contraceptives	Inconclusive • Some studies report on a decreased risk of RA
Breast feeding	Inconclusive • A reduced risk of RA has been observed in women after long-term breast feeding.

Table 1 | ***Currently discussed environmental risk factors for RA.*** [4(p26)]

Further risk factors discussed by other authors are:

- Obesity or dietary factors have been linked to RA, with the risk for RA rising with lower intake of vitamin D and antioxidants and higher doses of sugar, red meat, sodium, iron and protein. Additionally, a large-scale prospective study of nurses in the United States associated a generally healthy diet to lower risks of RA, yet they did not describe specific dietary factors. [2(p660),12(p6)]
- Gender. There are studies suggesting, that the outcome of RA in women is worse than in men, with men being more likely to enter spontaneous remission. When compared with age- and sex-matched samples of the general population, the mortality ratios seem to be lower for men with RA than for women. [13(pp2-3)]

In addition, Dutch researchers[14] developed a clinical rule to predict the risk of RA in patients with undifferentiated arthritis, ranging from 0 – 14, with an increasing risk of developing RA in patients with high scores, as shown in the following table.

Patients characteristics	**Number of points**
1. Age	Years × 0,02
2. Female sex	1,0
3. Distribution of involved joints (more than one possible)	
a. In case small joints of hands or feet	0,5
b. In case symmetric	0,5
c. In case upper extremities	1,0
d. In case upper and lower extremities	1,5
4. Score for morning stiffness on a 100-mm VAS	
a. 26 – 90mm	1,0
b. > 90mm	2,0
5. Number of tender joints	
a. 4 - 10	0,5
b. ≥ 11	1,0
6. Number of swollen joints	
a. 4 - 10	0,5
b. ≥ 11	1,0
7. CRP level	
a. 5 - 50 mg/L	0,5
b. ≥ 50 mg/L	1,5

8. Patient is positive for rheumatoid factor	1,0
9. Patient is positive for anti-citrullinated protein antibody	2,0
	Total score:

Score	**Likelihood ratio of developing RA**	**% with RA in one year**
0 – 3,5	0	0
3,51 – 6,5	0,42	16
6,51 – 8,5	3,0	57
> 8,5	12,7	85

*Table 2 | **Clinical rule for predicting risk and likelihood of developing RA in patients with undifferentiated Arthritis**. Total score ranges from 0 – 14 and should be rounded to the nearest number ending in .0 or .5. Additionally, the likelihood ratio of developing RA and the percentage of patients likely to develop RA within a year depending on the reached score.* [14(pp436-437),15(p1248)]

3.1.3.4 *Systemic autoimmunity associated with RA*

It seems possible to detect RF or ACPA in patients, before the clinical phase of RA, which was suggested when examining patients' blood from biobanks in Finland and Northern Sweden, years before developing RA.[16] In addition, acute phase reactants and bone degradation has been found to be increased in some patients in the preclinical stage of RA, indicating, that, in a certain patient collective, the onset of the disease precedes the onset of the clinically detectable RA.[4(pp22-23)]

3.1.3.5 *Symptoms without clinical arthritis*

This phase was defined to address patients presenting with arthralgia or joint stiffness and positive RF and/or ACPA, yet without any signs of arthritis on physical examination, like local inflammation. Those patients might, after a period of time, progress into a stage of classical RA.[11(p163)]

3.1.3.6 *Unclassified arthritis*

As the name suggests, unclassified or undifferentiated arthritis describes a diagnosis for a patient with arthritis without fulfilling the whole criteria of classification of RA which are described in "Classification of rheumatoid arthritis" or any other connective tissue disease. It is a diagnosis of exclusion, with a considerable probability of developing into RA with suggestions of 30%-40% of patients with unclassified arthritis progressing into patients with RA.[11(pp163-164)]

With this, I would like to conclude with the topic of pre-rheumatic arthritis. One might notice, that the five stages of pre-RA are formulated quite broadly and open for further development. It was a quite conscious decision to not restrict those categories to specific risk factors or

features known at the time, because in a field this rapidly advancing, the nomenclature of pre-RA would have been rendered redundant very soon.[10(pp571-572)]

3.1.3.7 *Clinical rheumatoid arthritis*

The presentation of RA is extremely variable and differs in the clinical course of each patient, ranging from mild arthritis to a rapidly progressive multisystemic inflammation with severe mortality and morbidity. [17]

In the early stage of rheumatoid arthritis, mainly the synovial lining, also called synovial membrane becomes infiltrated with immune cells, responsible in causing an inflammation, while the outermost layer of the joint, the joint capsule, remains unaffected. [1,2,4,17] The following image from *"Atlas of Rheumatoid Arthritis by Emery, Paul"*[4] pictures the destruction of joint structure in RA in comparison to a healthy joint.

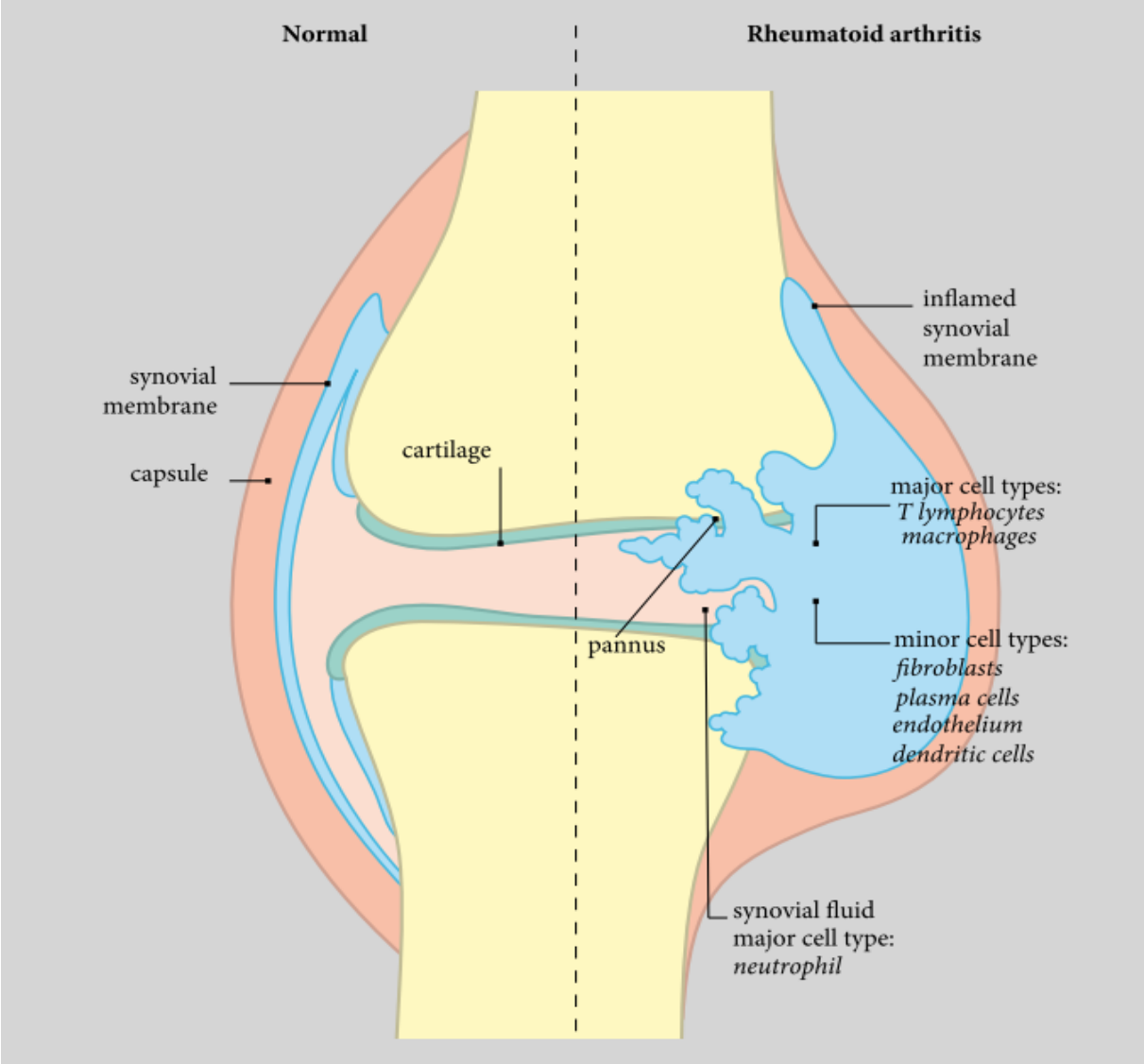

Figure 2 | ***Synovial joint in health and rheumatoid arthritis*** *indicating cellular components and sites of destruction in diseased joint.* Reproduced with permission from Springer Nature [4(p45)]

Although heterogenous, disease onset is insidious in most cases, with symptoms usually developing gradually. Main symptoms include pain, swelling and stiffness of peripheral joints, typically occurring in the morning (=morning stiffness), especially in small joints of hands and feet, typically including the MCP (metacarpophalangeal) and PIP (proximal interphalangeal) joints. Those joints may present themselves warm and soft with a fluctuant sensation, due to an increased amount of fluid and tissue at the place of inflammation. In addition, general symptoms such as weakness and fatigue, subfebrile temperatures, weight loss and illness may occur. [1(p792),2(p660),4(pp69-72),17(pp903-905)]

As the disease progresses further, the chronic inflammation leads to progressive synovial thickening and hypertrophy as well as advancing destruction of articular surface resulting in functional impairments, such as hyperextensions, subluxations and swan-neck deformities. Some patients develop a multisystemic inflammation, commonly called an extra-articular involvement of RA. Depending on the location of the extra-articular manifestation of RA, the appearing symptoms may differ greatly: [1(p792),2(p660),4(pp69-72),17(pp903-905)]

- Cervical spine involvement – A manifestation of RA in cervical spine typically presents itself as nocturnal pain in the involved area, radiating into the back of the head. Other sings of cervical involvement are paraesthesia and movement disorders, especially in upper extremities as well as gait disorders and incontinence.
- Skin and vascular involvement – rheumatic vasculitis, resulting in manifestations ranging from skin ulcerations and necrosis to purpura and rheumatoid nodules. Rheumatoid nodules are especially common at areas exposed to pressure.
- Respiratory involvement – presenting itself with Pulmonary fibrosis and pleural effusion
- Cardiac involvement – with pericardial inflammation and myocarditis as well as the formation of valvular nodes.
- Others, such as hepatic involvement with increased hepatic transaminases; ocular involvement with keratoconjunctivitis; oral involvement with salivary inflammation or haematological manifestations such as anaemia, thrombocytosis, leucocytosis, etc. [1(p792),2(p660),4(pp69-72),17(pp903-905)]

The main goal of the treatment of rheumatoid arthritis is, to prevent the manifestation of extra-articular RA and the increasing joint dysfunction emerging from the ongoing inflammation. To achieve this goal, early diagnosis and therapy is crucial, yet if managed correctly, RA may reach a stage of remission or at least minimal disease activity.

3.1.4 Diagnosis of rheumatoid arthritis

There are typical signs indicating the presence of RA, like pain and morning stiffness in multiple joints, with wrists, proximal interphalangeal joints and metacarpophalangeal joints being most commonly involved. Also, synovial thickening or boggy swelling may be visible and palpable as well as systemic symptoms like fatigue, weight loss and low-grade fever, especially in active disease.[15(p1246)]

Also, it is possible to witness the formation of autoantibodies against the Fc-fragment of Immunoglobulin G. Those antibodies, commonly called rheumatic factor (RF-positive), do play an important part in the diagnosis of the RA. However, the existence of rheumatic factors does not prove the diagnosis RA, it is only a major component of diagnosis. Another component that seem to take part in the pathogenesis of the RA are antibodies against cyclic citrullinated peptides (aCCP-antibodies) which might possibly be formed within inflammatory processes in the lung of the patient. This could also explain the risk factor of smoking in the epidemiology of RA. [2]

Although most APCA-positive patients are also RF-positive, APCA seems to be more specific and sensitive in early diagnosis of RA and seems to be correlated with poor prognostic factors like progressive joint destruction. [3]

As rheumatoid arthritis is a quite heterogenous disease, it differs in the clinical course of each patient. Especially in its early stages, RA has a rather insidious onset, therefore resulting in the major challenge of diagnosing RA as soon as possible. Nevertheless, in the past years it has become the intention to detect and correctly diagnose RA as soon as possible, in order to induce therapy in early stages of RA. Therefore, classification tools were established to include early onset RA patients into studies.[18]

3.1.4.1 *Classification of rheumatoid arthritis*

Considering that the process of development of rheumatoid arthritis proceeds rather gradually, it is difficult to determine a definite onset of clinically relevant RA.

Thus, in 1987, the American College of Rheumatology (ACR) established classification criteria for RA in order to differentiate it from other rheumatic diseases. Due to their limitations and inability to classify RA in its early stages, those criteria were deemed to be out of date. They were replaced by the new criteria proposed by the ACR and the EULAR in 2010, as an approach to classify RA as early as possible and thereby avoiding long-term damage such as bone destruction and extra-articular disease. [3,19]

The new criteria were shown to be more sensitive than the 1987 ACR criteria, additional examination of anti-CCP titres were suggested to further increase sensitivity.[20,21] Although

those criteria simplify classification standards, they should not be followed blindly. Points of critique include the absence of other methods for diagnosis of synovitis besides clinical examination, as well as the inclusion of serology into the criteria. Although RF and anti-CCP have a high specificity for detecting RA, both, can be detected in variety of other diseases. Also, there are patients suffering from RA, without major changes of their acute phase reactants. Considering this, the inclusion of serology may lead to false positive results or even more, false negative exclusion of patients with RA, yet no major changes of RF or ACPA. [19]

3.1.4.2 *Criteria*

The classification criteria may be applied to every patient fulfilling two requirements: first, evidence of active clinical synovitis in at least one joint, with the exception of the distal interphalangeal (DIP) joints, the first metatarsophalangeal (MTP) joint and the first carpometacarpal (CMC) joint. Those restrictions were made, due to the fact that those joints are frequently involved in osteoarthritis. As second requirement, the criteria may not be applied to patients whose synovitis can be better explained by another diagnosis, such as lupus erythematosus, psoriatic arthritis and others. Both requirements need to be assessed by a specialist. [22]

Four criteria were named to be applied onto potential patients, whose application provides a score from 0-10, where a score of 6 or more indicates the presence of RA. A patient with a score bellow 6 cannot be classified with RA, but might meet the necessary criteria at a later time point. The four classification criteria received from *"The 2010 Criteria for the classification of rheumatoid arthritis by D. Aletaha, T. DNeogi, A. Silman et al"* and additionally, the number of points they award, are:

A. **Joint involvement**, with:	
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 1 small joint)	5
B. **Serology** (at least 1 test result is needed for classification)	
a. Negative **RF** and negative **ACPA**	0
b. Low positive **RF** or low positive **ACPA**	2
c. High positive **RF** or high positive **ACPA**	3
C. **Acute phase reactants** (at least 1 test result is needed for classification)	
a. Normal **CRP** and normal **ESR** 0	0

b. Abnormal **CRP** or normal **ESR** 1	1
D. **Duration of symptoms**	
a. < 6 weeks	0
b. ≥ 6 weeks	1

*Table 3 | **The 2010 Criteria for the classification of rheumatoid arthritis** - An American College of Rheumatology/European League Against Rheumatism collaborative initiative.*[22]

3.1.4.3 *Other diagnostic tools*

In addition to the described criteria catalogue, imaging plays a significant role in the diagnosis, assessment of remission and follow up of rheumatoid arthritis in order to monitor progressive joint damage.[23]

3.1.4.4 *Conventional radiography*

Despite the rise of ultrasound and MRI, conventional radiography still remains important in the diagnosis and monitoring of joint involvement in patients with RA. In daily routine it provides a basic assessment of joint involvement and damage, although it is showing mainly bony changes. While being cheap, widely available and easy to perform and interpret, the most relevant limitation is its low sensitivity, especially in early RA. Radiographic signs of RA include soft tissue swelling, joint effusion, juxta-articular osteoporosis, cysts, bone erosions, joint subluxations and malalignment. Besides its potency to aid in the assessment of joint involvement, there are other scoring systems to assess the radiographic damage in evaluating RA, mainly focusing on hands and feet. [23,24]

3.1.4.5 *Computed tomography (CT)*

Similar to the conventional radiography, computed tomography allows evaluation of bone damage in patients with RA. Computed tomography has a higher sensitivity in detecting changes in the bone structure than conventional radiography, ultrasound and MRI, being able to depict even small bone erosions and other signs of RA described in the above chapter "conventional radiography". Yet, its high exposure to ionizing radiations limits the CTs use.[24,25]

3.1.4.6 *Magnetic resonance imaging (MRI)*

In comparison to a CT, an MRI has the added ability to provide information about synovial or soft tissue involvement and cartilaginous damages. Due to being able to detect active inflammation like bone marrow edema, synovial hypertrophy and pannus formation before the onset of bony defects, MRI is a very sensitive measurement. In accordance, studies have shown superiority of MRI in the early detection of RA compared with CTs and radiographs.

Routine use of MRI in diagnosis of RA is limited by cost, accessibility and difficulty of imaging multiple joints at a time.[23–25]

3.1.4.7 *Ultrasound (US)*

Ultrasound is regarded as the most sensitive imaging modality available in daily rheumatology practice. Its ability to detect early inflammatory processes and soft tissue changes make it superior to clinical examination only. Its main advantages compared to other imaging techniques consist of the absence of radiation, good visualisation capability of soft tissue, real time imaging and low costs. Also, it can be performed quickly and multiple times. Main limitations are the operator dependent quality of ultrasound imaging, its low resolution regarding cartilage. [23–25]

3.1.5 Therapy of RA

The main goal in the management of RA is to treat rheumatoid arthritis to low disease activity or, if possible, complete remission. Receiving and keeping a stage of remission is the principal objective as curing rheumatoid arthritis is currently not possible.

The therapy of rheumatoid arthritis varies depending on the present stage and inflammatory activity of RA, yet, as RA is a heterogenous disease, its management can be quite complex. Therefore, the American college of rheumatology (ACR) and the EULAR specify guidelines and recommendations for the management of rheumatoid arthritis.

In 2015, the ACR defined recommendations for the treatment of either patients with early RA or patients with established RA.[26] Additionally, in 2016, the EULAR published overarching principles and an algorithm for the management of patients with RA. [27]

3.1.5.1 *Recommendations for the treatment of patients with early RA*

The following chapter depicts recommendations for treatment of patients with symptomatic early arthritis, presented by the ACR in 2015. The early RA is defined as rheumatoid arthritis with a disease duration under 6 months.[26]

Recommendations for patients with symptomatic <u>early</u> RA
1. Regardless of disease activity level, use a treat-to-target strategy rather than a non-targeted approach.
2. If the disease activity is low, in patients who have never taken a DMARD: • **Use DMARD monotherapy (MTX preferred) over double therapy** • **Use DMARD monotherapy (MTX preferred) over triple therapy**
3. If the disease activity is moderate or high, in patients who have never taken a DMARD:

• Use DMARD monotherapy over double therapy. • Use DMARD monotherapy over triple therapy.
4. If disease activity remains moderate or high despite DMARD monotherapy (with or without glucocorticoids), use combination DMARDs or a TNFi or a non-TNF biologic (all choices with or without MTX, in no particular order of preference), rather than continuing DMARD monotherapy alone.
5. If disease activity remains moderate or high despite DMARDs: • Use a TNFi monotherapy over tofacitinib monotherapy. • Use a TNFi + MTX over tofacitinib + MTX.
6. If disease activity remains moderate or high despite DMARD or biologic therapy, add low dose glucocorticoids.
7. If disease flares, add short term glucocorticoids at the lowest possible dose and for the shortest possible duration.

Table 4 | ***Summary of 2015 ACR recommendations for the treatment of early rheumatoid arthritis.*** [26]

Green and bold sections describe strong recommendations, meaning that the authors were confident that the desirable effects of following the recommendations outweigh the undesirable effects.

Yellow sections describe conditional recommendations, meaning that the desirable effects of following the recommendations probably outweigh the undesirable effects.[26]

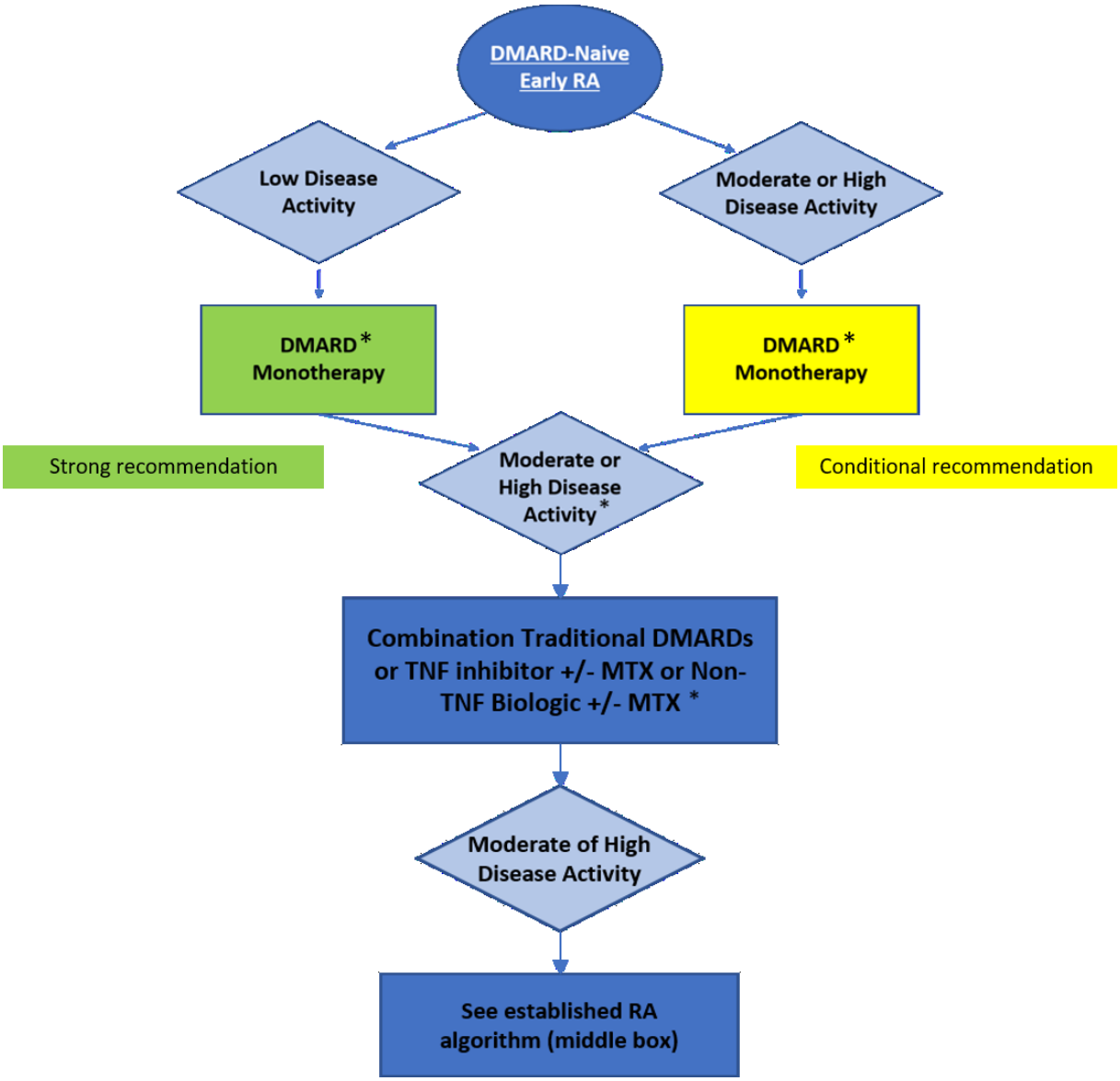

Figure 3 | **2015 ACR recommendations for the treatment of early RA,** defined as disease duration <6 months.
* = consider adding low-dose glucocorticoids (< 10mg/day prednisone or equivalent) in patients with moderate or high RA disease activity when starting disease-modifying antirheumatic drugs (DMARDs) and in patients with DMARD failure or biologic failure, also consider using short-term glucocorticoids (defined as <3 months treatment) for RA disease flares. Glucocorticoids should be used at the lowest possible dose and for the shortest possible duration.[26]
DMARD = disease modifying antirheumatic
Green box/strong recommendation due to: Missing evidence in favour of triple DMARDs therapy, and DMARD monotherapy is usually better accepted and tolerated by low disease RA patients than DMARD combinations.
Yellow box/ conditional recommendation due to: Low quality of evidence, only minor difference in the use of double DMARD therapy over monotherapy, and triple therapy may be preferred by patients with need for a rapid short-term benefit with potential added risk. [26]

The last years have seen a drastic paradigm shift from initial treatment of RA with nonsteroidal anti-inflammatory drugs (NSAIDs) followed by progressive addition of disease-modifying antirheumatic drugs (DMARDs), to the current approach of aggressive treatment with DMARD soon after diagnosis of RA. This changes in treatment of RA resulted from

data, supporting improved outcome and prognosis with early DMARD therapy with rapid escalation to combination therapy including biologics. [17,28]

Also, as mentioned before, growing data suggest that early identification and especially intensive early treatment of RA leads to an easier course of disease with improved outcomes, improved rates of drug-free remission and radiological outcomes. Early treatment may also prevent the development of early, subtle joint damages into significant disabilities with lost opportunity of damage regression. Although the effectiveness of these therapeutic changes still needs further investigation, it seems that early treatment of RA plays a key role in the optimal management of rheumatoid arthritis.[28,29]

3.1.5.2 *Recommendations for the treatment of patients with established RA*

The 2015 American college of rheumatology[26] recommendations for the treatment of patients with established RA, defined as disease duration > 6 months.

Recommendations for patients with <u>Established</u> RA
1. Regardless of disease activity level, use a treat-to-target strategy rather than a non-targeted approach.
2. If the disease activity is low, in patients who have never taken a DMARD, use DMARD monotherapy (MTX preferred) over a TNFi.
3. If the disease activity is moderate or high in patients who have never taken a DMARD: • Use DMARD monotherapy (MTX preferred) over tofacitinib. • Use DMARD monotherapy (MTX preferred) over combination DMARD therapy.
4. If disease activity remains moderate or high despite DMARD monotherapy, use combination of traditional DMARDs or add a TNFi or a non-TNF-biologic or tofacitinib (all choices with or without MTX, in no particular order of preference), rather than continuing DMARD monotherapy alone.
5. If disease activity remains moderate or high despite TNFi therapy in patients who are currently not on DMARDs, add one or two DMARDs to TNFi therapy rather than continuing TNFi therapy alone.
6. If disease activity remains moderate or high despite use of a single TNFi: • Use a non-TNF biologic with or without MTX, over another TNFi with or without MTX. • Use a non-TNF biologic, with or without MTX, over tofacitinib with or without MTX

7. If disease activity remains moderate or high despite use of a single non-TNF biologic, use another non-TNF biologic, with or without MTX, over tofacitinib, with or without MTX
8. If disease activity remains moderate or high despite use of multiple (2+) sequential TNFi therapies, first use a non-TNF biologic, with or without MTX, over another TNFi or tofacitinib (with or without MTX).
9. If disease activity remains moderate or high despite the use of multiple TNFi therapies, use tofacitinib, with or without MTX, over another TNFi, with or without MTX, if use of a non-TNF biologic is not an option.
10. If disease activity still remains moderate or high despite use of at least one TNFi and at least one non-TNF biologic: • First use another non-TNF biologic, with or without MTX, over tofacitinib. • If disease activity remains moderate or high, use tofacitinib, with or without MTX, over another TNFi.
11. If disease activity remains moderate or high despite use of DMARD, TNFi, or non-TNF biologic therapy, add short-term, low dose glucocorticoid therapy.
12. If disease flares in patients on DMARD, TNFi, or non-TNF biologic therapy, add short-term glucocorticoids at the lowest possible dose and the shortest possible duration.
13. If the patient is in remission: • Taper DMARD therapy. • Taper TNFi, non-TNF biologic, of tofacitinib therapy.
14. If disease activity is low: • Continue DMARD therapy • Continue TNFi, non-TNF biologic or tofacitinib therapy rather than discontinuing respective medication.
15. If the patient's disease is in remission, DO NOT discontinue all RA therapies.

Table 5 | ***Summary of 2015 ACR recommendations for the treatment of Established rheumatoid arthritis.*** [26]

Green and bold sections describe strong recommendations, meaning that the authors were confident that the desirable effects of following the recommendations outweigh the undesirable effects. [26]

Yellow sections describe conditional recommendations, meaning that the desirable effects of following the recommendations probably outweigh the undesirable effects.[26]

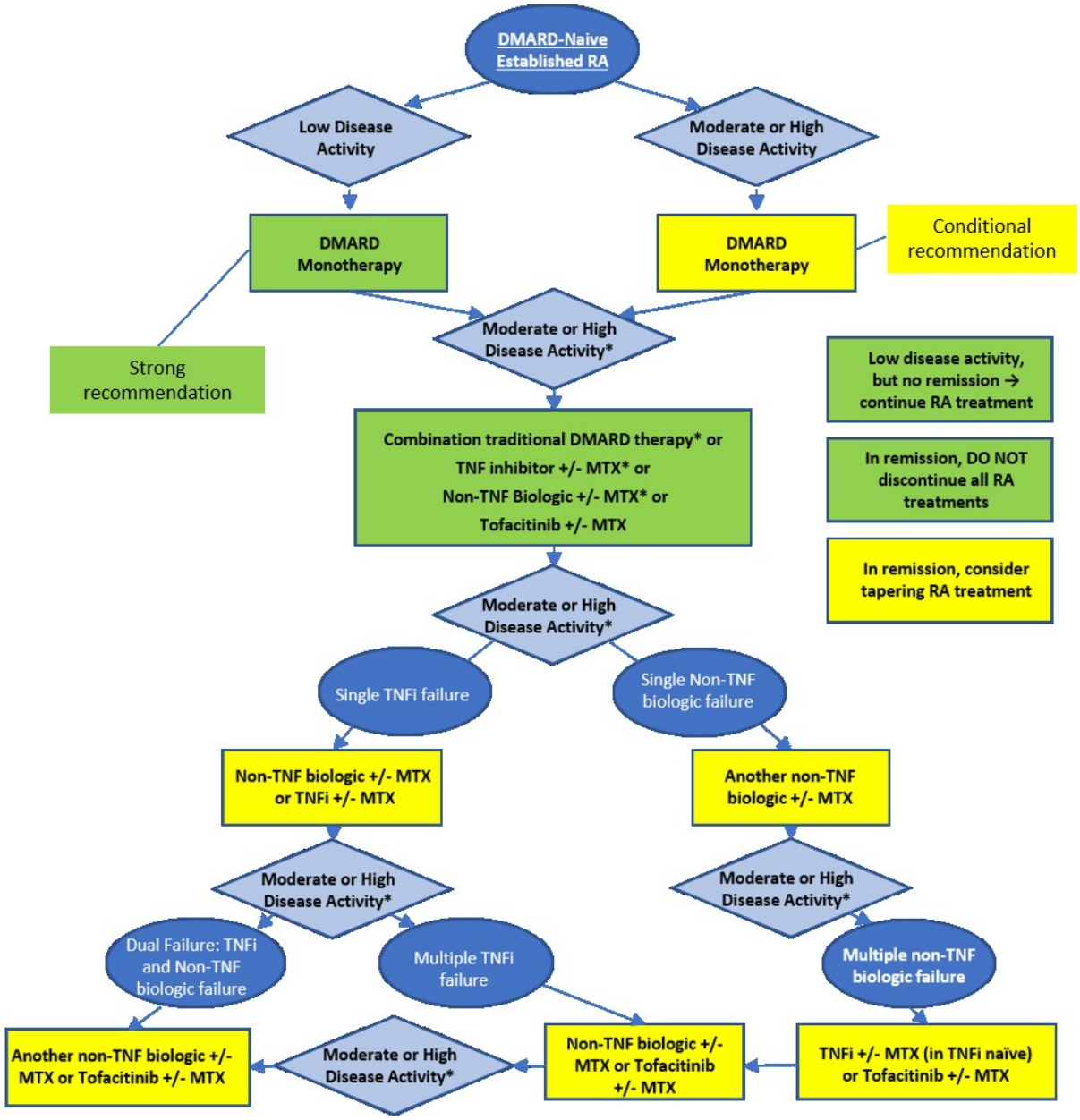

*Figure 4 | **2015 ACR recommendations for the treatment of Established RA,** [26] defined as disease duration >6 months. * = consider adding low-dose glucocorticoids (< 10mg/day prednisone or equivalent) in patients with moderate or high RA disease activity when starting disease-modifying antirheumatic drugs (DMARDs) and in patients with DMARD failure or biologic failure, also consider using short-term glucocorticoids (defined as <3 months treatment) for RA disease flares. Glucocorticoids should be used at the lowest possible dose and for the shortest possible duration.* [26]

3.1.5.3 *Principles for the management of patients with RA*

2016 EULAR updated recommendations for management of patients with RA [27]
Overarching principles
○ Treatment of patients with RA should aim at the best care and must be based on a shared decision between the patient and the rheumatologist

○ Treatment decisions are based on disease activity and other patient factors, such as progression of structural damage, comorbidities and safety issues
○ Rheumatologists are the specialists who should primarily care for patients with RA
○ RA incurs high individual, medical and societal costs, all of which should be considered in its management by the treating rheumatologist
Recommendations
• Therapy with DMARDs should be started as soon as the diagnosis of RA is made
• Treatment should be aimed at reaching a target of sustained remission or low disease activity in every patient
• Monitoring should be frequent in active disease (every 1–3 months); if there is no improvement by at most 3 months after the start of treatment or the target has not been reached by 6 months, therapy should be adjusted
• MTX should be part of the first treatment strategy
• In patients with a contraindication to MTX (or early intolerance), leflunomide or sulfasalazine should be considered as part of the (first) treatment strategy
• Short-term glucocorticoids should be considered when initiating or changing csDMARDs, in different dose regimens and routes of administration, but should be tapered as rapidly as clinically feasible
• If the treatment target is not achieved with the first csDMARD strategy, in the absence of poor prognostic factors, other csDMARDs should be considered
• If the treatment target is not achieved with the first csDMARD strategy, when poor prognostic factors are present, addition of a bDMARD or a tsDMARD should be considered; current practice would be to start a bDMARD.
• bDMARDs and tsDMARDs should be combined with a csDMARD; in patients who cannot use csDMARDs as comedication, IL-6 pathway inhibitors and tsDMARDs may have some advantages compared with other bDMARDs
• If a bDMARD or tsDMARD has failed, treatment with another bDMARD or a tsDMARD should be considered; if one TNF-inhibitor therapy has failed, patients may receive another TNF-inhibitor or an agent with another mode of action

• If a patient is in persistent remission after having tapered glucocorticoids, one can consider tapering bDMARDs, especially if this treatment is combined with a csDMARD
• If a patient is in persistent remission, tapering the csDMARD could be considered

Table 6 | ***2016 EULAR updated recommendations for the management of rheumatoid arthritis.***
DMARD = disease-modifying antirheumatic drug, csDMARD = conventional synthetic DMARD, bDMARD = biological DMARD, tsDMARD = targeted synthetic DMARD, MTX = methotrexate. [27]

3.1.5.4 *Algorithm for the management of patients with RA*

Based on their 2016 recommendations [27], EULAR published a comprehensive algorithm for management of RA[27]. The 2016 EULAR recommendations and algorithm differ to the 2015 ACRs recommendations and algorithm as they do not subdivide therapeutic aspects into early and established RA but use only one algorithm with different phases. Apart from that, the resulting algorithms are quite similar.

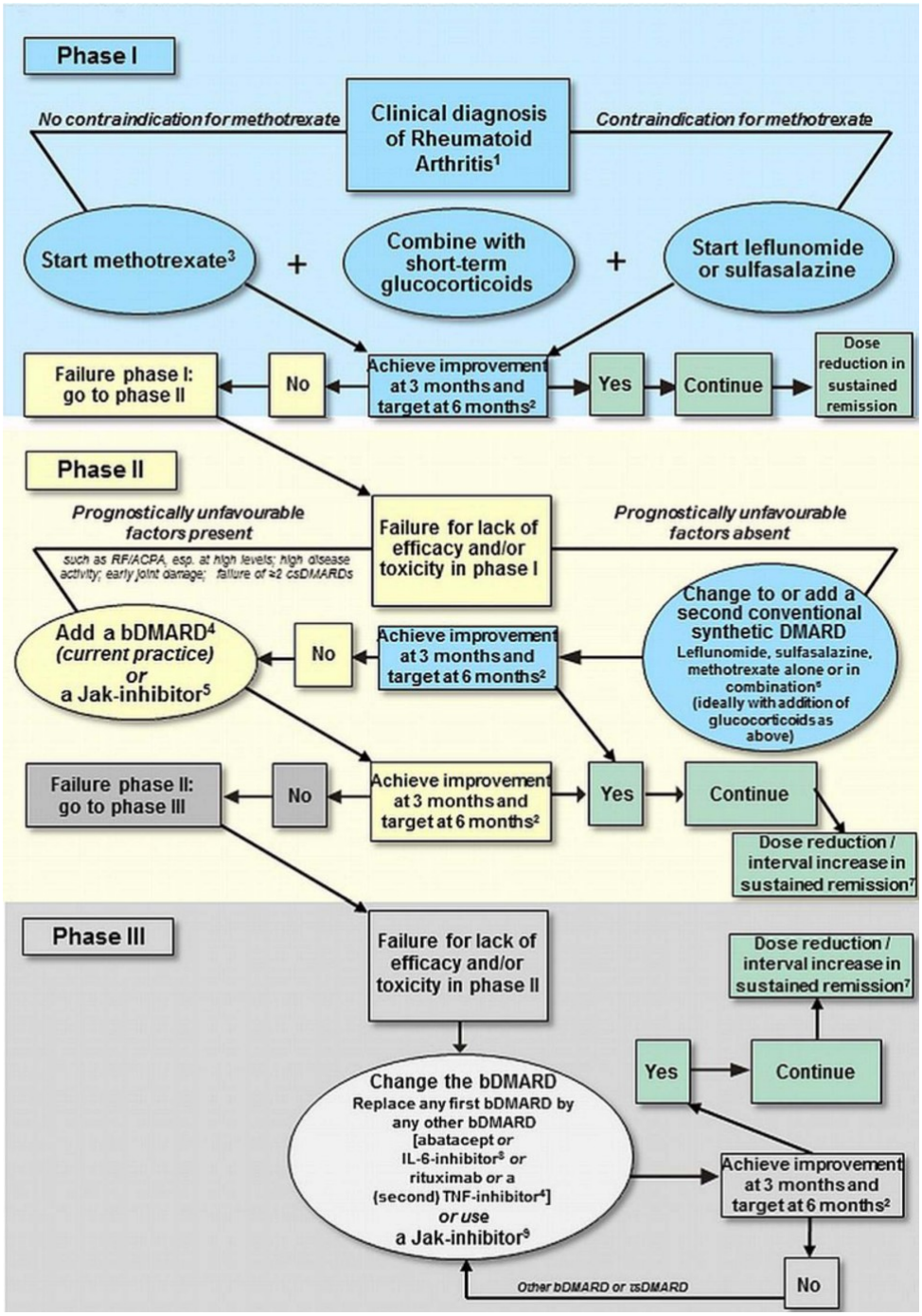

Figure 5 | ***Algorithm based on the 2016 EULAR recommendations for management of RA.***

[1] 201β ACR/EULAR classification criteria can support early diagnosis. [2] The treatment target is set as clinical remission EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update. according to ACR/EULAR definition or, if remission is unlikely, at least low disease activity. The target should be reached after 6 months, yet therapy should be adapted if there is no apparent improvement after 3 months. [3] MTX should be part of the first treatment strategy [4] biological DMARD. [5] Current practice suggests to start with a bDMARD prior to tsDMARD (Jak-inhibitor) due to superior long-term experience. [6] most common combination is MTX, sulfasalazine and hydroxychloroquine. [7] Dose reduction/interval increase can be done with all bDMARDs with little risk of flares. [8] Efficacy and safety of bDMARDs after Jak-inhibitor failure is unknown as well as efficacy and safety of IL-6-pathway inhibitors after failure of another IL-6-inhibitor [9] Efficacy and safety of Jak-inhibitor after insufficient response to another Jak-inhibitor is unknown. Reproduced with permission from BMJ Publishing Group Ltd. [27]

3.1.5.5 *Medication in rheumatoid arthritis*

3.1.5.5.1 Corticosteroids and NSAIDs

Additionally, therapy of RA my include oral, intramuscular or intra-articular corticosteroids and NSAIDs in the early management of pain and inflammation. Glucocorticoids are extremely potent anti-inflammatory agents, especially used to treat the occurrence of flares, but there is also evidence that early application of low-dose glucocorticoids can help delaying the radiologic progression of rheumatoid arthritis. While NSAIDs used to be the first-line therapy in RA, they have been replaced by early initiation of DMARDs and are used only as adjuncts in treatment of RA. NSAIDs and corticosteroids should only be used for short-term management of RA and additional to the preferred DMARDs.[15,25]

3.1.5.5.2 Disease-modifying antirheumatic drugs – DMARDs

The term disease-modifying antirheumatic drugs (DMARDs) describes a heterogenous group of drugs, defined by their role of reducing disease activity in rheumatoid arthritis and other autoimmune disorders. DMARDs serve as immunosuppressives designed to inhibit or slow progression of joint damage in order to induce or maintain remission and reduce the frequency of flare ups. They also suppress acute phase responses, decrease autoantibody levels and improve long-term function. [3,15,30]

As a heterogenous family, DMARDs act through different mechanisms in the treatment of rheumatoid arthritis. The following table describes the mechanism of effect triggered by the most common synthetic and biologic DMARDs as well as their adverse effects.[15]

Name of DMARD	***Effect mechanism***	***Adverse effects***
Synthetic DMARDs		
Methotrexate	Inhibits dihydrofolate reductase	Liver effects, teratogenesis, hair loss, oral ulcers, myelosuppression
Leflunomide	Inhibits pyrimidine synthesis	Liver effects, gastrointestinal effects, teratogenesis
Hydroxychloroquine	Antimalarial, blocks toll-like receptors	Rare ocular toxicity
Sulfasalazine	Folate depletion, other mechanisms unknown	Anaemia in G6PD deficiency, gastrointestinal effects
Minocycline	Antimicrobial, other mechanisms unknown	Drug-induced lupus erythematosus, Clostridium difficile colitis

Gold sodium	Inhibits antigen processing, decreases cytokines (TNF, interleukin-6)	Skin, heme, renal effects
Penicillamine	Chelates metal, other mechanisms unknown	Heme, renal effects Infertility,
Cyclophosphamide	Nitrogen mustard alkylating agent, cross-links DNA	Infertility, cancer, haemorrhagic cystitis
Cyclosporine	Calcineurin inhibitor, decreases interleukin-2	Hypertension, renal effects, hirsutism
Biologic DMARDs		
Anti-TNF agents		
Adalimumab	Anti-TNF-α	Tuberculosis (TB), infection
Certolizumab pegol	Anti-TNF-, pegylated	TB, infection
Etanercept	Anti-TNF-α	TB, infection
Golimumab	Anti-TNF-α	TB, infection
Infliximab	Anti-TNF-α	TB, infection, infusion reaction
Other biologic agents		
Abatacept	Costimulator blocker, cytotoxic T lymphocyte antigen 4	infection
Anakinra	Anti-interleukin-1 receptor	infection, injection site pain
Rituximab	Anti-CD20, eliminates B cells	Infusion reaction, infection, progressive multifocal leukoencephalopathy
Tocilizumab	Anti-interleukin-6 receptor blocker	infection
Targeted synthetic DMARDs		
Tofacitinib	Selective JAK-inhibitor, preferentially JAK1 and JAK3.	Infection, TB, herpes zoster, lipid elevation, decrease in neutrophils, lymphocytes and anaemia, rare side effects of cardiovascular events and malignancies

Baricitinib	Selective JAK-inhibitor, preferentially JAK1 and JAK2	Infection, TB, herpes zoster, lipid elevation, rare side effects of cardiovascular events and malignancies

*Table 7 | **Mechanism of synthetic and biologic DMARDs**. Synthetic DMARDs are listed in order of priority, with bolded DMARDs more commonly used than others.* [15,31–33]

The ongoing progress in introducing new therapeutic aspects for the management of RA have expanded the family of DMARDs by adding biological compounds. Therefore, a new nomenclature was designed to classify DMARDs adequately. The new nomenclature proposed to divide the family of DMARDs into synthetic DMARDs and biological DMARDs. Subsequently, synthetic DMARDs present themselves with two subclasses, conventional synthetic DMARDs (csDMARDs) and targeted synthetic DMARDs (tsDMARDs), as well as the biological DMARDs, who are divided into biological originator DMARDs (boDMARDs) and biosimilar DMARDs (bsDMARDs).[30] It should be mentioned, that, although this new nomenclature was proposed in 2014, many authors still classify DMARDs into synthetic and biologic DMARDs only. The following table shows the described nomenclature in detail.

Disease modifying anti-rheumatic drugs			
Synthetic DMARDs (sDMARDs)		**Biological DMARDs (bDMARDs)**	
Conventional synthetic DMARDs (csDMARDs) *Targeted*	*Targeted synthetic DMARDs (tsDMARDs)* *Biological*	*Biological originator DMARDs (boDMARDs)* *Biosimilar*	*Biosimilar DMARDs (bsDMARDs)*

*Table 8 | **Proposal for a new nomenclature of disease-modifying antirheumatic drugs.*** [30]

3.1.5.5.3 Targeted synthetic DMARDs

Targeted synthetic DMARDs are of particular interest, due to their mode of action, the selective inhibition of JAKs. Several JAK-inhibitors have been developed as new therapies for patients with RA. These oral synthetic DMARDs inhibit JAK1, JAK2 and JAK3. Two JAK-inhibitors are currently been approved in many countries, namely tofacitinib, a JAK1+2-inhibitor, and baricitinib, a JAK1+3-inhibitor. The mechanism of these JAK-inhibitors compromises on the selective regulation of JAKs and their interaction with specific STAT proteins. [34,35]

JAK1 correlates with the signalling of the γc and gp130 cytokine family as well as class II cytokine receptors. There is also evidence suggesting that JAK1 modulates the signalling of TNFα via TNFR-1 receptors. JAK2 is activated by the γc cytokine family, but also by other cytokines like βc family cytokines, thrombopoietin and erythropoietin. Activation of JAK3 is induced relatively selectively by γc cytokines IL-2, IL-4, IL-7, IL-9, IL-15, IL-21.
Generally speaking, JAK-inhibitors interrupt and reduce the JAK-STAT signaling cascade, thereby leading to modulated immune responses. In the future, JAK-inhibitors capable of a selective inhibition of certain JAK isoforms are expected to present an enhanced therapeutic effect with fewer side effects than the currently used JAK-inhibitors. [34,35]

3.2 The JAK-STAT signalling pathway

3.2.1 Cytokines

While numerous cellular and humoral components of the immune system are involved in the aetiology of RA, the inter-cellular signaling messengers called cytokines seem to play a major part. Cytokines are proteins whose task it is, to regulate and mediate the proliferation and differentiation of cells, thereby regulating inflammatory and immune responses to pathogens and other antigens. [6] Unlike hormones, cytokines cause their effect over a much shorter distance (few micrometres), autocrine as well as paracrine. Cytokines can be categorized according to their biological function:

- Growth factors
- Interleukins
- Interferons
- Chemokines

Cytokines are of special significance due to the fact that interferons as well as interleukins use receptors with associated tyrosine kinases in order to trigger their signalling cascade, in which the JAK-STAT signalling pathway plays an important part.[7(p778),8(p1)]

3.2.1.1 *Interferons*

Interferons are a group of glycoproteins that belong to the cytokine-family. They are released by numerous cells as a response to infectious causes and mediate cell-to cell communications, during which they induce immunomodulatory changes. Besides, they also serve as one of the immune systems first line defences against bacterial and viral infections. Interferons can be put into three categories, which can be determined by their unique complimentary receptor complex: [36–39]

- interferon type I: Binding to an IFN-alpha receptor complex, and consisting of the following interferon classes: IFN-α, IFN- β, IFN- ε, IFN- κ, IFN- ω.

- interferon type II: binding to an IFNGR receptor complex, consisting only of IFN-γ.
- interferon type III: binding to a receptor complex consisting of IL10R2 and IFNLR1, consisting of INF-λ. Some years ago, the type III interferons were referred to as interferon-like cytokines, with different subtypes of INF-λ, yet meanwhile most authors summarize them as interferon type III. [36–39]

The main activities of interferons include:

- Antiviral activity – potent, but non-specific activity induced by inhibiting viral entry, viral protein synthesis and viral maturation as well as the induction of assembly defects.
- Anti-tumor activity – numerous effects modulation of cell proliferation and differentiation, cytostatic effects and induction of apoptosis as well as other effects.
- Immunomodulating activity – interferons unfold their immunomodulating activity via different ways:
  - differentiation and activation of NK-cells, monocytes, macrophages and dendritic cells
  - increasing the activity of cytotoxic cells and the production of immunoglobulins
  - and also increasing the production of TNF (tumor necrosis factor)
- Other activities- such as anti-angiogenic effects and inhibition of keratinocyte proliferation. [37]

3.2.1.2 *Interferon alpha*

Interferon alpha (IFN α) has antiviral, immunomodulating and antiproliferative effects. It is produced in NK cells, macrophages, myelomonocytic cells, TH1 cells, cytotoxic T lymphocytes an B cells. Major function aside from the already named include promotion of cytotoxic activity, TH1 differentiation, upregulation of MHC I and II, cell growth inhibition and proapoptotic effects alongside control of activation induced cell death. Interferon alpha contributes to many autoimmune diseases such as type 1 diabetes, multiple sclerosis, systemic lupus erythematosus and finally RA, where reports have shown correlations between IFN α levels and disease activity of RA. Also, IFN α has been detected in synovial fluids of patients with RA. [37(p52),40(p58),41(p12)]

3.2.1.3 *Interferon gamma*

Interferon gamma (IFNγ), the only type II interferon, mediates antiviral, antibacterial and proapoptotic effects, promotes cytotoxic activity and differentiation of TH1 cells, NK cells, monocytes and macrophages and inhibits cell growth. It also inhibits IL-4 synthesis, yet

mediates synthesis of IL-2 and TNF alpha. Main source are NK-cells and activated T cells. IFNγ activates JAK1, JAK2 and a is used in the treatment of chronic granulomatous disease, malignant osteopetrosis, leishmaniasis and also in the off-label treatment of bladder and renal cancers. [37,41,42,43]

3.2.1.4 *Interleukins*

Interleukins are a vaguely defined sub-class of cytokines, where every secreted protein which is binding to its specific receptors and thereby playing a role in intercellular communication among leukocytes is named interleukin. As the nomenclature has been continuously evolving, as well as the research about inflammation and immune cell functions, the list of identified interleukins grew constantly, resulting in numerous interleukins with currently known interleukins from IL-1 to IL-40. As mentioned, the group of Interleukins is hardly homogenous, but rather heterogenous, with some interleukins acting as pro-inflammatory immunomodulators, whereas other interleukins having an anti-inflammatory effect.[6,8,41]

3.2.1.5 *Interleukin 2*

Interleukin 2 is a proinflammatory cytokine mainly produced by activated CD4+ and CD8+ T cells through activation of the TCR (T cell receptor). IL-2 plays a central part in T cell-dependent immune responses, as evidently, one of the most rapid consequences of T cell-activation is the de novo synthesis of IL-2. Its main biological activities include extensive clonal expansion of T cells, such as induction of CD4+ and CD8+ - expansion and promotion of NK cells proliferation. It also exerts anti-apoptotic and pro-metabolic signals in T-cells as well as signals for enhanced antibody secretion of B cells. [44–46]

3.2.1.6 *Interleukin 4*

Interleukin 4 is a pleiotropic cytokine produced by TH2 type helper T cells, mast cells and eosinophils. Its major functions include IgE class switching and effects associated with allergies and asthma.[47] It seems to be connected to tissue adhesion and inflammation, as high IL-4 levels have been found in rheumatoid arthritis patients. Yet, it also mediates anti-inflammatory effects.[8(p4),41(p6)]

3.2.1.7 *Interleukin 6*

Interleukin 6 is produced by various cells such as endothelial cells, fibroblasts, macrophages, T cells, B cells, smooth muscle cells, osteoblasts and many more. It responds to infections and tissue damages, aids in host defence through activation of acute phase proteins, haematopoiesis and immune reactions mediated by activation and differentiation of T cells, B cell differentiation and production of IgG, IgM and IgA. In patients with RA, high IL-6 mRNA is found in the synovium. [8(p4),41(p7),48(pp1-3)]

3.2.1.8 *Interleukin 12*

Interleukin 12 is a proinflammatory cytokine produced by monocytes, macrophages, neutrophils, DCs (dendritic cells) and B cells. Its main function consists of differentiation and maintenance of TH1 cells, but also on other proinflammatory processes such as activation of NK cells, induction of DC maturation and induction of cytotoxicity. IL-12 has been found in high level in the synovial tissue of patients, also exogenous supply of IL-12 seems to worsen rheumatoid arthritis. [8(p6),41(p8),49]

3.2.1.9 *JAK/STAT pathway*

The JAK/STAT signalling pathway starts with binding of cytokines to their corresponding receptors. Cytokines are able to bind to a particular set of receptors, capable of activating transcriptional regulators who are kept in an idle stat inside the cell, close to the cellular membrane. As soon as those regulators, called STAT-proteins (= signal transducers and activators of transcription), are being activated, they move towards the nucleus, triggering the transcription of certain genes. [50(p603),51(p3),52(p1),53]

As cytokine receptors possess no own enzymatic activity, they use cytoplasmatic tyrosine kinases, so called JAKs (= Janus kinases), to whom they are connected to.

As soon as a certain messenger substance, in this case type I/II cytokines, binds to its receptor, the signaling cascade is initiated. Type I/II cytokine receptors are built up by distinct chains, which oligomerize once cytokines bind to them. Oligomerization induce the separation of intracellular subunits of the cytokine receptors, thereby moving receptor-associated JAK-domains apart from inhibitory pseudokinase domains, causing their activation. Those JAKs are being phosphorylated and thereby activated, resulting in their phosphorylation as well as the intracellular portion of receptors serving as docking sites for STAT-proteins. In result, inactive cytosolic STAT-monomers are activated and phosphorylated as well. Phosphorylation begins with activation of JAKs by cytokines, which mediates phosphorylation on of receptor tyrosine residues. Through this, specific STATs are recruited and tyrosine-phosphorylated as well. Resulting STAT-monomers detach from the receptor complex and together, build a dimeric structure. STAT-dimers translocate towards the nucleus where they activate the transcription of specific genes. [50(p603),51(p3),52(p1),53]

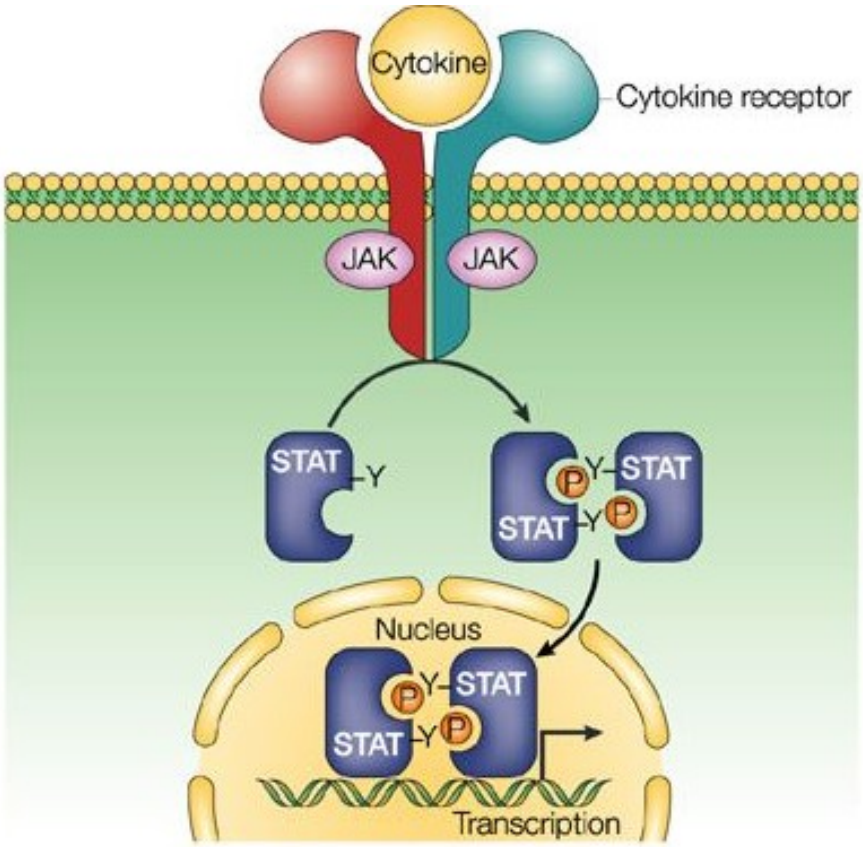

Figure 6 | ***The JAK–STAT pathway.*** *A schematic representation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. The activation of JAKs after cytokine stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription.* Reproduced with permission from Springer Nature [54]

In mammals, the Janus family of kinases consists of four JAKs, namely JAK1, JÁK2, JAK3 and TYK2 (tyrosine kinase 2), with seven STAT molecules serving as their signal transducers, STAT1, STAT 2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Those JAKs and STATs, creating the JAK-STAT pathway, are used by more than 50 cytokines and growth factors, with different cytokine receptors signalling through different JAKs. Each receptor is built by different subunits, with the subunits associating with a JAK. However, some receptor subunits associate selectively with one JAK, while others are less selective. Thus, the degree of how much a cytokine receptor relies on a JAK, depends on the different subunits the cytokine receptor is made of. Therefore, certain cytokines are transmitted selectively through one JAK, while others can be transmitted through more than one JAK. For example, IL2, IL4, IL7, IL9, IL15 and IL21 only use JAK3. It is important to keep this in mind when regarding therapeutic inhibition of the JAK-STAT pathway in order to stop the inflammatory cascade. [51,52,55(p118)]

As mentioned above, there are seven members of the STAT family, whereas normally, STAT-proteins are inactive cytoplasmic proteins, yet, after cytokine activation, STAT-proteins are

recruited to the signalling pathway - complex by becoming phosphorylated, promoting the formation of homodimers, heterodimers and also tetramers, which is necessary for them being translocated to the nucleus, where they bind to STAT-responding motifs, thereby acting as transcriptional factors. [51,55(p118)]

Another fact necessary to be addressed is, that there seem to be differences on how certain cytokines affect one and the same signalling pathway. For example, when looking at IL6 and IL10, both potent activators of STAT3 in myeloid cells, yet with IL6 provoking mostly proinflammatory effects whereas IL10 presents mostly anti-inflammatory features.

The following table, established from findings of George Stark et al[56] and Christian Schindler[53] shows the currently known cytokines and proteins to use the JAK-STAT signalling pathway and their associated JAKs as well as STATs. It should be mentioned, that the following data are still matter of research and discussion.

STATs	JAKs	Major cytokines
STAT1	JAK1, JAK2, TYK2	Type I, II, and III IFNs
STAT2	JAK1, TYK2	Type I and III IFNs
STAT3	JAK1, JAK2, JAK3, TYK2	IL-6 family cytokines, IL-10, IL-11, IL-27, IL-21, OSM, LIF, CNTF, NNT-1/BSF-3, G-CSF, CT-1, Leptin,
STAT4	JAK2, TYK2	IL-12, IL-23
STAT5 a/b	JAK1, JAK2, JAK3	IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, EPO, TPO, GM-CSF, GH, PRL,
STAT6	JAK1, JAK2, JAK3, TYK2	IL-4, IL-13

Table 9. ***Summary of cytokines and IFNs using JAK tyrosine kinases and STAT transcription factors to connect their cell-surface receptors to the activation of specific gene targets.*** [53(p1134),56(p3)]

(STATs - Signal transducers and activators of transcription; JAKs - Janus kinases; IFN - interferon; IL - interleukin; OSM - Oncostatin M; LIF - leukaemia inhibitory factor; CNTF - ciliary neurotrophic factor; NNT-1/BSF-3 - novel neurotrophin-1/B cell–stimulating factor-3; CT-1 - cardiotropin-1; EPO - erythropoietin; TPO - thrombopoietin; GM-CSF - granulocyte macrophage colony-stimulating factor; GH - growth hormone; PRL - prolactin)

3.2.1.10 *Signal transducer and activator of transcription 1 (STAT1):*

STAT1is the first transcription factor in the STAT family, its phosphorylation site is Tyr701. Similar to STAT3 and STAT5, Stat1 is shown to be activated by many different cytokines, such as IFNα, IFNγ and cytokines from the IL-6 family. [57,58] Studies suggest that the expression of STAT1, alongside with the expression of STAT6, is upregulated in synovial lymphocytes, macrophages and fibroblasts in inflammatory arthritis and also declines with

successful response to DMARDs.[59] Its antiviral effects will be discussed below together with STAT2.

3.2.1.11 *Signal transducer and activator of transcription 2 (STAT2):*

STAT2, the second member of the STAT family has its phosphorylation site on Tyr690. Unlike other STATs, it is almost uniquely phosphorylated and activated by IFNα/β, cytokines responsible for activation of the ISGF-3 (IFN-stimulated gene factor 3) complex which is also activated by STAT1 through IFNα and IFNγ.[58] Thereby, STAT1 and STAT2 are the main mediators for responses to type I and III IFN. Together with the interferon regulatory factor (IRF) 9, they are capable of controlling viral infections as well as intracellular bacteria and parasites.[60,61]

3.2.1.12 *Signal transducer and activator of transcription 3 (STAT3):*

The STAT3 transcription factor is an important signaling molecule and is used by numerous cytokines and growth factors, therefor STAT3 has multiple biological roles with transcriptional functions such as the mediation of growth factors, IL-6 cytokines, G-CSFs and many more, as well as non-transcriptional activities regarding cellular respiration, autophagy, cancer development, foetal development and metabolism. STAT3 may play an essential role in anti-inflammatory responses induced by IL-10. Also, inadequate function of STAT3 seems to be linked to immune disease yet extended insight into cell type specific activities is still needed[58,62,63]. A 2017 study from Takatsugu Oike et al [64] stated STAT3 as a main promoter of inflammation and joint erosion in mice and suggested STAT3-inhibitors as promising drugs for RA therapy.

3.2.1.13 *Signal transducer and activator of transcription 4 (STAT4):*

STAT4 is primarily activated by IL-12, a cytokine playing a major role in in controlling T helper cell differentiation along the Th1 pathway.[58] Other activators of STAT4 are IFNα, IL-2, IL-18, IL-21, IL-23 and IL-35. STAT4 plays a crucial part in regulating immune cells such as differentiation of T helper 1 cells and their ability to inhibit follicular cells as well as its important role for the function of NK and CD8+ cells, for the differentiation of B cells and regulatory T cells. Unsurprisingly, inadequate function und dysregulation of STAT4 have been found to be linked to autoimmune diseases and their severity.[65]

3.2.1.14 *Signal transducer and activator of transcription 5 (STAT5):*

STAT5 is a unique STAT family member as it is comprised of two different STAT5s. STAT5a and STAT5b are highly homologous proteins with different phosphorylation sites. STAT5a is phosphorylated at Tyr 694 and STAT5b at Tyr699, with numerous activating cytokines including growth hormone (GH), erythropoietin (EPO) prolactin (PRL) and several ILs like

IL-2, -3, -5, -7, -9 and-15. STAT5a has been found to be more present in mammary tissue whereas STAT5b is more prevalent in muscle and liver. Their functions include modulation of cell differentiation, lipid mobilization, lymphocyte development and oncogenesis. A dysregulation of STAT5 proteins is found promote tumours activity by inhibiting antitumor activity due to their role in expanding regulatory T cells. STAT5 also seems to play a part in maintaining normal immune function and homeostasis of T-cells and NK- cells which are regulated by specific IL-2 members. [58,66,67]

3.2.1.15 *Signal transducer and activator of transcription 6 (STAT6):*

STAT6 is mainly activated by IL-4 and the highly related IL-13. STAT6 seems to be necessary for IL-4 to induce the differentiation of Th2 cells.[58] An increased expression of STAT6 is found in synovial lymphocytes, macrophages and fibroblasts in inflammatory arthritis. It declines with successful response to DMARDs.[59]

3.2.2 Cluster of differentiation

Leucocytes can be divided into numerous subgroups, which can be differentiated either by their expression of cytokines or by their Cluster of differentiation. Cluster of differentiation (CDs) antigens refer to cell surface molecules expressed on leukocytes among other immune active cells. Certain monoclonal antibodies are capable of recognising same cell surface antigens and therefor labelled as Cluster of differentiation(e.g. CD3, CD4,…). [68,69]

3.2.2.1 *Cluster of differentiation 3 (CD3):*

The CD3 antigen is a complex composed of major and minor glycoproteins. Those glycoproteins, depending on their size, are identified as alpha, beta, gamma, delta or epsilon chain. CD3 is expressed by thymocytes in their developmental stages and by all mature T cells. Together with the TCR and the so called zeta chains, CD3 forms the TCR complex, which serves as the primary trigger for activation in T-Lymphocytes and the clonal expansion of antigen-specific cells from the T cell reservoir.[70–72]

3.2.2.2 *Cluster of differentiation 4 (CD4):*

The CD4 glycoprotein is a cell surface receptor expressed on the surface of immune cells such as T helper cells and thymocytes and in low levels on monocytes, macrophages and dendritic cells. T helper cells are a subpopulation of mature T cells, due to their surface expression of CD4, they are also called $CD4^+$ cells. Likewise, $CD4^+$ cells present a series of distinct cell populations with different functions and can be divided into different subsets, Th1, Th2, Th17 and iTreg cells. They play a major part in protective immunity and autoimmunity due to their capability to produce cytokines and chemokines which induce development of enhanced microbiological activity in macrophages and recruit neutrophils, basophiles and eosinophils to

a focus of infection and inflammation. $CD4^+$ cells thereby orchestrate the full range of immune response. [73–75]

Th1 cells are responsible for mediating immune responses against intracellular pathogens by stimulating macrophages, cytotoxic $CD8^+$ T cells and the promoted production of IgG via their main cytokines IFNγ, IL-2 and LTα (lymphotoxin α). Particularly important is their role against mycobacterial infections and their role in the CD8 memory formation.

Th2 cells are important in the host defence against extracellular parasites, leading to a humoral immune response. Also, Th2 cells are important in the pathology of asthma and other allergic diseases, mediating induction and persistence of named diseases. They display their main effects on B cells, mast cells and eosinophils via IL-4, -5, -9, -10, -13 and IL-25.

Th17 cells induce proinflammatory immune responses against extracellular bacteria and fungi mainly via IL-17 but also IL-21 and IL-22. [73–75]

Treg cells, CD4+ cells stimulated by TGFβ, seem to play a critical role in maintaining immune self-tolerance as well as regulating immune responses. Through IL-10, Treg cells seem to be capable of switching B cell´s production of immunoglobulins to another type of immunoglobulins, for example from IgM to IgG. This mechanism is called Ig-class switching. It is not surprising that CD4+ cells are crucial for an adequate immune response and also for the pathogenesis of many inflammatory and autoimmune diseases. Some examples are multiple sclerosis, psoriasis and asthma. [73–76]

3.2.2.3 *Cluster of differentiation 16 (CD16):*

CD16 is expressed as two forms, CD16a and CD16b. CD16a is expressed on the surface of NK cells, activated monocytes, macrophages and placental trophoblasts. CD16b on the other hand is specifically expressed on neutrophils. Its main function evolves around the binding of IgG or IgG-antigen-complexes which results in NK cell activation, phagocytosis and antibody-dependent cell mediated cytotoxicity.[77] Studies have found increased activity of CD16 alongside CD14 in patients with rheumatoid arthritis as well as other inflammatory diseases. In patients with RA, increasing activity was found to correlate with erythrocyte sedimentation rate, CRP and rheumatic factor. The rise was most pronounced in active disease and reduced in patients with well responding therapie.[78–80]

3.2.2.4 *Cluster of differentiation 19 (CD19):*

The CD19 antigen, another member of the immunoglobulin family, is expressed only on B lymphocytes and serves as important co-receptor for the B cell antigen receptor (BCR) signal transduction and plays a crucial role in B cell maturation. As CD19 is critical for an adequate B-cell response, abnormal expression of CD19 leads to B-cell related diseases and immune-

deficiency. For example autoimmune diseases, such as systemic sclerosis, systemic lupus erythematosus and experimental autoimmune encephalomyelitis.[81]

3.2.2.5 *Cluster of differentiation 45RA (CD45RA):*

CD45 is a receptor linked protein tyrosine phosphate expressed on leucocytes. CD45 is characterised by its expression of several isoforms, specific to a certain cell type. In T cells, the two main isoforms of CD45 are CD45RO, the low molecular weight form of CD45, expressed predominately on T memory cells and CD45RA, the high molecular weight form, mainly expressed on native T cells. CD45 is one of the key players in the induction of T cell receptor signaling through activation in the TCR-CD3 complex and on CD4/CD8 -cells and the adhesion molecule CD2. Therefore, deficiency in CD45 results in B-and T lymphocyte dysfunction presenting as immune deficiency, autoimmune diseases and cancer development.[82,83]

3.2.3 Transcription factors

The term transcription factor (TF) designates proteins regulation the rate of genetic transcription. By recognizing distinct DNA sequences regarding transcription, TFs guide the genomes expression. The specific mechanism of TFs and their distinct regulation of transcription is not yet fully surveyed, yet currently over 1.600 likely human TFs are catalogued. Certain TFs, like T-bet, may be related to autoimmune disease.[84]

3.2.3.1 *T-bet:*

T-bet, an immune cell-specific member of the T-box family of transcription factors is recognized to have an important role in many cells of the adaptive and innate immune system, where it is essential for the survival, development and proper function of those cells. Thereby T-bet is a key molecule in the coordination of effective and appropriate type 1 immunity. As a result, inadequate function T-bet may be causally connected to autoimmune diseases, transplant rejection, cancer development and infectious diseases.[85] *The following figure from "T-bet: A bridge between innate and adaptive immunity" by Lazarevic, Glimcher and Lord* describes the expression and function of T-bet in immune cells.

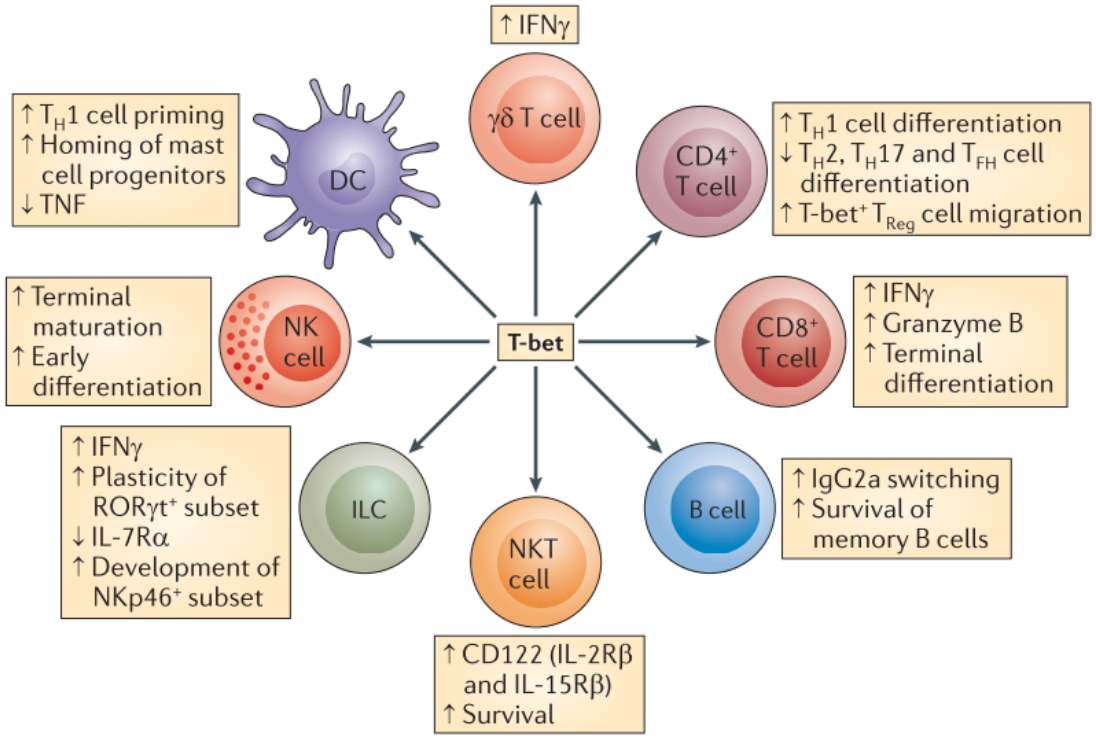

Figure 7 | **Expression and function of T-bet in immune cells.** Reproduced with permission from Nature Reviews Immunology [85(p2)]

3.3 The role of JAK/STAT-signaling in rheumatoid arthritis

In patients with RA, the physiologic synovial membrane undergoes hyperplastic and inflammatory changes, due to an increased influx of immune and nonimmune cells. This migration is influenced by elevated levels of certain proinflammatory cytokines and adhesion proteins, for example TNFα, various interleukins and growth factors. As a result, activated immune cells lead to a clinical progression of RA with the destruction of articular cartilage and subchondral bone. Thus, these factors and their signaling pathway are important components in the development and aggravation of RA. [51,55]

Although the mechanisms responsible for the development of RA are still a matter of further research, several signaling pathways have been suggested to be crucial in this progression of RA. Examples would be the activation of the stress-activated, mitogen-activated protein kinase (SAPK/MAPK) pathway, the PI3K/Akt/mTOR pathway and the JAK/STAT pathway, all predominantly activated by cytokines essential in the pathogenesis of immune-mediated diseases like RA. [51,55] In this context, research has focused increasingly onto signaling pathways in immune disease.

Focusing on the JAK/STAT pathway, under normal circumstances this pathway is regulated by feedback mechanisms, for example a negative-feedback inhibition of cytokine signaling. In patients with rheumatoid arthritis, those mechanisms are malfunctional, resulting in

continuous activation of the JAK/STAT pathway and elevated levels of pathogenic factors as metalloproteinase genes and apoptotic chondrocytes. In addition, the affected synovial tissue seems to develop a resistance against apoptosis. [55]

A study from 2016 showed a significantly higher expression of STAT1, STAT3, STAT4, STAT5 and STAT6 in peripheral blood leucocytes and synovial fluid in RA patients, compared to control groups.[86] Another 2015 study suggested an elevated expression and phosphorylation of STAT3 in T cells and monocytes in patients with RA, compared to healthy controls. Also, IL-6 levels correlated with the activation of STAT3 in RA.[87]

Further approval for the importance of the JAK/STAT pathway in RA can be established by the recently approved JAK-inhibitors, tofacitinib and baricitinib. Tofacitinib a JAK1/3-inhibitor, and baricitinib, a JAK1/2- inhibitor were currently approved and integrated into the therapy of RA. In a 2016 report from a phase III randomized controlled trial, Tofacitinib in combination with conventional DMARDs led to statistical significant improvements compared to placebo treatment at month 3 of therapy [88]. As a future prospect, next-generation JAK-inhibitors may be more selective than current JAK-inhibitors, resulting in fewer adverse effects such as cytopenia.

4 Aim of the study

The binding of certain cytokines to cell-surface receptors activates JAK molecules, which, thereupon, phosphorylate STAT molecules intracellular. Thus, the phosphorylation of STAT molecules allows conclusion onto the contact of cells with cytokines and growth factors. In rheumatoid arthritis, this is of further interest, as the pathogenesis of RA is connected closely to cytokines. However, the measurement of cytokines from blood is limited, due to local distribution and a short half-life.

We expected the phosphorylation of STAT molecules to be a superior indicator for the distribution of cytokines. Apart from a better understanding of the pathogenesis of RA, this indicator could be used in the detection of RA as well as the monitoring of therapy and remission. Regarding the therapy of RA, it might show the response of patients to medication, especially in case of selective targeted JAK-inhibitors (targeted synthetic DMARDs).

We also assumed the presence of certain endotypes within the RA, with different pathogenesis and varying response to therapy. The definition of patients STAT-phosphorylation and consequently their cytokine milieu could help to characterise this endotypes. This characterisation would either provide further insight into the pathogenesis of RA, but also into different therapeutic strategies

Subsequently, we proposed the following hypotheses:

- There is a difference between patients with rheumatoid arthritis and healthy patients regarding the phosphorylation of STAT-molecules in leucocytes.
- There are subgroups within the patients collective (so-called endotypes).

In order to focus on these issues, we aimed to examine the phosphorylation of STAT molecules in leucocyte populations of patients with RA and healthy individuals. To obtain the required data, we established a laboratory protocol capable of depicting the JAK-STAT signaling pathway, which was performed by measuring the mean fluorescent intensity (MFI) of leucocytic cells. MFI would represent phosphorylation in STAT-molecules, thereby showing the activity of the JAK-STAT pathway. The established laboratory protocol was also planned to further enlarge patient's data set for additional research.

5 Material and Methods

5.1 Patients

We defined one patients' group with 30 patients and a control group with 10 individuals. The main inclusion criterion for the patients' group was defined as the current presence of rheumatoid arthritis. No exclusion criteria were defined for a certain age. Both genders were included equally.

The control group was defined as individuals without the current presence of RA. Again, both genders were included.

In our prospective study 17 patients with the diagnosis of rheumatoid arthritis were recruited at the rheumatology outpatient clinic of the Medical University of Graz. As control group, 6 healthy persons from the medical and non-medical staff were used. In total, we received blood samples from 23 person. Permission to perform this survey was obtained from the Review Board of the Medical University, Graz. Participants were required to sign a written informed consent.

One EDTA- tube of venous peripheral blood was collected from each participant.

Participants blood was tested for a number of present cytokines by the in-house laboratory of immunology. The following cytokines were analysed:

- Interleukin 1RA (IL1RA)
- Interleukin 5 (IL5)
- Interleukin 8 (IL8)
- Interleukin 10 (IL10)
- Interleukin 17A (IL17A)

- Interleukin 18 (IL18)
- Tumor necrosis factor α (TNFα)
- Interferon α (IFNα)
- Interferon γ (IFNγ)
- Interferon gamma-induced protein 10 (IP-10)

5.2 Procedure

The procedure to obtain participants cellular data for statistical evaluation included:

- Collection of one tube of venous peripheral blood.
- Flow Cytometry - Preparing and stimulating participants blood sample prior to a staining process followed by flow cytometric analysis with FACS (fluorescence activated cell sorting)
- Analysis of received cellular data with the FlowJo10 program

The following chapters are going to depict the stated procedure in detail.

5.2.1 Analysing pSTATs

The staining of static molecules does not provide data about dynamic cellular events, as it does not regard signaling events involved in cellular stimulation. Thus, in order to gain data about dynamic events, additional to using fluorochrome conjugated antibodies, antibodies specific to the phosphorylated form of certain cells are applied. This is performed due to the knowledge that protein phosphorylation is a frequent part of signaling cascades. [89,90]

In this case, we used STAT1-6 -specific antibodies to analyse the distribution of phosphorylation, and thereby activity, of each STAT-protein in immune cells. As described above, staining was performed with two different panels. Panel 1 contained antibodies specific to STAT1, STAT2 and STAT6. Panel 2 contained antibodies for STAT3, STAT4 and STAT5.

5.2.2 Flow Cytometry

Flow cytometry is a laser-based technology, used for the measurement of physical and biochemical parameters from heterogenous populations of biological particles such as whole cells as well as cellular components. A flow cytometer scans those particles individually and one at a time while they flow in a carrier medium past an excitation light source, a laser beam. As the laser beam strikes the moving individual cells, two phenomena occur, light is scattered and fluorescence is emitted, both events are recorded.

The scattering of light is directly related to morphological and structural characteristics. The occurrence of fluorescence emission happens when the measured particles are attached to fluorescent probes. Those probes are typical monoclonal antibodies conjugated to

fluorochromes, targeting specific antigens of the cell particle. In order to achieve fluorescence in a flow cytometric measurement, those fluorescent probes must attached to the particles of interest prior to the flow cytometric analysis. [91,92]

The process of cell marking by targeting cell specific antigens with antibodies conjugated to a fluorochrome is called antibody staining, its idea is to bring fluorochrome-conjugated antibodies in contact with their specific antigens and thereby staining those antigens and their related cell particle. In the flow cytometric analysis, the stained antigens are excited by laser light resulting in fluorescent emission. Depending on the used fluorochrome, different wavelengths of lasers are required. Currently, up to seven lasers with wavelengths between 325 to 650 nm are used to achieve adequate emission of fluorescence. [93]

As the main question of this paper evolves around leucocytes, we used antibodies specific to different subgroups of leucocytes. Leucocytes can be divided into numerous subgroups, which can be differentiated either by their expression of cytokines or by their Cluster of differentiation. Cluster of differentiation (CD) antigens refer to cell surface molecules expressed on leukocytes among other immune active cells. [68,69]

Beside the staining of different CD antigens, we also used antibodies specific to various STAT proteins. Those antigens, commonly used as a target for antigen staining and therefore used as cell markers for the identification of leucocyte population are described in detail later.

5.2.2.1 *Principles of Flow cytometric Instrumentation*

The flow cytometric analysis was performed by the FACS Canto II from BD Bioscience. FACS stands for fluorescence activated cell sorting, and should only be used for BD technologies, as it is a BD trademark. The more general term would be “flow cytometry cell sorting”. It describes a renowned tool in immunology used to define and count cell types by using light scattering and fluorescence emitted by fluorochrome labelled antibodies against cell-surface proteins or intracellular proteins. [69,93]

Basically, flow cytometers consist of four systems:

- *The fluidic system*, used to transport particles through the instrument
- *The illumination system*, for intercepting and exciting particles with laser light, leading to light scattering and fluorescent emission.
- *The optical/electronic system*, used for detection, collection and translation of scattered and fluorescent light caused by the *illumination system*.
- *The computer/ data storage system*, finally interpreting translated light, converting it into meaningful data. [91]

Inside the flow cytometer, the cell suspension containing antibody stained cells is carried along within a rapidly flowing stream of liquid (*fluidic system*), this stream is forced through a nozzle, resulting in a fine stream. This process, called hydrodynamic focusing, makes sure that the cells surpass the measuring station one by one. This measuring station consists of focused lasers (*illumination system)* and associated detectors (*optical/electronic system)*.
When passing through the focus laser, cells or cell-particles scatter light according to the cells size, form and granularity. Also, fluorescent dyes bound to antibodies are stimulated, thereby emitting fluorescence.
After the laser beam strikes the particles, light is scattered and emitted in all directions (360°). The according detectors (*optical/electronic system)* sense at least five types of parameters from the scattered and emitted light output. Two of these parameters refer to light-scattering, *a forward scatter detector* measures the amount of light emitted when the laser strikes the cell, which is usually proportional to the cells size, whereas the *side scatter detector* usually receives information about the cell's granularity. Together with additional detectors, it quantifies the fluorescence emission. Afterwards, the collected data referring light scattering and fluorescence are converted into electrical signals and converted into digital data, which are interpreted and visualized by *the computer/ data storage system.* Thereby every cell is assigned its size, granularity and fluorescence emission, making it possible to identify them as specific subtype of leucocytes.[91,92]
The following *Figure 8* depicts the above described structure of a FACS cantometer.

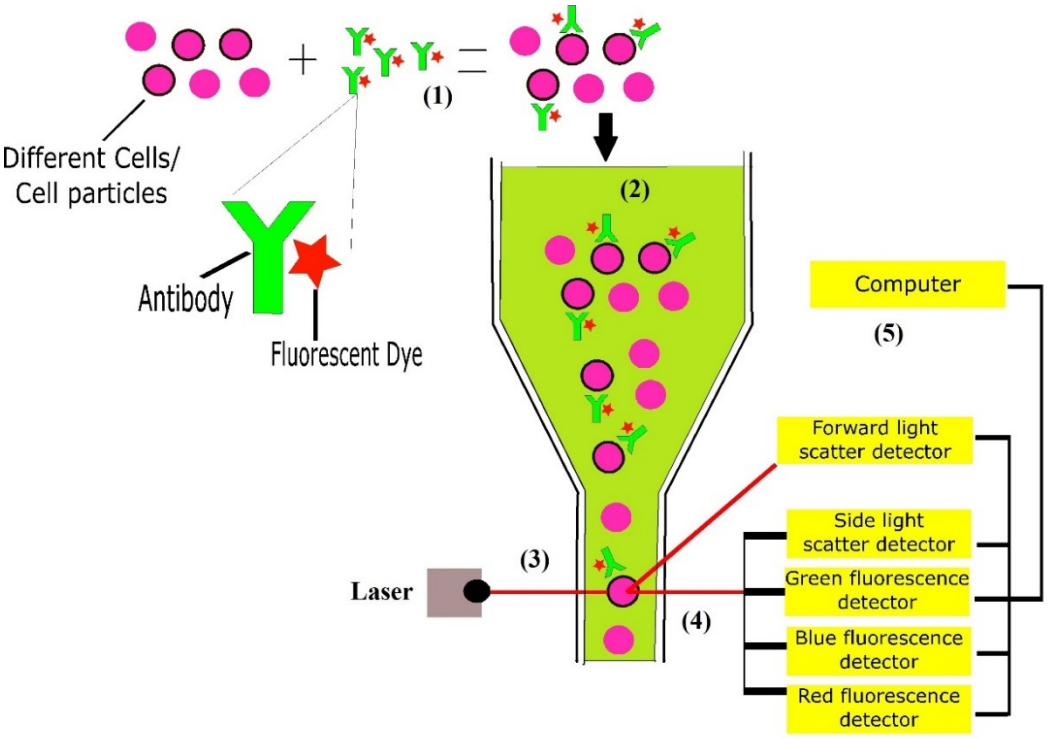

Figure 8 | **Basic structure of FACS (fluorescence activated cell sorting) cantometer.**
First (1) comes the staining of particles of interest with fluorescent dye conjugated to specific antibodies. The received cell suspension enters the fluidic system (2), the particles are individually transported through a nozzle, there they intercepted by laser light (3), the resulting fluorescence and light shattering is collected by specific detectors (4), from there, the collected data are interpreted and visualized by a computer (5).

5.2.3 Laboratory protocol

We established our STAT-signaling protocol based on the BD Phosflow Staining Protocols. In order to add the possibility of stimulating the patient's blood before the staining process, BD Phosflow Staining protocols were modified. The idea was to split every patient's blood into one sample which would not be stimulated and one sample which would be stimulated with the following cytokines prior to applying the Staining protocol to the patient's blood:

- Interleukin-2 (Recombinant Human IL-2, BD Pharmingen™, Catalog No.554603, 200 µg/ml)
- Interleukin-4 (Recombinant Human IL-4, BD Pharmingen™, Catalog No. 554605, 100 µg/ml)
- Interleukin-6 (Recombinant Human IL-6, BD Pharmingen™, Catalog No. 550071, 200 µg/ml)
- Interleukin-12 (Recombinant Human IL-12(p70), BD Pharmingen™, Catalog No. 554613, 100 µg/ml)
- Interferon-α (Purified NA/LE Mouse Anti-Human IFNα (2b), BD Pharmingen™, Catalog No. 551795, 1000 µg/ml)

Used laboratory equipment:

- Vortex (Heidolph REAX 2000)
- Centrifuge (Megafuge 1.0R from Heraeus)
- BD FACSCanto™ II

Reagent list:

- 5X Lyse/Fix Buffer (BD Phosphoflow Cat. No.558049, Lot. No. 8088847) [94]
- Perm Buffer III (BD Phosphoflow Cat. No. 558050, Lot. No. 8299805) [95]
- PBS (Phosphate Buffered Saline) pH 7,4 1X (gibco Ref. No. 10010-015, Lot. No. 2104339)
- Staining Buffer (invitrogen by ThermoFisher Scientific Ref. No. 00-4222-26, Lot. No. 2059560) [96]

We started the STAT-signaling protocol by preparing 1X Lyse/Fix Buffer according to the TDS (Total Dissolved Solids) instructions by diluting 5X Lyse/Fix Buffer in distilled or deionized water (requires 2mL/sample). 1X Lyse/Fix Buffer was warmed to 37°C prior to use. Perm Buffer III was chilled to -20°C, Staining Buffer was chilled to 4°C, PBS was kept at room temperature. Frozen IL2, IL4 and IL6 (each at -80°C) and -20°C frozen IL12 and INFα (each on -20°C) was slowly defrosted on ice. Per each patient, two tubes were prepared, one tube labelled “stimulated” and one tube labelled “unstimulated”.
Each tube was filled with approximately 200µL of whole blood (EDTA).
While “Unstimulated” blood was kept on ice, “Stimulated” tubes were stimulated with each:

- 0,2 µg of IL2
- 0,1 µg of IL4
- 0,4 µg of IL6
- 0,05 µg of IL12
- 0,5 µg of IFNα

Afterwards, the “stimulated” tubes were incubate at 37°C for 5 minutes. Both “stimulated” and “unstimulated” cells were then fixed by adding 2mL of pre-warmed Lyse/Fix Buffer to the samples. Cells were mixed well by vortexing in order to ensure complete erythrocyte lysis. All cells were incubated at 37°C for 10 minutes. Panels were then centrifuged at 600g for 6 minutes, supernatant was removed (ca. 50 µL residual volume) and panels again vortexed to disrupt the remaining cell pellet.
2mL of PBS (equivalent to the volume of Lyse/Fix Buffer used) was added, again, panels were centrifuged at 600g for 6 minutes; supernatant was removed and cells vortexed. Cells were then permeabilized by adding 1mL of pre-chilled Perm Buffer III (for approximately 1-

10 x 10^6 cells). Cells were again vortexed and incubated on ice for 30 minutes. Cells were then washed by adding 3mL of Staining Buffer (at least 3mL Staining Buffer for every 1mL Perm Buffer used), centrifuging them at 600g for 6 minutes, removing supernatant and vortexing them. This washing procedure was performed overall three times by repeating the step. During all three washing procedures we ensured that Staining Buffer was put back into the refrigerator. After the washing process, cells were then resuspended in 100 µL Staining Buffer (ca. 0,5-1x10^6 cells/100 µL), each tube (both "stimulated" and "unstimulated") was split onto two new tubes (Panel 1 and Panel 2) with 50 µL each and stained with the following products, described in *Table 10* for Panel 1 and *Table 11* for Panel 2:

Panel 1 (P1)	**Dye**	**Company**	**Nr.**	**Concentration/ 50 µL test**
STAT1	AF647 (APC)	Cellsignaling	8009	25,0 µg
STAT2	PE	Cellsignaling	77366	0,1 µg
STAT6	AF488 (FITC)	BioLegend	68600	0,5 µg
CD3	PerCP	BD	345766	0,125 µg
CD19	V450 (PacificBlue)	BD	560353	0,0125 µg
CD16	APC-Cy7	BioLegend	302018	0,5 µg

Table 10 | ***Flowcytometric staining products used in the staining of Panel 1***

Panel 2 (P2)	**Dye**	**Company**	**Nr.**	**Concentration/ 50 µL test**
STAT3	AF647 (APC)	Cellsignaling	4324	0,05 µg
STAT4	PE	Cellsignaling	13223	0,003 µg
STAT5	AF488 (FITC)	Cellsignaling	3939	0,075 µg
CD3	APC-eF780 (APC-Cy7)	eBio	47-0036-42	0,125 µg
CD4	eF506 (AmCyan)	eBio	69-0049-42	0,125 µg
CD45RA	PE-Cy7	BD	337186	0,125 µg
Tbet	PerCP-Cy5.5	eBio	45-5825-80	0,5 µg

Table 11 | ***Flowcytometric staining products used in the staining of Panel 2***

After the staining process, cells were vortexed and incubate at room temperature for 60 minutes and protected from light.

Finally, cells were again washed by adding 3mL of Staining Buffer (at least 3mL Staining Buffer for every 1mL Perm Buffer used), centrifuging them at 600g for 6 minutes, removing supernatant and vortexing them. Subsequently, they were resuspended in 100 µL Staining Buffer prior to flow cytometric analysis. Flow cytometric analysis was performed with BD FACSCanto™ II.

5.2.4 Samples

We performed different sets of analyses, with samples either being stimulated or unstimulated and samples being either stained with dyes conjugated to specific antibodies from Panel 1 or Panel 2. Thus, 4 different sets of analysis were performed onto patient's blood:

- Stimulated Panel 1
- Unstimulated Panel 1
- Stimulated Panel 2
- Unstimulated Panel 2

The following *Figure 9* shows a brief summary of the performed staining.

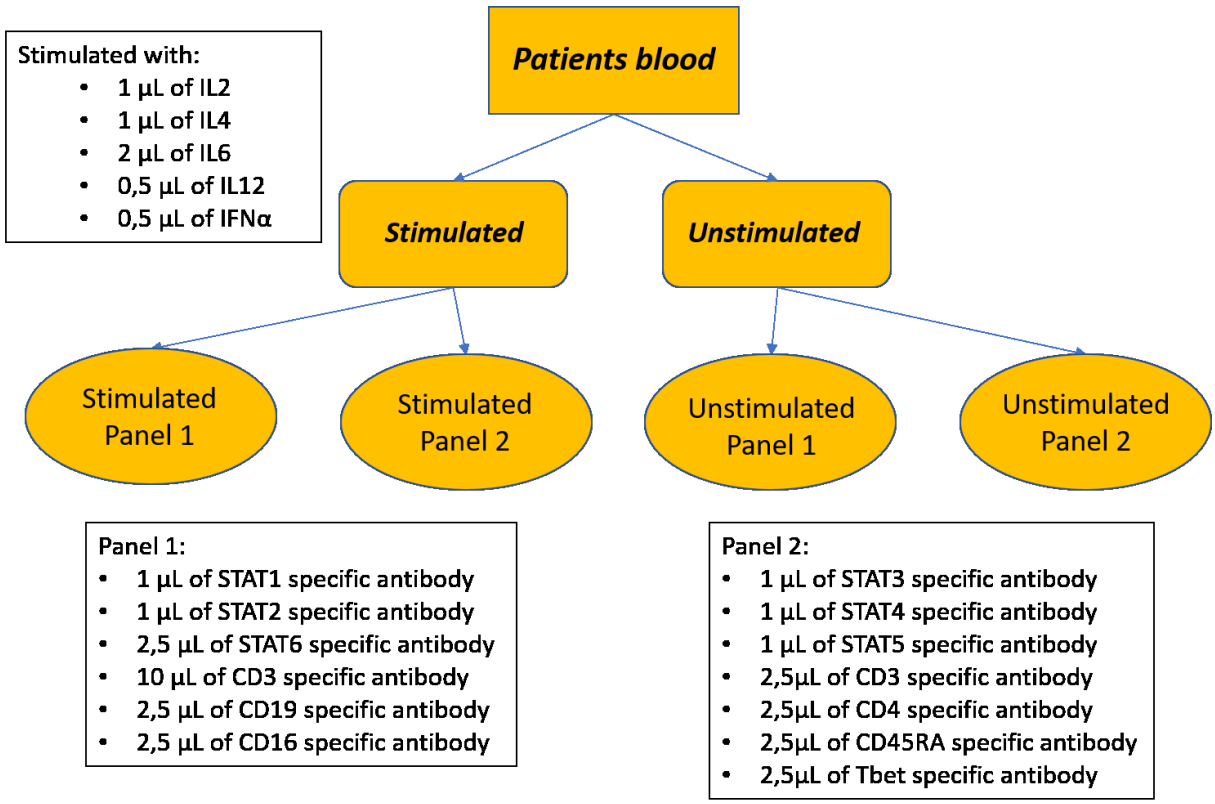

*Figure 9 | **Summary of performed sets of analysis***

5.2.5 Immunostaining

In the staining process described in the stat-signaling protocol in chapter *3.2.1. laboratory protocol,* the cells are stained with an antibody conjugated to a specific dye. The following

Table 12 and *Table 13* describe the used dyes and their conjugated antibodies from Panel 1 and Panel 2 as well as their functional mechanisms.

Products name/ company	***Obtained from:***	***Products conc.:***	***Functional mechanism***
AF647 (APC) Nr. 8009/ Cellsignaling	*Rabbit IgG*	*25.0 µg/µl*	Conjugated to STAT1-specific antibodies. Detects STAT1-antigen when phosphorylated at Tyr701. Detects Tyr701 of both STAT1α and STAT1β. [97]
PE Nr. 77366/ Cellsignaling	*Rabbit IgG*	*100.0 µg/ml*	Conjugated to a STAT2-specific antibody. Detects STAT2-antigen when phosphorylated at Tyr690. [98]
AF488 (FITC) Nr. 68600/ BioLegend	*Mouse IgG1*	*200.0 µg/ml, yet lot-specific; 5µl/ 100µl staining volume is recommended*	Conjugated to a STAT6-specific antibody. Detects STAT6-antigen when phosphorylated at Tyr641.[99]
PerCP Nr. 345766/ BD	*Mouse IgG1, κ*	*12.5 µg/ml*	Conjugated to monoclonal mouse anti-human antibodies. Detects CD3-antigen when reacting with the epsilon chain of the CD3antigen/T-cell antigen receptor (TCR).[100]
V450 (PacificBlue) Nr. 560353/ BD	*Mouse IgG1, κ*	*5.0 µg/ml*	Conjugated to HIB19 monoclonal mouse anti-human antibodies that react with the CD19 glycoprotein expressed during all stages of B-cell differentiation and maturation, except on plasma cells.[101]
APC-Cy7 Nr. 302018/ BioLegend	*Mouse IgG1*	*200.0 µg/ml, yet lot-specific; 5µl/ 100µl staining volume is recommended*	Conjugated to monoclonal anti-human CD16 antibodies. Recognises cells expressing both distinct forms of CD16. (CD16a/CD16b).[77]

Table 12 | ***Dyes and their conjugated antibodies used in the immunostaining process of Panel 1.***

Products name/ company	***Obtained from:***	***Products conc.:***	***Functional mechanism***
AF647 (APC) Nr. 4324/ Cell Signaling	*Rabbit IgG*	*50.0 µg/ml*	Conjugated to Phospho-STAT3 antibodies, detects STAT3-antigens at Tyr705. STAT3 isoform expressions may differ between STAT3α (86 kDa) and STAT3β (79 kDa). [63]
PE Nr. 13223/ Cell Signaling	*Rabbit IgG*	*3.0 µg/ml*	Conjugated to Phospho-STAT4 antibodies. Detects STAT4-antigens when phosphorylated at Tyr693. [102]
AF488(FITC) Nr. 3939/ Cell Signaling	*Rabbit IgG*	*75.0 µg/ml*	Conjugated to Phospho-STAT5 antibodies, Detects STAT5a-antigens at Tyr694 and STAT5b-antigens at Tyr699.[103]
APC-eF780 (APC-Cy7) Nr. 47-0036-42/ eBio	*Mouse IgG1, kappa*	*50.0 µg/ml*	Conjugated to SK7 monoclonal antibodies, Detects CD3e-antigens by reacting with human and chimpanzee CD3e. [72]
eF506 (AmCyan) Nr. 69-0049-42/ eBio	*Mouse IgG1, kappa*	*50.0 µg/ml*	Conjugated to RPA-T4 monoclonal antibodies, reacts with human CD4. [73]
PE-Cy7 Nr. 337186/ BD	*Mouse IgG_1, κ*	*50.0 µg/ml*	Conjugated to the CD45RA antibody, used for identifying cells expressing CD45RA antigen.[104]
PerCP-Cy5.5 Nr. 45-5825-80/ eBio	*Mouse IgG1, kappa*	200 µg/mL	Conjugated to eBio4B10 monoclonal antibodies reacts with mouse and human T-bet.[105]

Table 13 | ***Dyes and their conjugated antibodies used in the immunostaining process of Panel 2.***

5.2.6 Data analysis with FlowJo

To analyse the cellular data received from the flow cytometry, the leucocyte's subgroups were visualized and classified. This was performed with the program FlowJo10.

When opening FlowJo, the workspace window, shown in *Figure 10* and *Figure 11*, appears. Basically, it can be separated in three sections. At the top is the *"workspace ribbon"*, containing functions of FlowJo. Below the ribbon is the "*groups"* section, it contains groups

of samples and, after they are defined, their classification in groups and subgroups. Every sample added to the group is classified according to the defined classification of subgroups, thus making it easier to organise cell samples into subgroups. Last comes the *"samples"* section. Samples received from the flow cytometric analysis are added to this section, where they are listed as cellular groups as well as subgroups. In addition, it shows information about the sample, like cell count and it's percentual distribution. The visualizations of each sample and it`s subgroups are accessed from the *"samples"* section.

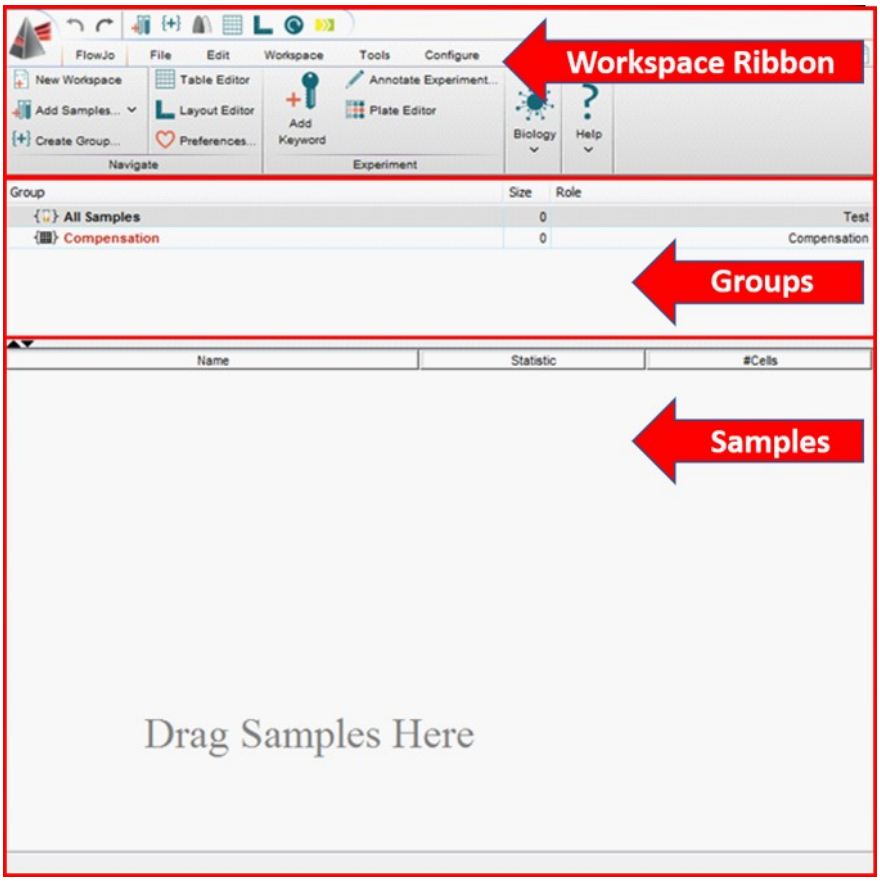

Figure 10 | ***The workspace window of FlowJo*** *and its three main sections.*

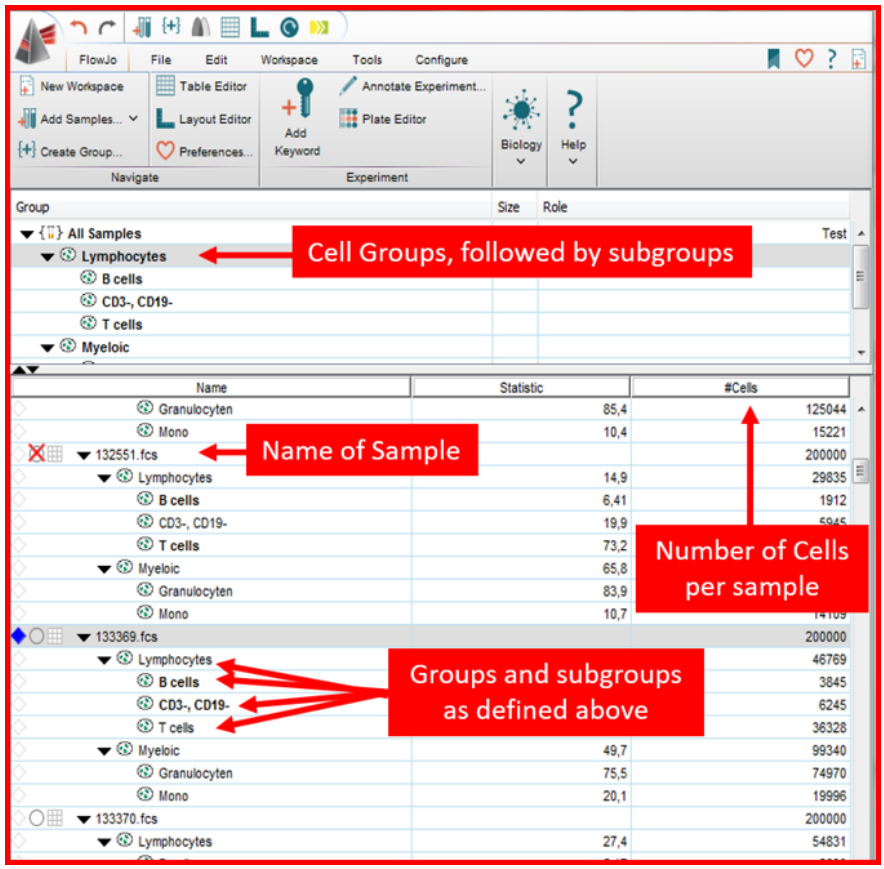

*Figure 11 | **The workspace window** after samples where added and their subgroups defined.*

5.2.6.1 *Double parameter graph*

As seen in *Figure 12*, when selecting a separate sample, the "*Graph window"* opens. In here, every cell of the according sample is represented as a dot and arranged to a graph along one or two cytometric parameters depending on its characteristics measured in the flow cytometric analysis.

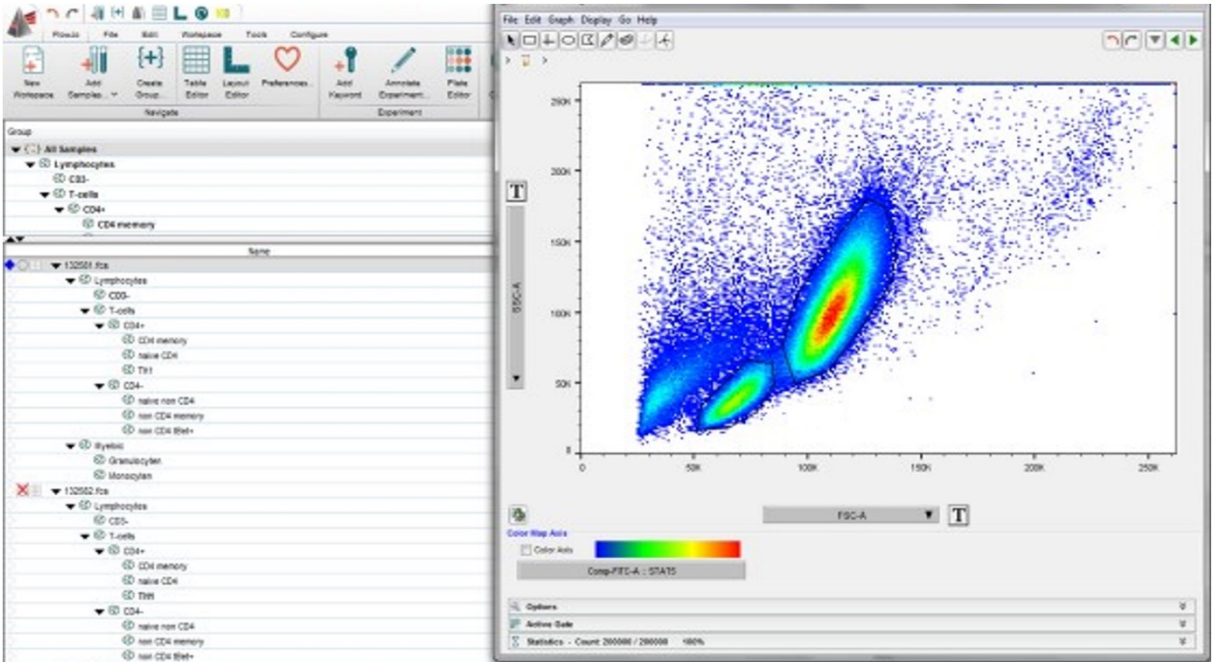

*Figure 12 | **The Graph window**, accessed by selecting a cell sample.*

The depicted graph is formed by the x-axis and the y-axis, each axis representing a chosen parameter. The cellular parameters generally refer to specific fluorescent pulse measurements collected in the flow cytometry. These parameters can represent cell-specific features such as size, structure, granularity or expression levels of cellular proteins or functional processes like cell signaling or metabolism. The desired parameter can be selected via the axis menu. When set to default, the two parameters are FSC-A (forward scatter parameter) and SSC-A (side scatter parameter), representing data about cellular size and granularity.[106] Other parameters depend on the used staining antibodies in Flow Cytometry. The staining antibodies used are described in detail in the *"Immunostaining*" chapter.

The more similar two cells are to each other in their cellular structure, the closer they stand together in the graph. Due to the great number of cells in each graph, it is necessary to demonstrate the number of cells close to each other by changing colours. A single cell is illustrated as a dot of certain colour. When more cells overlap in the graph, they are illustrated in a different colour. Thus, areas with a high number of events or large numbers of similar cells are represented in a different colour. In contrast, singe cell events differ greatly from the rest of the cells. Those could represent cell debris or other cellular abnormalities. Different areas with high numbers of similar cells can be identified as different subgroups. For example, in *Figure 12*, there are at least two areas with many similar cells. As the whole graph represents the whole group of leucocytes in this sample, the two subgroups can be identified as lymphatic and myeloic cells.

In *Figure 13,* the discussed *"Graph window"* is depicted in greater detail.

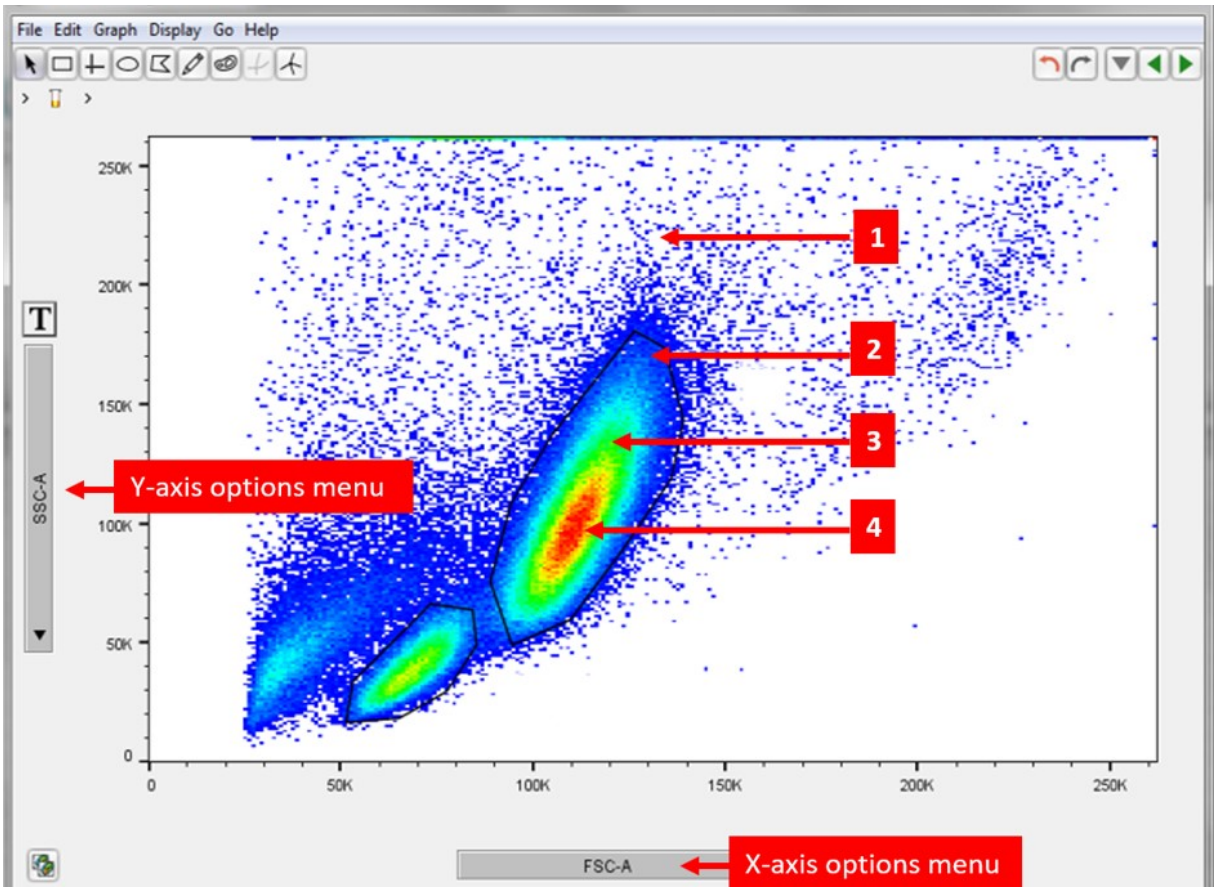

Figure 13 | ***The Graph window in detail:***
X-axis options menu. The menu to select the parameter representing the X-axis.
Y-axis options menu. The menu to select the parameter representing the Y-axis.
(1) single cells, with no prominent similarity.
(2) larger number of cells than in (1), yet still not exceptionally similar to each other.
(3) many overlapping cells, similar to each other.
(4) highest number of overlapping cells in this graph with great communality between each cell.

5.2.6.2 *Single parameter graph*

When analysing distinct cell populations, in order to isolate them further depending on their expression of specific markers, it is possible to examine only one parameter by applying the specific parameter and the requested cell population onto a histogram. In this case, as shown in *Figure 14*, blood was stained for the expression of CD3 and the received data inserted in a histogram. In this case, the Y-axis of *Figure 14* depicts the number of events within the lymphocyte's population. As can be seen, two peaks arise, which can be interpreted as the appearance of CD3 positive and CD3 negative cells. Due to the fact, that usually all T-cells express CD3, this lymphocyte population can be divided into $CD3^-$ cells and T cells.[107]

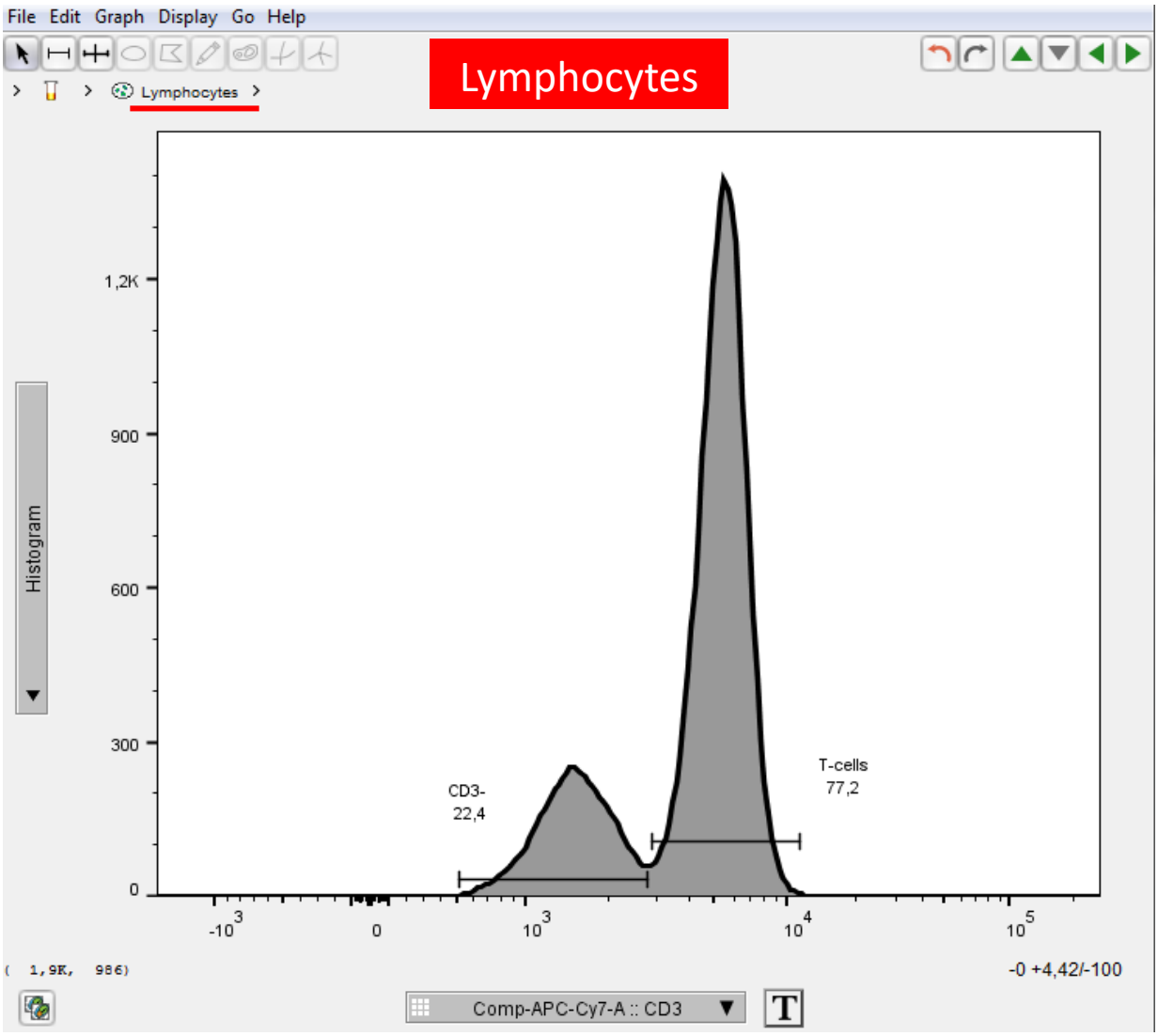

*Figure 14 | **A single parameter histogram.** The lymphocyte population of a patient's blood, examined onto its expression of a specific marker, in this case CD3. Subdivision of the leukocytes population in cells expressing CD3 and cells not expressing CD3. As a result, populations were isolated for CD3- cells and T-cells.*

5.2.6.3 *Gating*

In order to organize the samples cells into subgroups, a procedure called "*gating*" is used. *Gating* basically describes the digital isolation of different cellular subgroups from the main population. To achieve this isolation, it is required to localise areas representing higher-density regions of events. Those areas are gated by either the program or the user himself. There are different gating tools, as pictured in *Figure 15*, in this paper, the polygon and rectangle gating tool were used. After *gating* is completed, the program applies made settings to all samples in the same group, occasionally occurring discrepancies can be fixed manually.

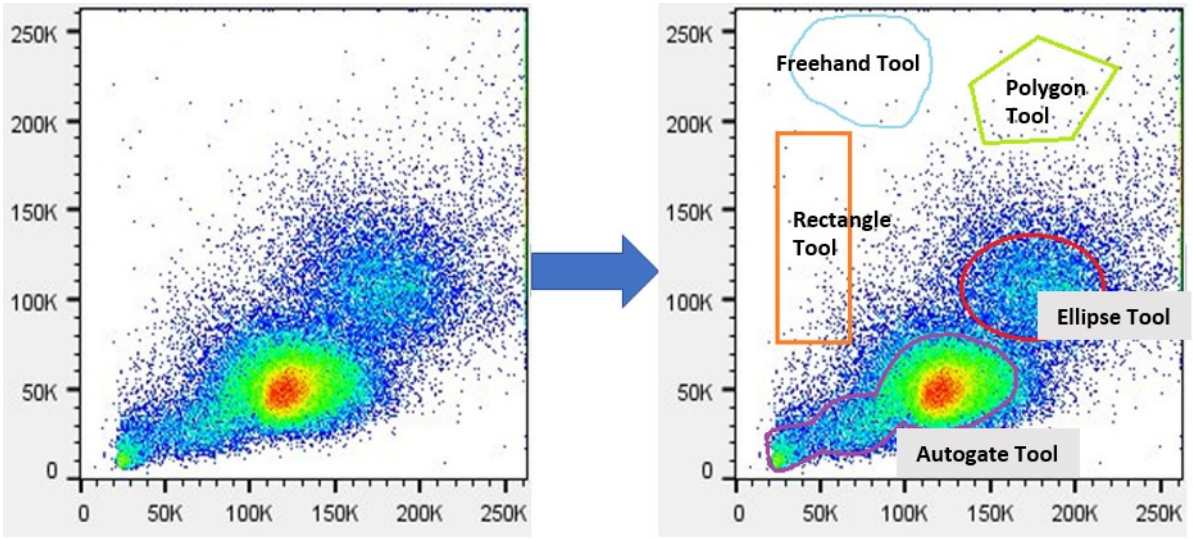

Figure 15 | ***Sample of leucocyte cells before and after being gated.*** *The gating tools are basically different tools for manual gating like "freehand, polygon, rectangle and ellipse tools" and an "autogate tool". In case of the autogate tool, gating is conducted by the program.*

The following images are going to depict the gating process of our flow cytometric data from patient's blood. We started by dividing each leukocyte population into myeloic and lymphatic cells. On that basis we subdivided those populations into each subpopulation. We used two different antibody staining panels, Panel 1 and Panel 2, each with different antibody staining for each blood sample. Both panels were applied to the flow cytometric procedure and the resulting data used for FlowJo- analysis. Due to the different antibody staining, it was possible to isolate different subpopulations, depending on the antibody staining used. Although the data from Panel 1 and Panel 2 were merged for the statistical analysis, they were inserted into FlowJo separated. Thus, the following images will depict populations of Panel 1 as well as Panel 2 separated. Samples will be labelled with "(P1)" and "(P2)".
Figure 16 describes the gating of a Panel 1 sample. Starting with a leucocyte population, it is possible to differentiate three areas of high density. The first gated area can be identified as myeloic cells, the second area as lymphatic cells. The third area can be taken as debris, and was therefore not gated. [107] Following the first gated area, the myeloic subpopulation, another graph window can be opened, presenting two areas of density. Again, both areas are gated, and identified as monocytes and granulocytes. The subdivision for myeloic subpopulations ends here. When following the lymphatic cells, yet another three areas are present. These subpopulations can be identified as T-cells, B-cells and cells lacking the expression of CD3 as well as CD19. This classification is possible due to the fact, that the CD3 antigen is present on the surface of all human T lymphocytes [70], whereas most human B-cells, except for plasma cells, express CD19. [81] In contrast, natural killer cells (NK-cells) do not express either CD19 or CD3 -antigens on their surface, thus $CD3^-/CD19^-$- cells can be regarded as NK-cells.[108]

In each cell population, we used different markers on X/Y-axis for gating:

	X-Axis	*Y-Axis*
Leucocyte population	FSC-A	SSC-A
Lymphatic population	Comp-Pacific Blue-A:: CD19	Comp-PerCP-Cy5-5-A::CD3
Myeloic population	Comp-APC-Cy7-A::CD16	SSC-A

*Table 14 | **Markers used for gating of cell populations from Panel 1.** For each population, we used different markers on the X/Y-Axis in FlowJo10.*

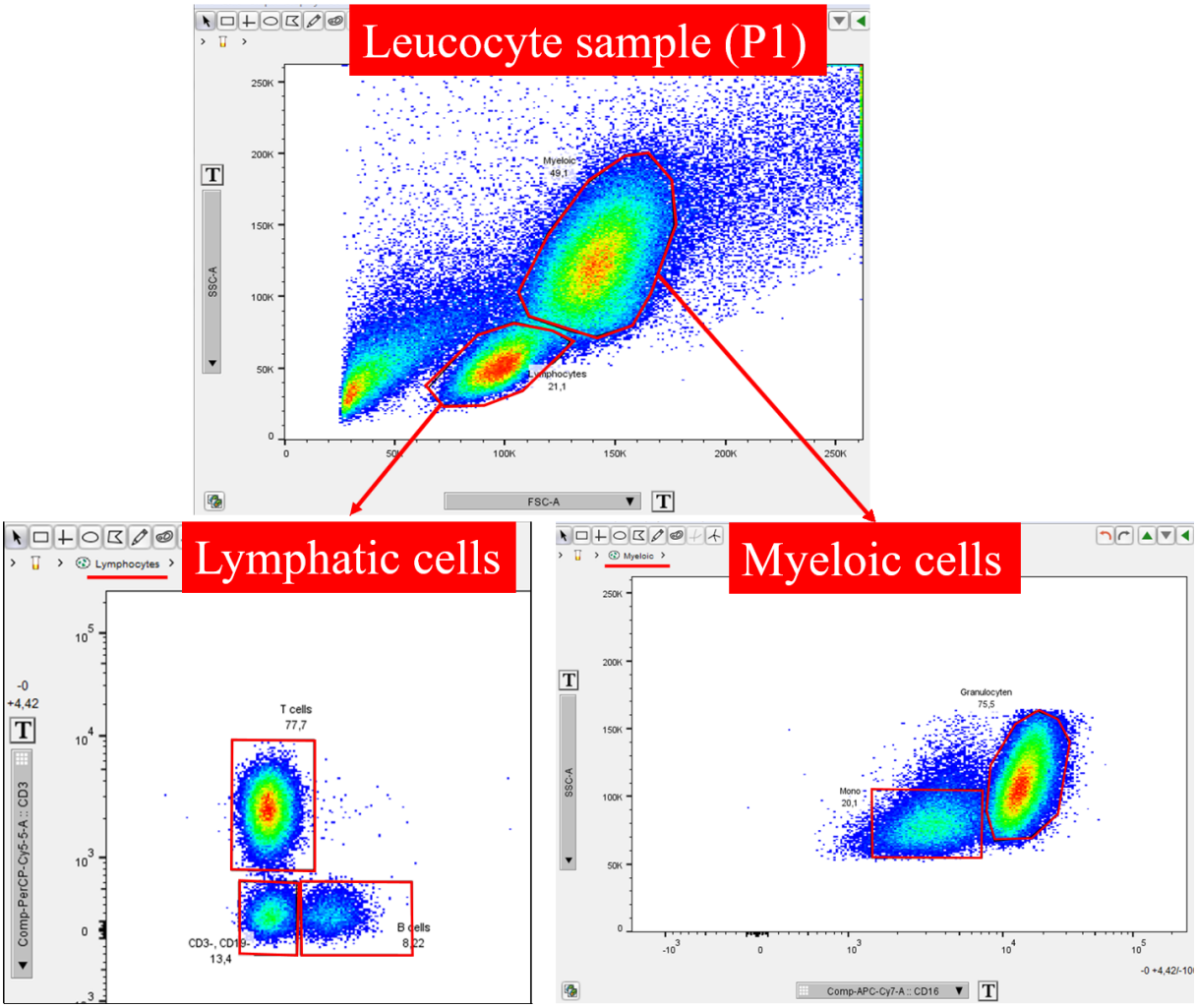

*Figure 16 | **Gating and subdivision of a leucocyte population from a Panel 1 sample.***
As seen in the first graph window, gating of a leucocyte population and identification of myeloic cells and lymphatic cells. The third, ungated area is regarded as cellular debris. Following the myeloic cell line, two subpopulations arise, monocytes and granulocytes. When following the lymphatic cell line, three subpopulations are present, T-cells, B-cells and cells not expressing either CD3 markers and CD19 markers, representing NK-cells.

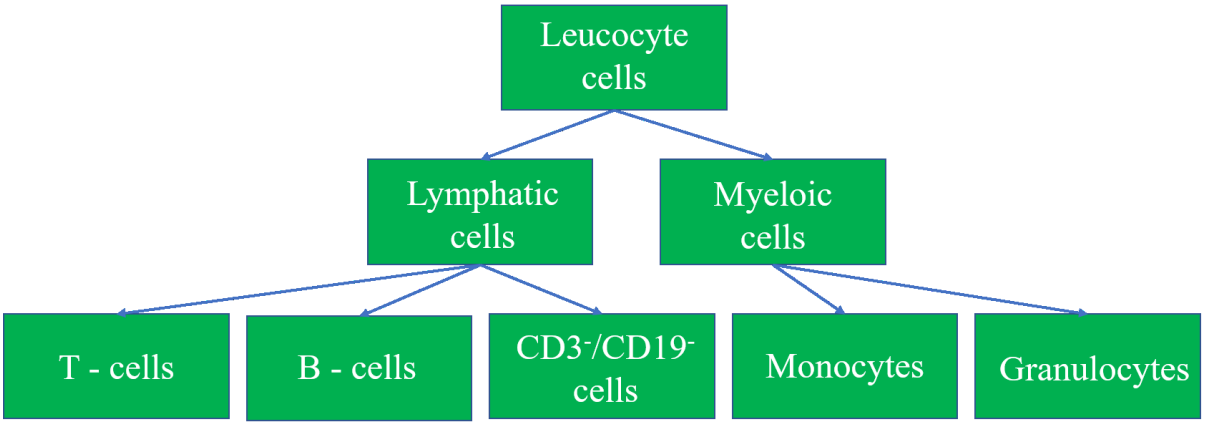

Figure 17 | ***Subpopulations received from gating Panel 1***

In the next image, *Figure 18,* a leucocyte population received from a Panel 2 sample is shown. Similar to the Panel 1 sample from *Figure 16*, the main leukocyte population can be subdivided into a myeloic cell line and a lymphatic cell line. Also, once again, there is an area of high density that is not gated, this area can be accepted as cell debris and will not be discussed. Same as in *Figure 16*, the myeloic cell line can again be subdivided into monocytes and granulocytes. In contrast, the lymphatic cell line presents itself in a different way to gate. As there were no CD19 targeting antibodies included into Panel 2, lymphatic cells cannot be divided into T-cells, B-cells and $CD3^-/CD19^-$-cells. Yet, as CD3 targeting antibodies are included into Panel 2, it is possible to create a histogram regarding the expression of CD3 antigens. Thus, the lymphatic cell line is divided into T-cells and $CD3^-$-cells, lymphatic cells not expressing the CD3 antigen. Beyond that, in Panel 2 samples, T-cells can be further subdivided, this will be described in another image, *Figure 19*.

Table 15 describes the markers used for the gating of cells from Panel 2 in *Figure 18*.

	X-Axis	*Y-Axis*
Leucocyte population	FSC-A	SSC-A
Lymphatic population	Comp-APC-Cy7-A::CD3	Histogram (number of events)
Myeloic population	Comp-PerCP-Cy5-5-A::Tbet	SSC-A

Table 15 | ***Markers used for gating of cell populations from Panel 2.***

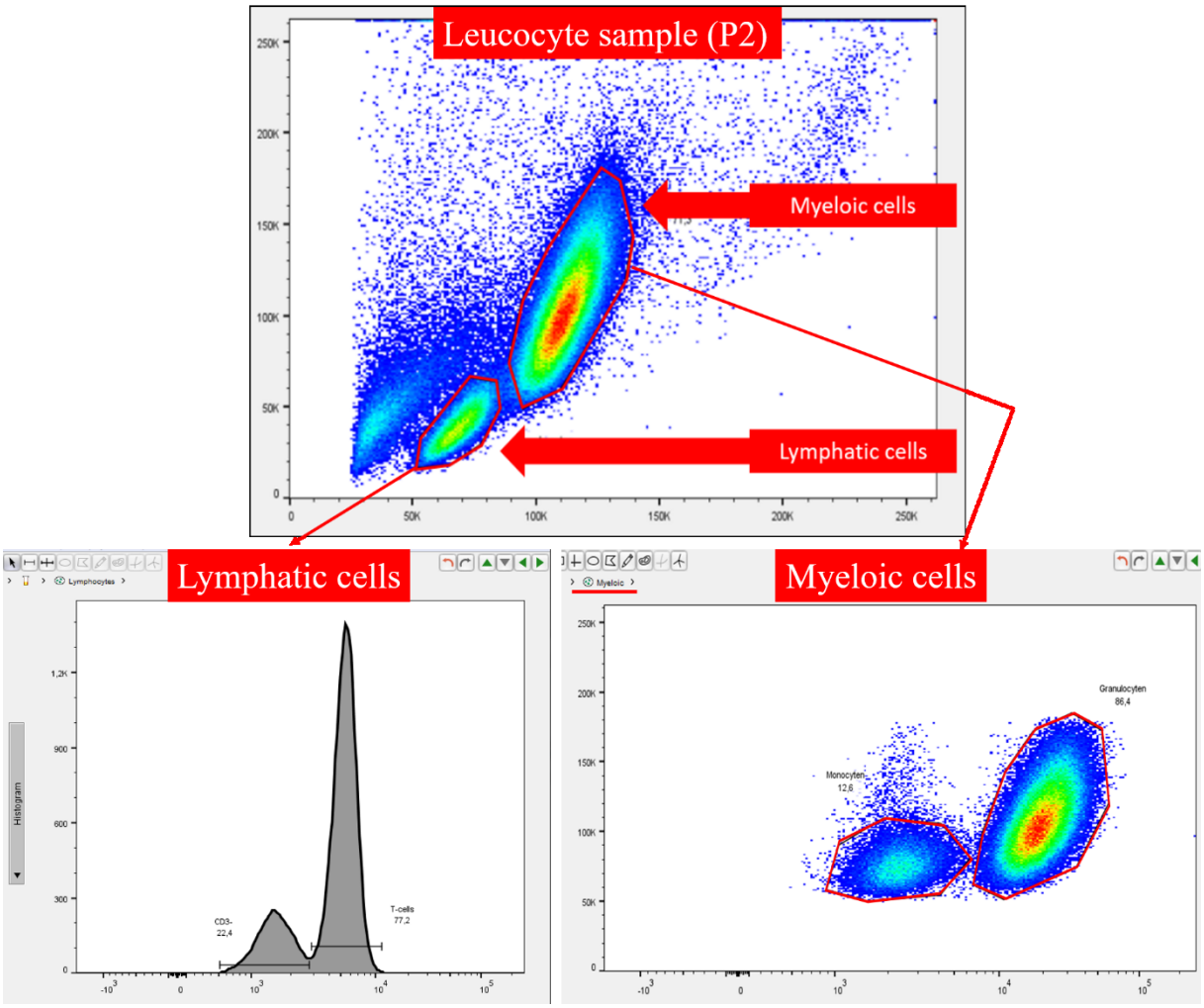

Figure 18 | ***Gating and subdivision of a leucocyte population from a Panel 2 sample.***

Similar to Figure 16, there are three graphs, the first graph showing the gating of a leucocyte sample and isolation of myeloic cells and lymphatic cells. The third ungated area is regarded as cell debris. The second graph picturing the myeloic cell line is once again divided into monocytes and granulocytes. The third graph describes a histogram regarding the expression of CD3 antigens in lymphatic cells. As illustrated in this graph, there are two peaks, one steel and high peaks and another peak clearly deeper. The first, high peak describes T-cells, the second peak CD3$^-$ -cells.

The Histogram in this image considers the expression of CD4 antigens, thus allowing the isolation of CD4$^+$-cells and CD4$^-$ -cells within the T-cell subpopulation. Following this digital isolation, the CD4$^+$ population is gated and subdivided into CD4-memory cells, naive CD4-cells and TH1 cells. This is accomplished by regarding the expression of CD45RA antibodies as well as Tbet antibodies. When applying the same expression requirements to the CD4$^-$ -cell line, it is split into non-CD4-memory cells, naive non-CD4-cells and non-CD4 Tbet$^+$ cells.

Table 16 describes markers used in the gating process of the T-cell population from Panel 2 in *Figure 19*.

	X-Axis	*Y-Axis*
T-cells	Comp-AmCyan-A::CD4	Histogram (number of events)
$CD4^+$-cells	Comp-PE-Cy7-A::CD45RA	Comp-PerCP-Cy5-5-A::Tbet
$CD4^-$-cells	Comp-PE-Cy7-A::CD45RA	Comp-PerCP-Cy5-5-A::Tbet

Table 16 | ***Markers used for gating of T-cell populations from Panel 2.***

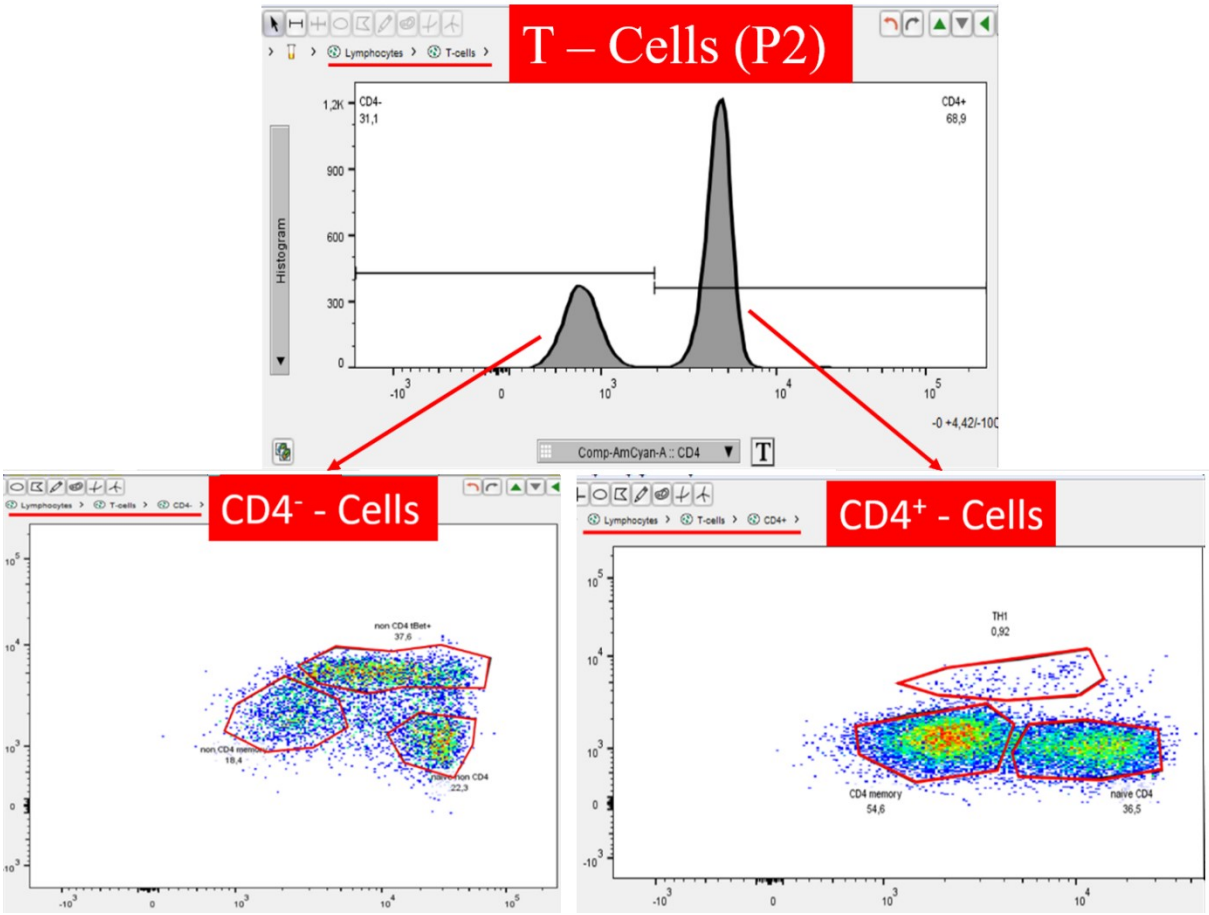

Figure 19 | ***Gating and subdivision of a T-cell population from a Panel 2 sample.***

Subsequently to the isolation of T-cells described in Figure 18, T-cells are again divided into CD4 positive cells and CD4 negative cells. The $CD4^+$ - cell line divisions into three subpopulations, CD4-memory cells, naive CD4-cells and TH1 cells. Similar to CD4 positive cells, CD negative cells are divided into non CD4-memory cells, naive non CD4-cells and non CD4 Tbet cells.

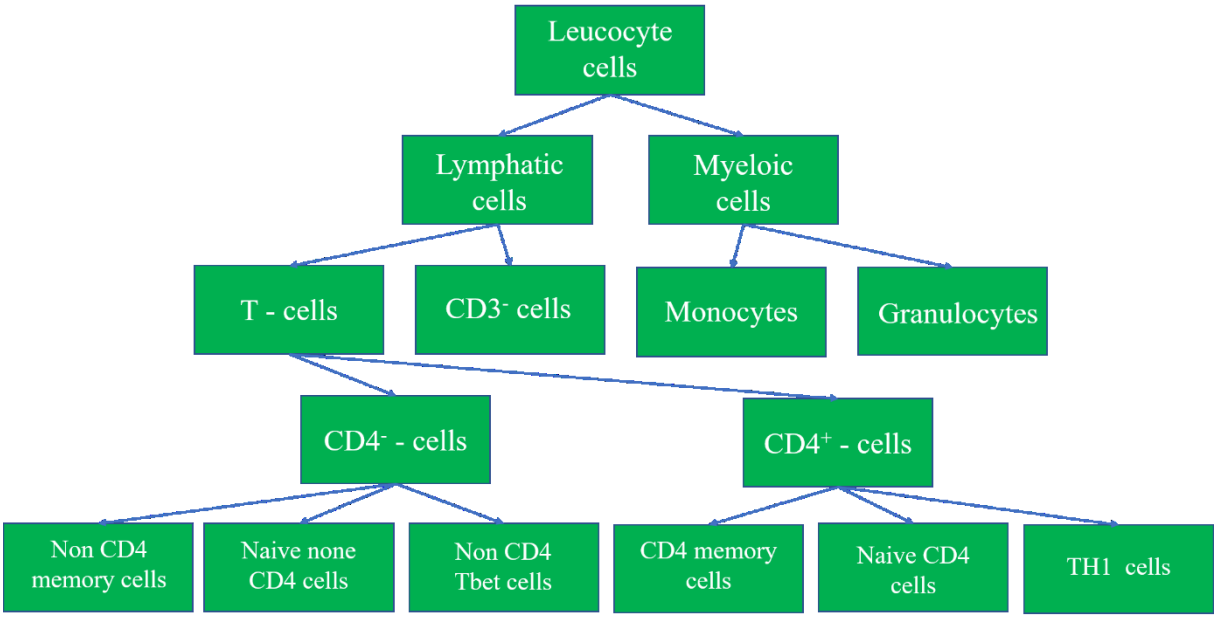

Figure 20 | **Subpopulations received from gating Panel 2**

Thus, gating the joined samples of Panel 1 and Panel 2 with FlowJo allows for the subdivision of the patient's leucocyte population into subpopulations and the counting of cells contained in each subpopulation. In summary, the following cell populations were isolated from the leucocyte population:

- Lymphatic cells
  - T- cells
    - CD4 positive cells
      - CD4-memory cells
      - naive CD4-cells
      - TH1 cells
    - CD4 negative cells
      - non CD4-memory cells
      - naive non CD4-cells
      - non CD4 Tbet cells
  - B-cells
  - CD3/CD19 negative cells
  - CD3 negative cells
- Myeloic cells
  - Granulocytes
  - Monocytes

Note that the supersets “Lymphatic cells” and “myeloic cells” were not used for further analysis, resulting in 14 subpopulations used for statistical analysis.

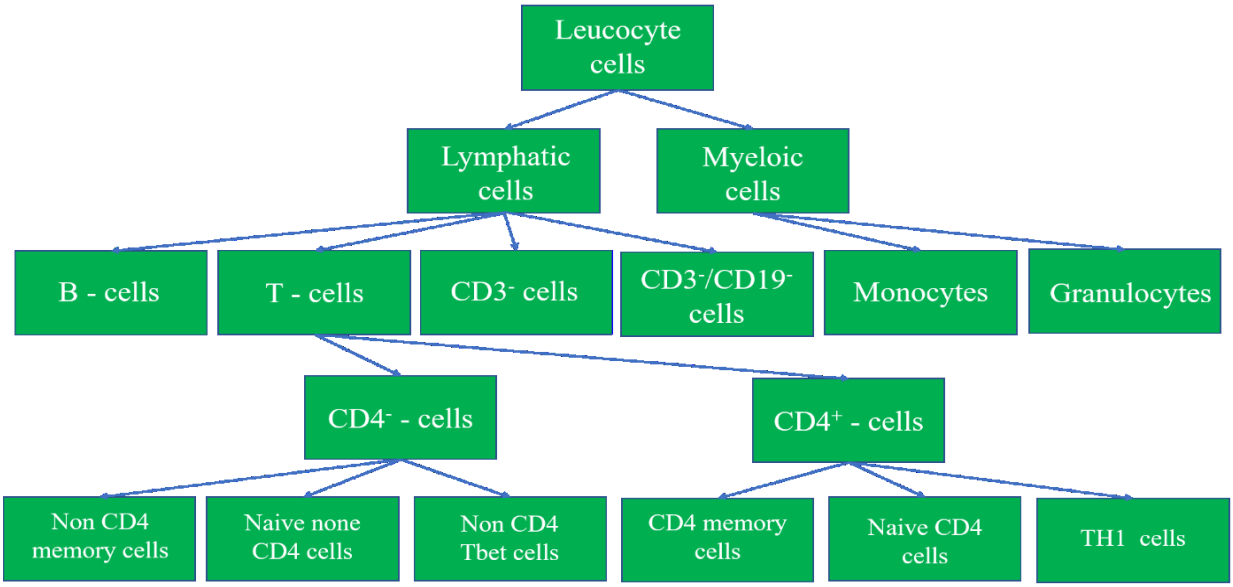

Figure 21 | ***Subpopulations received from both Panel 1 and Panel 2***

As Panel 1 contained STAT1, STAT2 and STAT6- specific antibodies and Panel 2 STAT3, STAT4, and STAT5- specific antibodies, each Panel and its defined subpopulations regarded certain STAT -proteins. Thus, each subpopulation of Panel 1 can be analysed upon its phosphorylation of either STAT1, STAT2 or STAT6. The same goes for Panel 2, but concerning STAT3, STAT4 and STAT5.

As a result, each defined cellular subpopulation defined three variables for statistical analysis, each regarding the phosphorylation of certain STAT-proteins. Thereby it is possible to examine, what part of a cell populations signaling cascade is transmitted by the certain STAT-molecule.

The following Figure 22 gives a short summary of pSTATs (phosphorylated STATs) and their associated Panels and subpopulations.

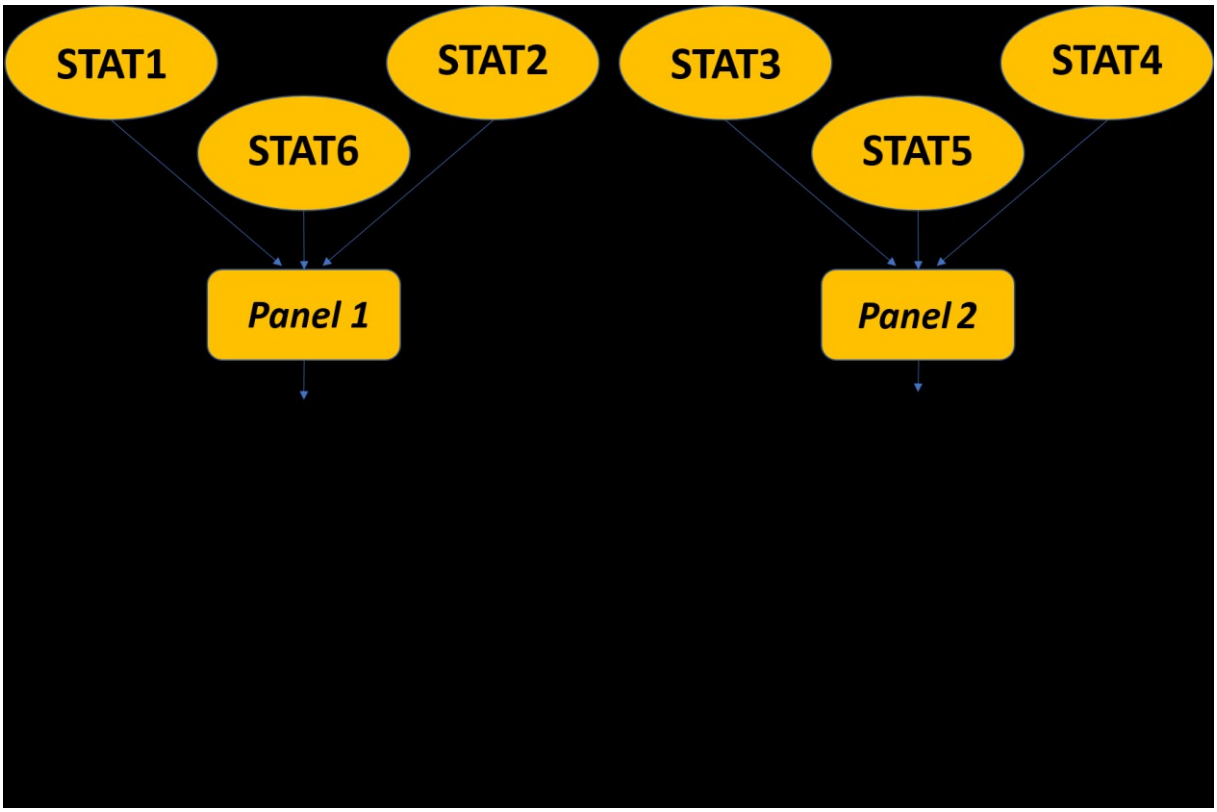

Figure 22 | ***pSTATs and their associated panels and subpopulations***

5.3 Statistics

From each of the above described cell populations, the MFI (mean fluorescence intensity) was determined on base of the gated cell panels. As described above, each subpopulation was examined for the phosphorylation of three STAT-molecules, depending on the populations panel. Therefore, each cell population contributed three variables for statistical analysis. Taking Panel 1 and Panel 2 together, each subject was analysed on the phosphorylation of STAT1-STAT6 in their associated cellular subpopulations, both stimulated with cytokines (IL-2, IL-4, IL-6, IL-12, IFNα) and unstimulated.

All in all, we received a total of 84 variables from 23 patients, 17 patients with diagnosed rheumatoid arthritis and 6 healthy patients as control group. 42 variables were contributed by unstimulated samples, 42 variables from stimulated samples, as described in the staining protocol. Each patient received a number, samples from healthy patients were labelled H(healthy) 1-6, samples from patients with RA were labelled P(patient) 1-17.

The received data about the cell populations were inserted into SPSS for statistical analysis, which was performed using *IBM SPSS Statistics 25.* For the analysis of the cell data, we performed descriptive statistics, hierarchical clustering and discriminant analysis.

5.3.1 Descriptive statistics

We used descriptive statistics for a general overview on the following issues:

- Similarities/Discrepancies between certain patients (P and H) in order to determine whether there are different endotypes within the overall patients collective.
- Differences between P (patients)-group / H (healthy)-group
- Differences between phosphorylation on STAT1-STAT6
- Differences between stimulated / unstimulated samples

For these issues, the mean and the standard deviation was calculated for every variable. Afterwards, variables with high standard deviation, compared to their mean, were sorted out. The limit for a high standard deviation was set at ½ of its mean, the limit for a moderately increased standard deviation at 1/3 of its mean.
For a better overview regarding cell types depending on distinct STATs, variables were depicted in diagrams for STAT1-STAT6.

5.3.2 Independent samples T-test

T-test was performed to analyse statistically significantly differences between healthy subjects and RA patients. Significance level was set at 0,05.

5.3.3 Hierarchical cluster analysis

The cluster analysis allows the division of patients into clusters, showing which other patients are most similar or most different to them. The Hierarchical clustering was performed using the Ward´s cluster method with the squared Euclidean distance as interval. Each cluster analysis was illustrated in a dendrogram, with the analysis of both stimulated and unstimulated variables as well as a general cluster analysis.
The following graphs depict the hierarchical clustering with its dendrogram and the following classification of patients into clusters. Three cluster options are shown, clusters with both stimulated and unstimulated variables, clusters with only unstimulated variables and at last clusters with stimulated variables.
The cluster analysis is necessary for performing the Kruskal-Wallis test, allowing to pinpoint certain variables, mainly responsible for the classification of clusters, therefore showing similarities/discrepancies between probands.

5.3.4 Kruskal-Wallis test

The Kruskal-Wallis test was performed to determine the variables responsible for the classification of the performed hierarchical clustering. Those variables would seem to be mainly responsible for discrepancies between subjects, indicating cell subclasses of interest for this paper.
The analysis was performed for both stimulated and unstimulated variables, with the null

hypothesis stating, that the distribution of each variable is the same across clusters of Ward Method in hierarchical clustering.

5.3.5 Cytokines compared to subjects

Additionally, subject´s cytokine values were analysed upon correlations with the STAT phosphorylation. Subject H3, H5 and H6 could only be analysed partially due to lack of data. Cytokines were not compared to "stimulated variables".

6 Results

6.1 Patients characteristics

Table 17 gives a short summary of the patient´s collective, Figure 23 describes patient's RA - medication at the moment of blood sampling.

	Patients group (n = 17)	***Control group (n = 6)***
Main inclusion criterion	Current presence of RA	Current absence of RA
Average age	62 a	49 a
Ratio male/female	3/14	3/3
Mean duration of disease	11a	/
Rheumatoid factor -positive	10 (~ 59%)	/
aCCP -positive	13 (~ 76%)	/
Mean clinical disease activity index (cDAI)	7,8 points	/
Mean disease activity score 28 (DAS28)	2,3 points	/

Table 17 | ***Summary of the patient´s collective.***

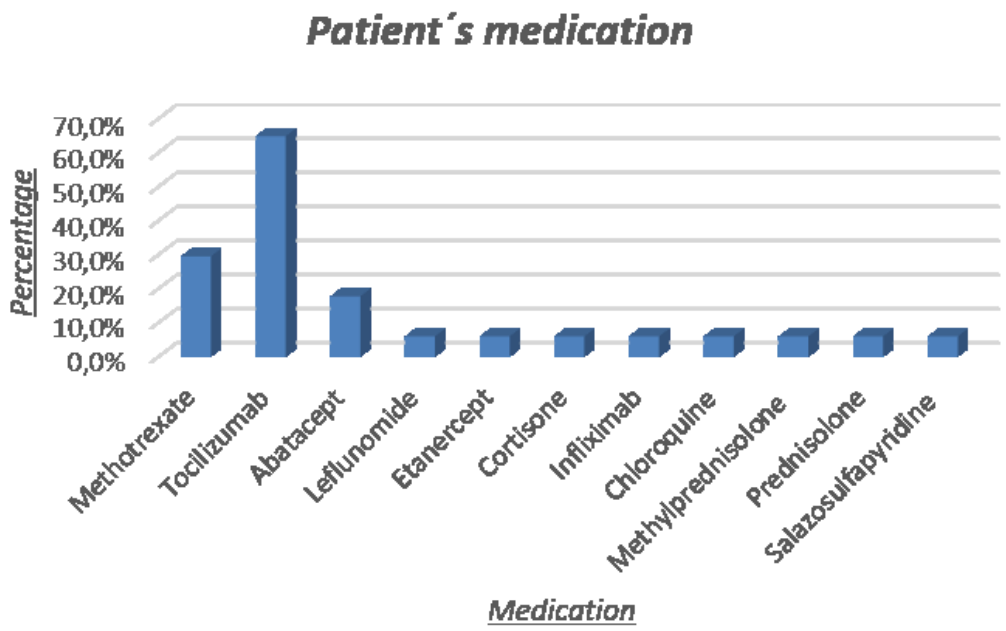

Figure 23 | ***Patients medication for treatment of RA during time of blood sampling.***

6.1.1 Descriptive statistics

The following *Table 18* and *Table 19* show the calculated mean and standard deviation from both patients and healthy samples.

Standard deviation	*Standard deviation higher than ½ of its mean*
Standard deviation	*Standard deviation higher than 1/3 of its mean*

variables	***mean***		***Standard deviation***	
	P1-17	***H1-6***	***P1-17***	***H1-6***
B-cells_ STAT1	428,6	305,5	150,5	79,0
B-cells_ STAT2	337,8	269,2	137,2	49,3
B-cells_STAT6	353,9	337,7	73,3	42,7
CD3_CD19_ STAT1	261,7	222,2	59,2	30,8
CD3_CD19_ STAT2	279,8	277,3	72,2	54,8
CD3_CD19_ STAT6	343,4	348,2	62,1	41,2
T-cells_ STAT1	418,8	357,0	78,8	29,0
T-cells_ STAT2	277,5	250,5	90,3	36,7
T-cells_ STAT6	324,9	324,3	61,5	37,2
CD3neg_ STAT3	432,5	365,8	65,5	29,5
CD3neg_ STAT4	323,1	352,3	39,9	23,0
CD3neg_ STAT5	440,8	456,0	87,5	26,7
CD4pos_memory_ STAT3	998,0	832,3	123,7	79,0
CD4pos_memory_ STAT4	184,7	179,2	36,6	23,3
CD4pos_memory_ STAT5	678,2	762,8	111,4	69,5
CD4pos_naive_ STAT3	1074,5	903,5	141,7	100,6
CD4pos_naive_ STAT4	306,9	319,3	50,4	52,7
CD4pos_naive_ STAT5	689,5	762,0	106,1	89,7
CD4pos_TH1_ STAT3	1753,5	1160,0	722,4	266,2

CD4pos_TH1_ STAT4	462,5	310,0	164,6	107,1
CD4pos_TH1_ STAT5	1480,1	1147,8	834,7	472,7
naive_non_CD4_ STAT3	950,1	839,2	138,3	101,3
naive_non_CD4_ STAT4	423,8	445,2	39,4	46,4
naive_non_CD4_ STAT5	487,2	504,3	69,8	70,9
non_CD4_memory_ STAT3	954,2	813,0	113,6	87,2
non_CD4_memory_ STAT4	167,5	156,0	33,1	21,3
non_CD4_memory_ STAT5	431,1	435,3	78,2	57,5
non_CD4_tBet_ STAT3	1007,0	853,8	110,2	99,6
non_CD4_tBet_ STAT4	337,4	319,7	92,4	88,3
non_CD4_tBet_ STAT5	474,2	478,5	63,7	60,3
Granulocytes_ STAT1	5877,4	5489,3	2630,0	1242,8
Granulocytes_ STAT2	1087,7	973,8	345,4	124,4
Granulocytes_ STAT3	6625,3	4964,5	2564,5	1042,3
Granulocytes_ STAT4	1480,9	1288,8	482,5	191,1
Granulocytes_ STAT5	5567,9	6134,0	1167,5	834,9
Granulocytes_ STAT6	1037,8	991,0	207,8	112,3
Monocytes_ STAT1	763,6	711,3	198,3	102,9
Monocytes_ STAT2	661,1	619,2	166,9	64,8
Monocytes_ STAT3	1473,9	1260,5	323,9	170,6
Monocytes_ STAT4	513,4	520,3	76,6	49,9
Monocytes_ STAT5	1664,4	1925,5	222,5	224,6
Monocytes_ STAT6	695,5	711,5	120,5	83,8

Table 18 | ***Mean and standard deviation in the patient's group and the healthy group, received from stimulated variables.***

Standard deviation	*Standard deviation higher than ½ of its mean*
Standard deviation	*Standard deviation higher than 1/3 of its mean*

variables	***mean***		***Standard deviation***	
	P1-17	***H1-6***	***P1-17***	***H1-6***
STIM_B-cells_ STAT1	1022,5	852,2	587,5	323,4
STIM_B-cells_ STAT2	708,5	603,8	281,1	103,6
STIM_B-cells_ STAT6	2332,1	2475,2	511,0	476,9
STIM_CD3_CD19_ STAT1	600,9	603,5	329,4	204,3
STIM_CD3_CD19_ STAT2	531,8	464,8	200,4	89,1
STIM_CD3_CD19_ STAT6	1353,9	1241,5	359,5	364,9
STIM_T-cells_ STAT1	1352,8	1472,7	852,7	412,7
STIM_T-cells_ STAT2	653,1	549,7	250,0	75,6
STIM_T-cells_ STAT6	2174,8	2168,5	491,4	440,7
STIM_CD3neg_ STAT5	1030,3	805,8	258,6	179,2
STIM_CD3neg_ STAT4	407,1	395,7	49,3	50,9
STIM_CD3neg_ STAT3	782,0	510,3	177,2	93,9
STIM_CD4pos_memory_ STAT5	3355,6	2664,2	1101,9	908,4
STIM_CD4pos_memory_ STAT4	442,9	393,0	109,6	99,3
STIM_CD4pos_memory_ STAT3	1874,0	1762,0	430,9	308,2
STIM_CD4pos_naive_ STAT5	2741,4	2578,8	856,1	1032,6
STIM_CD4pos_naive_ STAT4	495,9	495,5	84,7	117,7
STIM_CD4pos_naive_ STAT3	1987,1	1988,5	456,8	337,9

STIM_CD4pos_TH1_ STAT5	2144,7	1654,7	759,6	720,1
STIM_CD4pos_TH1_ STAT4	547,4	404,2	135,0	115,0
STIM_CD4pos_TH1_ STAT3	2350,4	1360,5	830,7	377,4
STIM_naive_non_CD4_ STAT5	1850,9	1626,2	474,2	526,4
STIM_naive_non_CD4_ STAT4	587,9	611,7	58,9	99,3
STIM_naive_non_CD4_ STAT3	1701,2	1715,0	316,3	218,5
STIM_non_CD4_memory_ STAT5	1721,9	1231,2	609,4	383,1
STIM_non_CD4_memory_ STAT4	364,2	302,0	100,0	66,6
STIM_non_CD4_memory_ STAT3	1413,4	1136,2	266,3	146,2
STIM_non_CD4_tBet_ STAT5	923,0	748,5	228,4	158,4
STIM_non_CD4_tBet_ STAT4	461,6	390,7	94,4	91,2
STIM_non_CD4_tBet_ STAT3	1245,6	938,3	171,6	120,1
STIM_Granulocytes_STAT1	6277,3	6342,0	2800,8	1504,4
STIM_Granulocytes_STAT2	1167,8	1075,2	471,3	126,7
STIM_Granulocytes_STAT3	7603,1	5883,8	2100,3	1374,4
STIM_Granulocytes_STAT4	1505,2	1358,2	418,3	190,0
STIM_Granulocytes_STAT5	5901,6	6738,3	1123,4	589,8
STIM_Granulocytes_STAT6	1802,9	2066,7	264,3	421,5
STIM_Monocytes_STAT1	2909,4	3673,0	2037,0	2336,9
STIM_Monocytes_STAT2	2073,5	1688,0	1035,8	529,3
STIM_Monocytes_STAT3	3353,5	3084,2	814,7	897,6
STIM_Monocytes_STAT4	653,8	633,3	90,2	79,0
STIM_Monocytes_STAT5	3760,1	3265,3	944,6	737,1
STIM_Monocytes_STAT6	4957,3	5262,2	1105,7	1508,9

Table 19 | ***Mean and standard deviation in the patient's group and the healthy group, received from stimulated variables.***

Mean in the patient's group was statistically significantly higher in 5 of 9 STAT3 variables ($CD3^-$ cells, $CD4^+$ memory cells, CD4+ naïve cells, nonCD4 memory cells, nonCD4 tBet cells) than mean in the healthy group. In 42 unstimulated variables, 2 non-STAT3 variables (STAT5 monocytes and STAT4 CD4+TH1 cells) were statistically significantly higher in RA patients. Significance level was set at 0,05.

The following Figure 24 shows a summary of the performed independent samples t-tests´ significances in unstimulated variables.

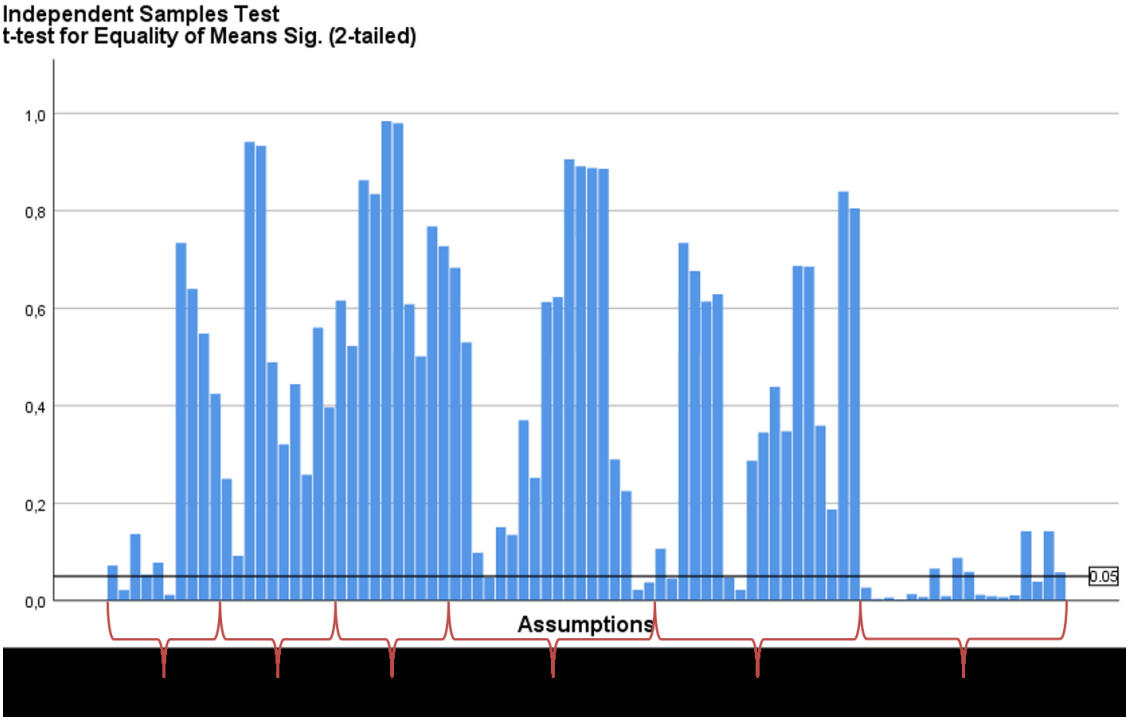

*Figure 24 |**Independent samples T-test performed for MFI of unstimulated variables.***

The following *Figure 25* again shows a summary of the performed independent samples t-tests´ significances in stimulated variables.

T-test for MFIs mean in stimulated variables shows similar results. Four STAT3 variables ($CD3^-$ cells, $CD4^+$ TH1 cells, nonCD4 memory cells, nonCD4 tBet cells)
mean seems to be statistically significantly higher in the patient's group, compared to healthy controls. One non-STAT3 variable (STAT4 $CD4^+$TH1 cells) was statistically significantly higher in RA patients.

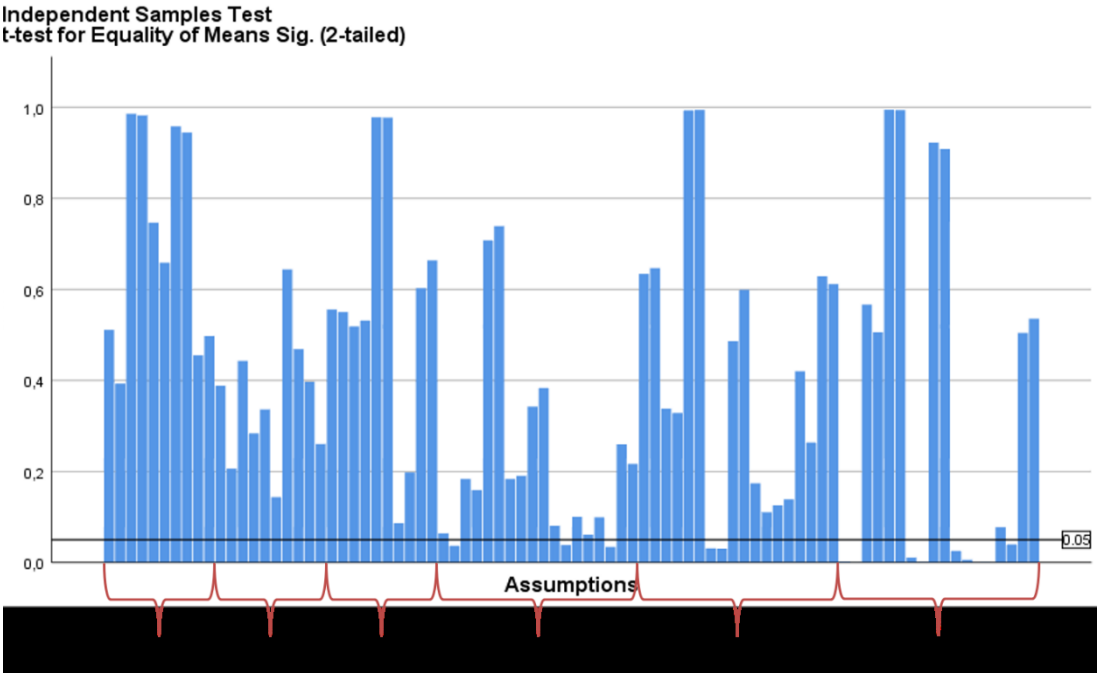

*Figure 25 | **Independent samples T-test performed for MFI of stimulated variables.***

Table 20 depicts variables with increased standard deviation compared between healthy subjects and patients with RA. Healthy group seems to have fewer variables with elevated standard deviation than the patient´s group regarding both high standard deviation and moderately increased standard deviation. This might suggest a larger diversity of STAT-phosphorylation in patients with RA compared to healthy subjects.

	Healthy group (H1-6)	*Patient´s group (P1-17)*
Number of variables with high standard deviation	1	5
Number of variables with moderately increased standard deviation	5	9
Total number	6	14

Table 20 | ***Number of variables with increased standard deviation compared between the healthy group and the patient´s group.***

When comparing elevated standard deviations from the phosphorylation on STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6, it seems that most discrepancies are present in the MFI of STAT1, followed by STAT5 and later STAT3 and STAT2. STAT4 and STAT6 show no particularly elevated standard deviation.

	pSTAT1	*pSTAT2*	*pSTAT3*	*pSTAT4*	*pSTAT5*	*pSTAT6*
Number of variables with high standard deviation	5	0	0	0	1	0
Number of variables with moderately increased standard deviation	4	2	3	0	5	0
Total number	9	2	3	0	6	0

Table 21 | ***Number of variables with increased standard deviation compared between phosphorylation on STAT1-STAT6.***

When comparing unstimulated variables to stimulated variables, stimulated variables show a notable higher ratio of elevated standard deviations in its variables, suggesting more statistical outliners within stimulated variables, perhaps resulting from intensified cytokine stimulation.

	Unstimulated variables	*Stimulated variables*
Number of variables with high standard deviation	1	5
Number of variables with moderately increased standard deviation	4	10
Total number	5	15

Table 22 | ***Number of variables with increased standard deviation compared between unstimulated variables and stimulated variables.***

To allow for a better overview regarding the distribution of cell types depending on distinct STATs, variables were depicted in diagrams for STAT1-STAT6.

As can be seen, certain cell populations tend to differ greatly from other cell lines and also from itself, when comparing each subject. Especially Granulocytes show striking differences in each STAT1-6. In STAT2 and STAT6, cell lines show a rather correlated performance to each other in comparison to STAT1,3,4 and 5.

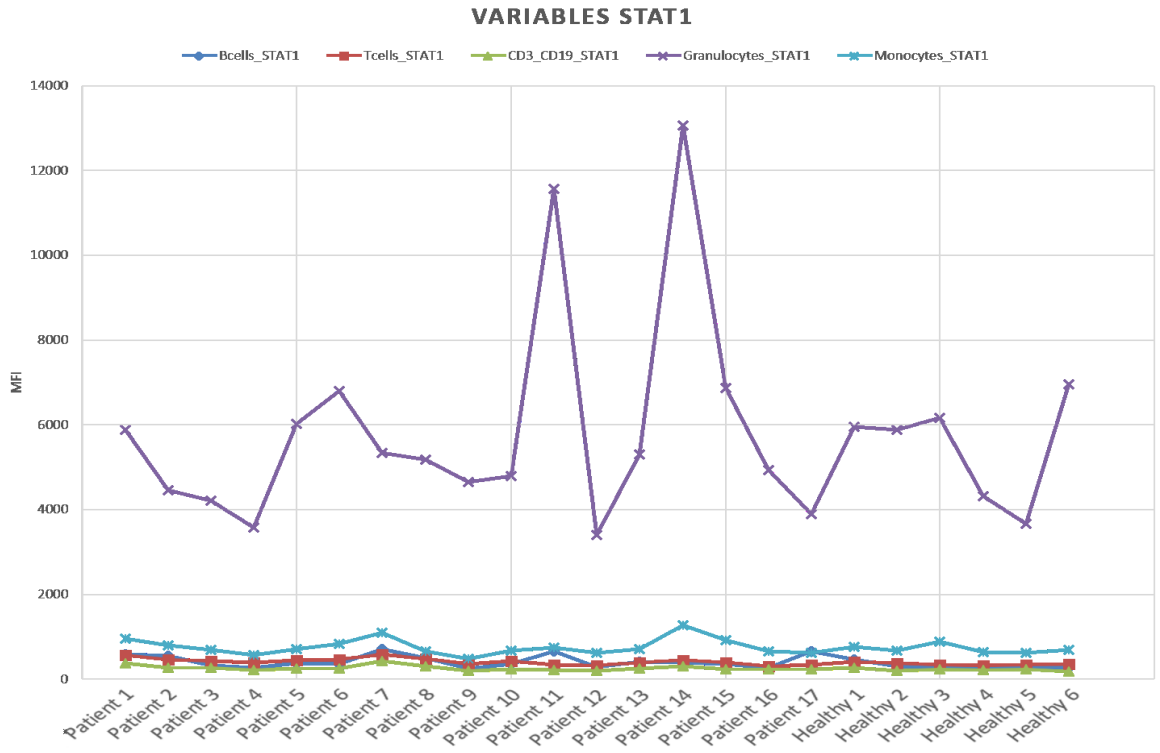

Figure 26 | ***Variables distribution on probands for STAT1***

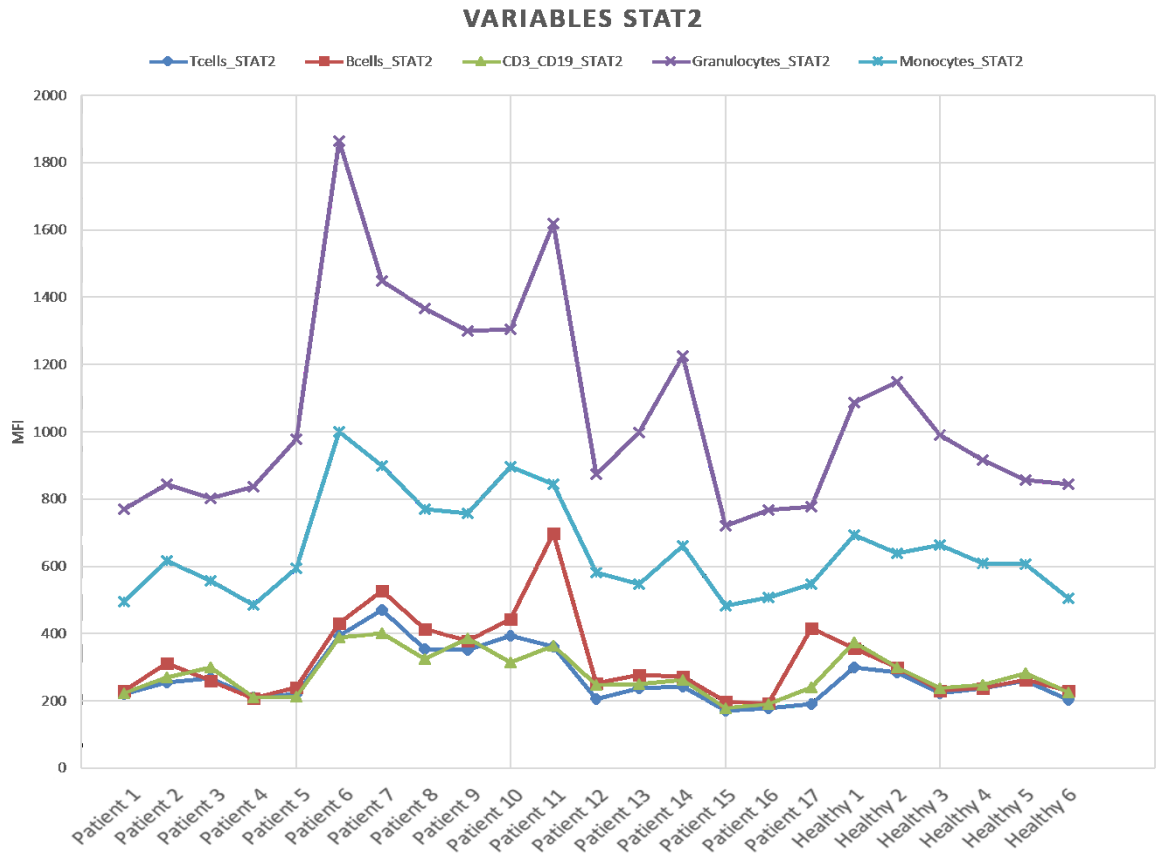

Figure 27 | ***Variables distribution on probands for STAT2***

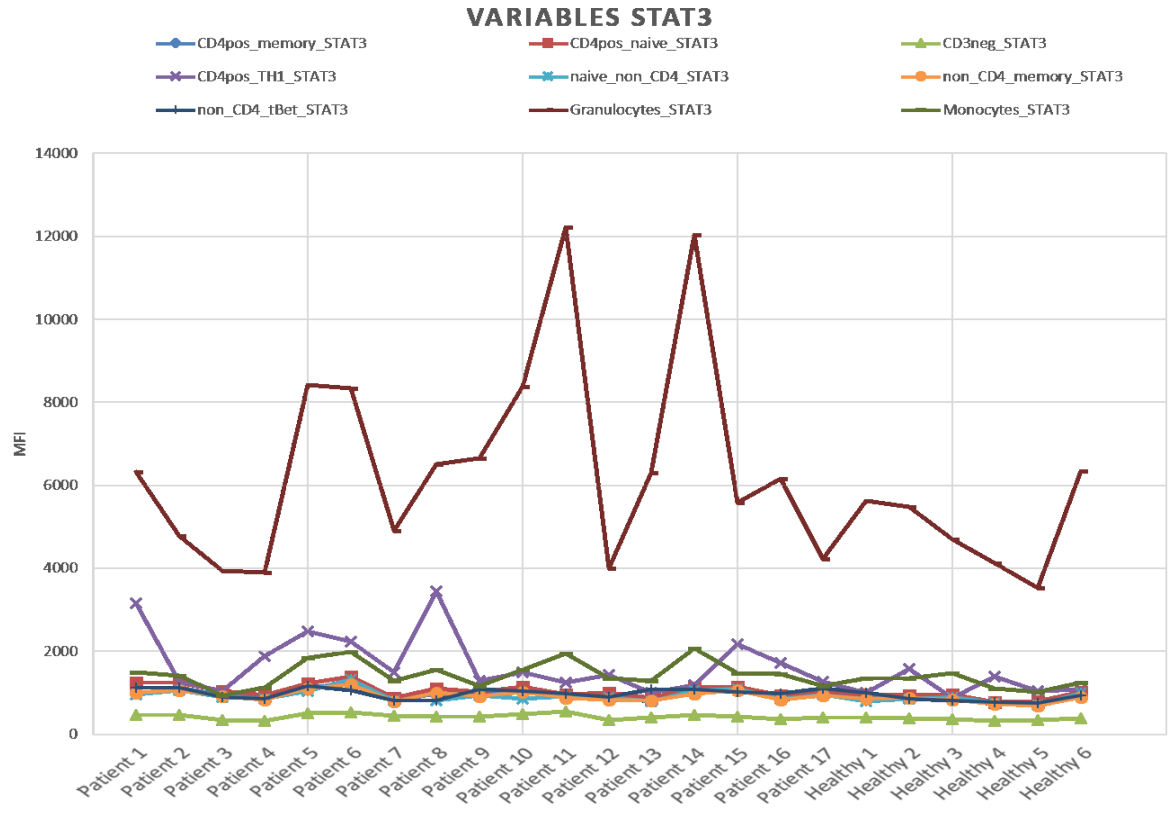

Figure 28 | ***Variables distribution on probands for STAT3***

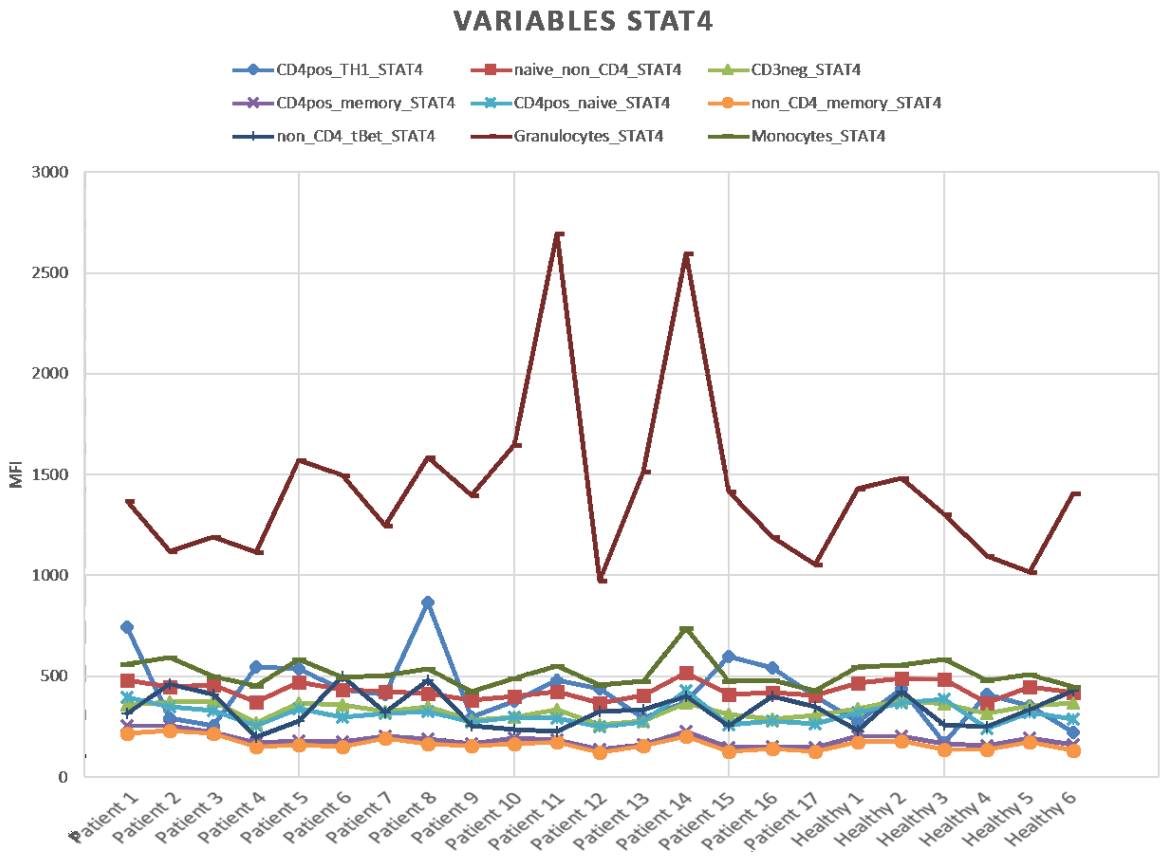

Figure 29 | ***Variables distribution on probands for STAT4***

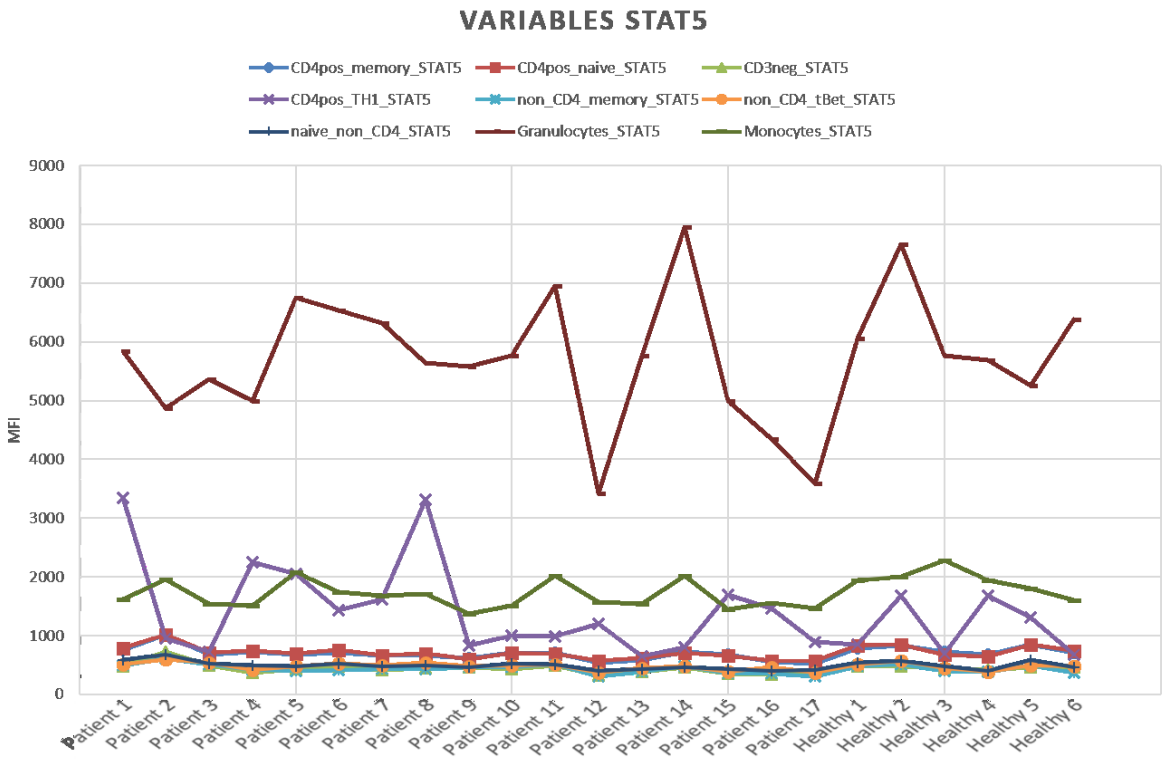

Figure 30 | ***Variables distribution on probands for STAT5***

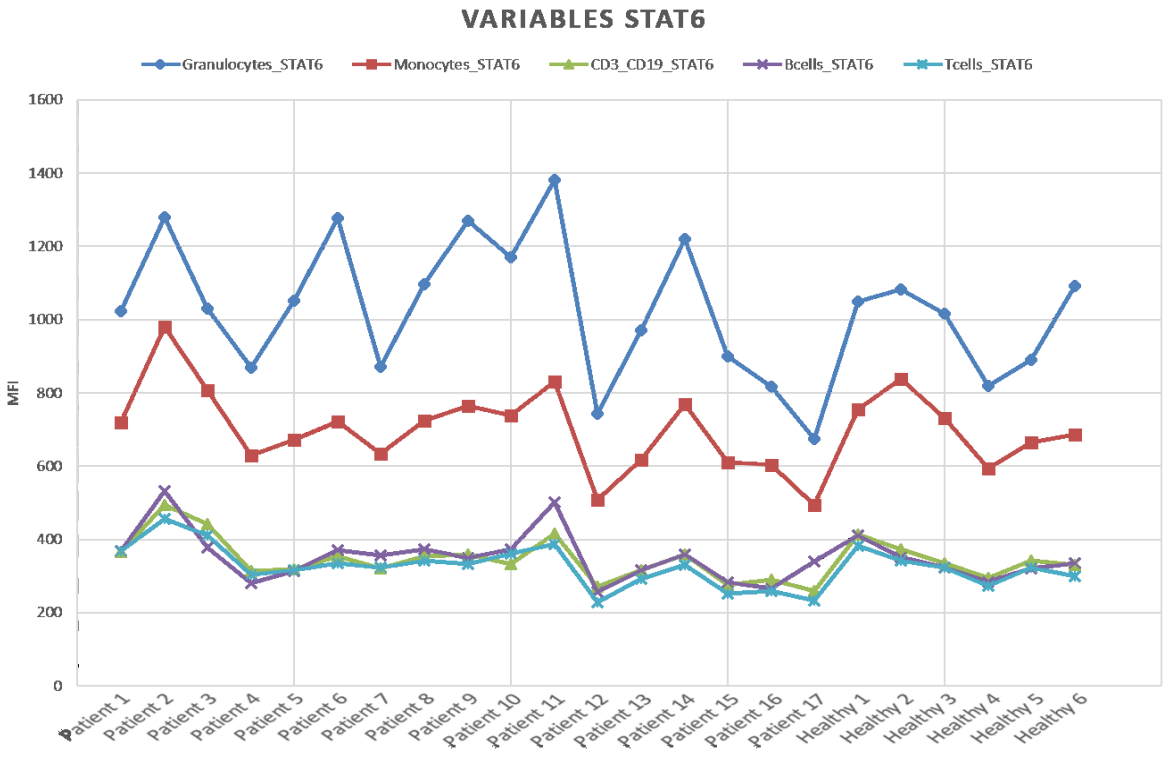

*Figure 31 | **Variables distribution on probands for STAT6***

6.1.2 Hierarchical cluster analysis

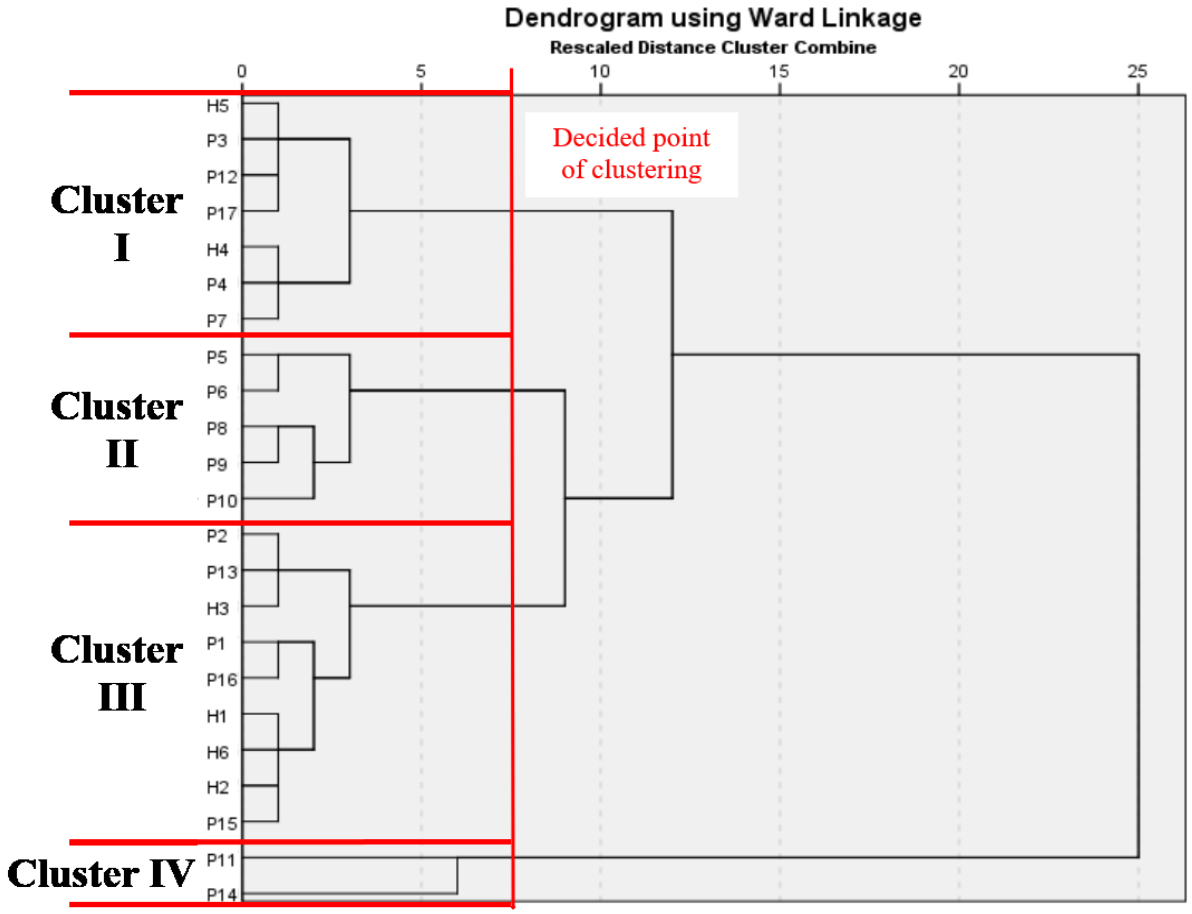

*Figure 32 | **Dendrogram using Ward linkage for both stimulated and unstimulated variables.***

The first hierarchical clustering, depicted in *Figure 33,* shows the subdivision of variables both stimulated and unstimulated. Depending on the set point of clustering, which is marked as a red line, it is possible to divide into a various number of clusters, in this case, the point we chose, results in four different clusters.

Notably, patient 11 and patient 14 vary widely from the other subjects, each form an own cluster. The rest of the subjects is more similar to each other, yet still different enough to form six clusters. Although H4 and H5 are clustered quite apart from H3 and H1,2,6, it is not possible to separate healthy subjects and patients from each other. Also, similar subjects responsible for divergent standard deviations, discussed in the descriptive statistics (*Table 18+19*), are often clustered into adjacent clusters or the same cluster, while opposing subjects tend to be clustered apart from each other. This confirms certain similarities/differences between probands.

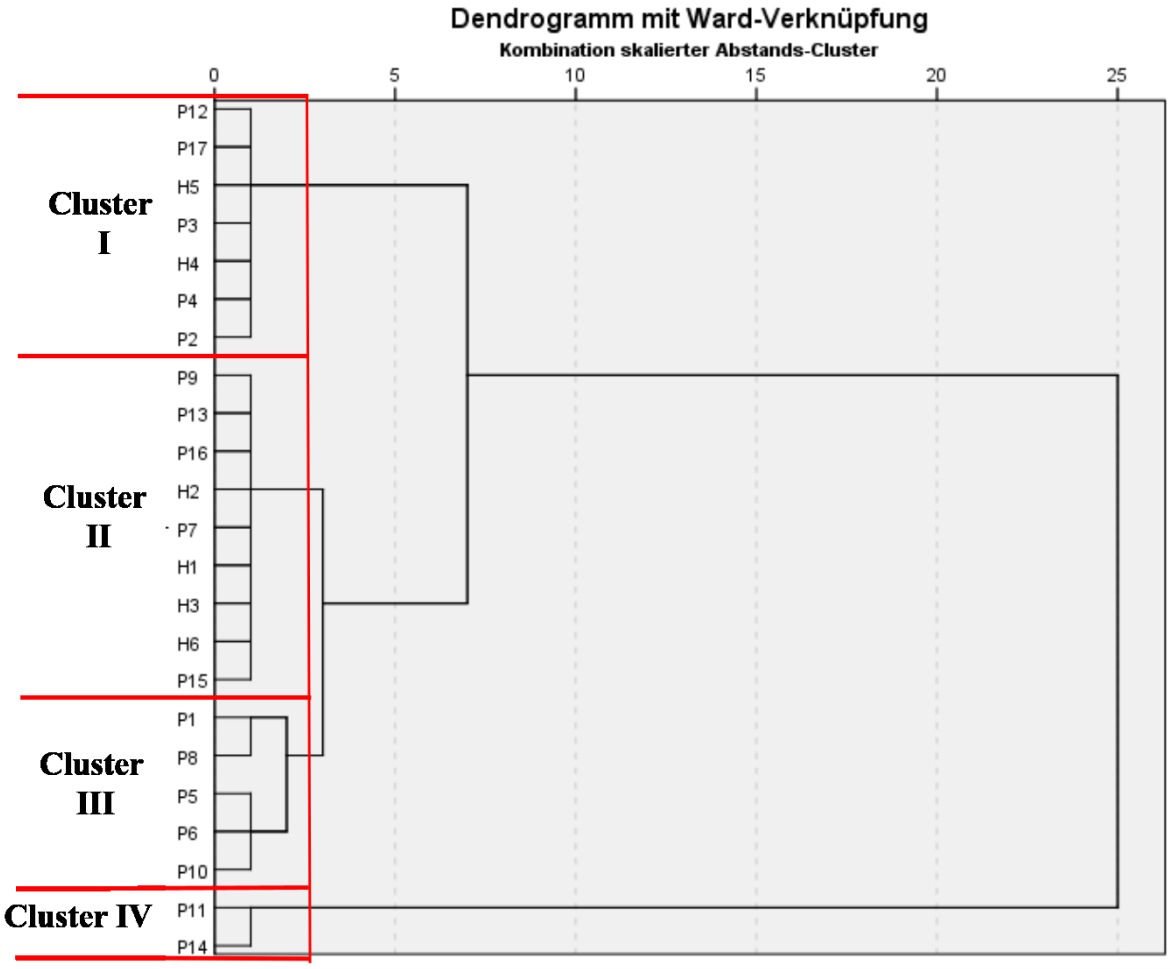

Figure 33 | ***Dendrogram using Ward linkage for unstimulated variables only.***

The cluster analysis for unstimulated variables only, depicted in *Figure 33,* results in four clusters. Again, P11 and P14 build one own cluster very different from each other cluster. Due to them differing strongly from the rest of the probands, they are responsible for the flat

dendrogram-clusters and the low number of clusters. In order to further subdivide the probands into more clusters, it would have been necessary to exclude P11 and P14. Same as the significant difference of P11 and P14 to the rest of the probands, cluster results are similar to the first cluster analysis, healthy probands H4 and H5 differ from H1, H2, H3 and H6, yet it is not possible to separate them from the other patients. Again, probands similar in the descriptive analysis form clusters, opposing probands form far apart clusters.
When comparing clusters of all unstimulated subjects *(Figure 33)* to only unstimulated RA subjects *(Figure 34)*, we found no alterations in clustering.

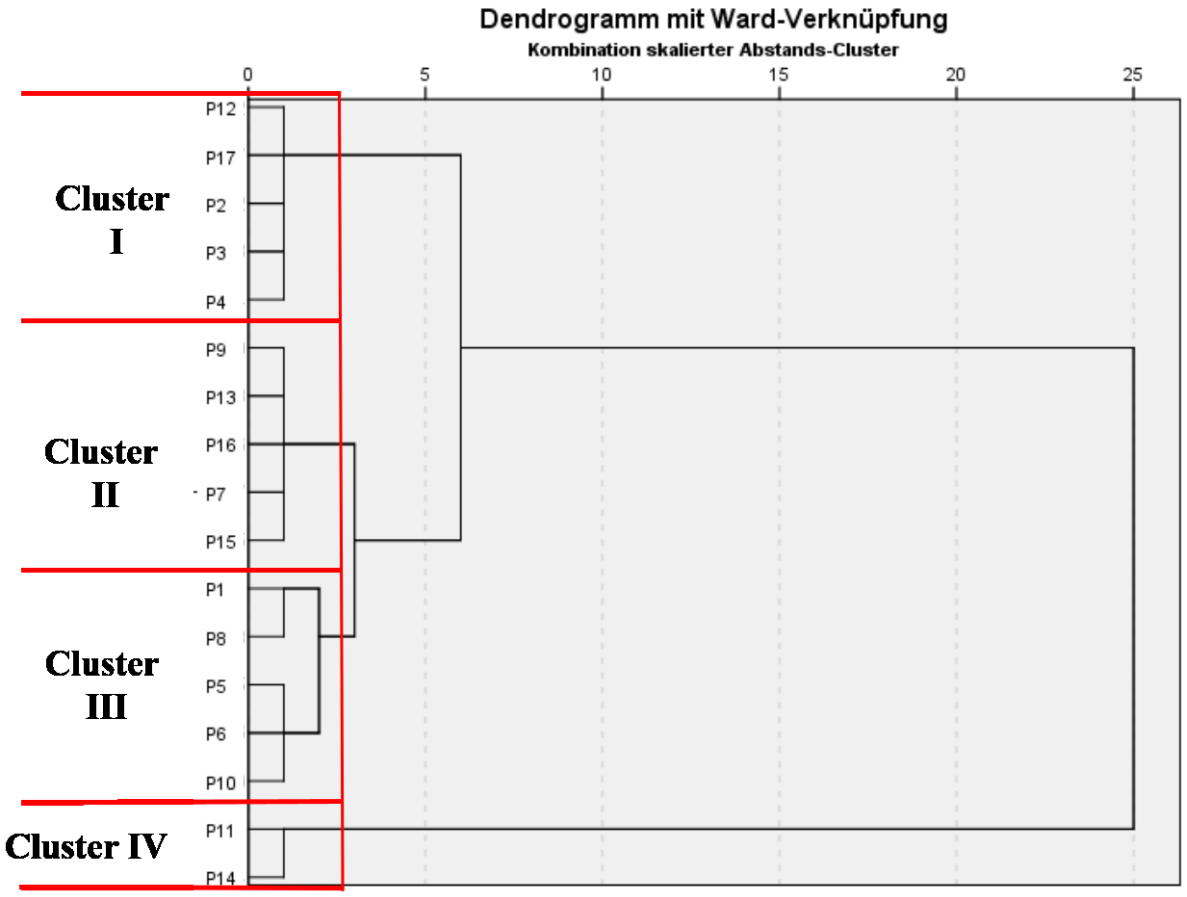

Figure 34 | ***Only RA patients classified into clusters according to unstimulated variables.***

The next cluster analysis depicts only stimulated variables, seen in *Figure 35*. This time, P11 and P14 no longer differ greatly from the rest of the subjects, P14 still builds its own cluster, yet does not differ from the other subjects as much as in with the cluster analysis with unstimulated variables. As before, results from the descriptive analysis match with the formation of clusters.
Comparison of all stimulated subjects *(Figure 35)* to only stimulated RA subjects *(Figure 36)* show noticeable alterations in clustering, yet similar subjects from *Figure 35* still cluster likewise.

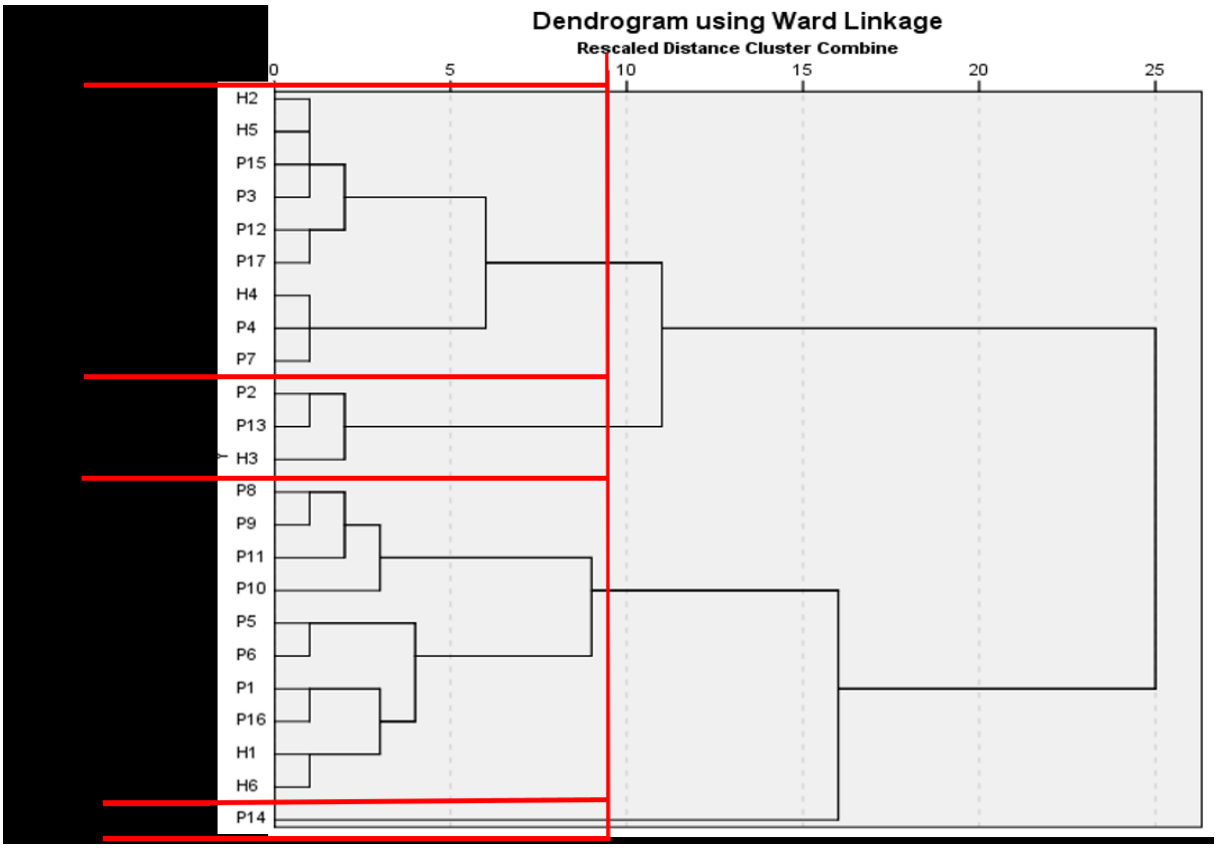

*Figure 35 | **Dendrogram using Ward linkage for stimulated variables only.***

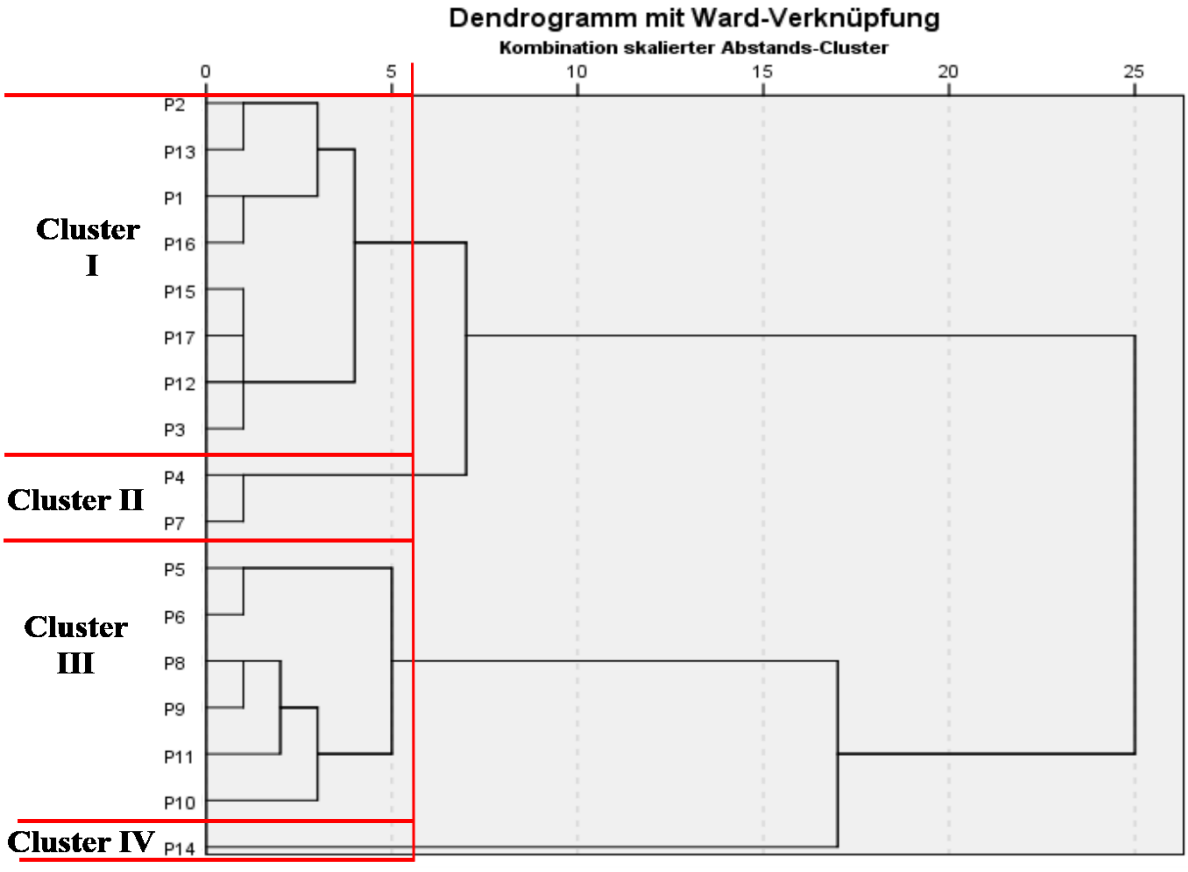

*Figure 36 | **Only RA patients classified into clusters according to stimulated variables.***

6.1.3 Kruskal-Wallis test

The following *Table 23* depicts a summary of the hypothesis tests, showing variables for whom the null hypothesis was rejected. Those variables were not equally distributed; thus, they are responsible for the subdivision of subjects into clusters. The column headings "*Stimulated variables*" and "*Unstimulated variables*" refer to the hierarchical-cluster dendrograms from Figure 33 and 35. Variables were organized according to their referring STAT.

Variables responsible for clustering decisions	
Unstimulated variables	
pSTAT1	***pSTAT3***
Granulocytes_STAT1	Granulocytes_STAT3
pSTAT4,5 and 6	Monocytes_STAT3
Granulocytes_STAT4	CD3neg_STAT3
Granulocytes_STAT5	CD4pos_memory_STAT3
Granulocytes_STAT6	CD4pos_naive_STAT3
	CD4pos_TH1_STAT3
	Non_CD4_memory_STAT3
Stimulated variables	
pSTAT1	***pSTAT4***
STIM_CD3_CD19_STAT1	STIM_CD4pos_memory_STAT4
STIM_Granulocytes_STAT1	STIM_Granulocytes_STAT4
STIM_Monocytes_STAT1	STIM_Monocytes_STAT4
pSTAT2	***pSTAT5***
STIM_Bcells_STAT2	STIM_CD4pos_naive_STAT5
STIM_CD3_CD19_STAT2	STIM_naive_non_CD4_STAT5
STIM_Tcells_STAT2	STIM _non_CD4_memory_STAT5
STIM_Granulocytes_STAT2	STIM_CD3neg_STAT5
STIM_Monocytes_STAT2	STIM _non_CD4_tBet_STAT5
pSTAT3	STIM_CD4pos_memory_STAT5
STIM_CD3neg_STAT3	STIM_Monocytes_STAT5
STIM _non_CD4_memory_STAT3	***pSTAT6***
STIM _non_CD4_tBet_STAT3	STIM_CD3_CD19_STAT6
STIM_Granulocytes_STAT3	STIM_Granulocytes_STAT6

Table 23 | ***Summary of variables responsible for the cluster classification in the hierarchical clustering analysis.***

As can be seen, it seems that there are certain cell subclasses and pSTATs, notably responsible for the subdivision of probands into clusters. Within the "unstimulated variables", pSTAT3

seems to make up most variables of interest for clustering, while the pSTATs responsible for the clustering of the “stimulated variables” are distributed more evenly. In both cases, Monocytes and especially Granulocytes seem to play an important role, alongside different cells from the lymphatic-cell-line.
To visualize the distribution of those key-variables onto each cluster, the following two images *Figure 37* and *38* show box-plots for “unstimulated variables”.

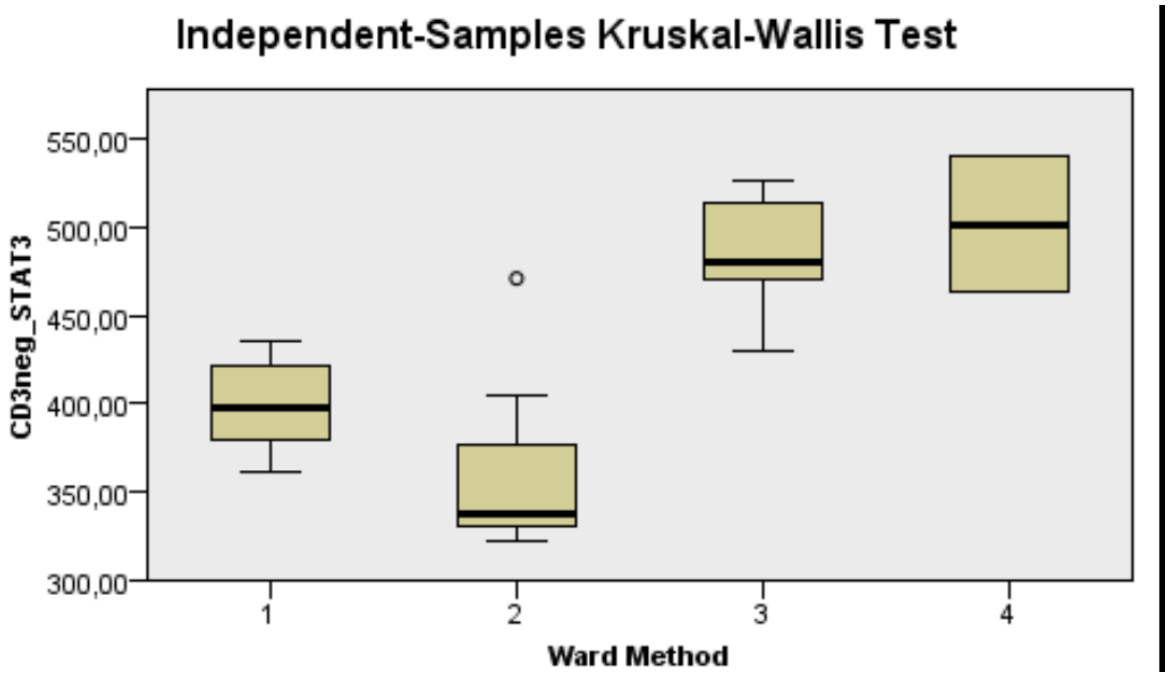

Figure 37 | ***Box-Plot received from Kruskal-Wallis test regarding variable “CD3neg_STAT” and it´s distribution onto cluster 1 to Cluster 4.*** *The box-plot describes the distribution of the MFI (mean fluorescent intensity) onto each cluster. X-axis depicts cluster 1 to cluster 4. The Y-axis presents the MFI level.*

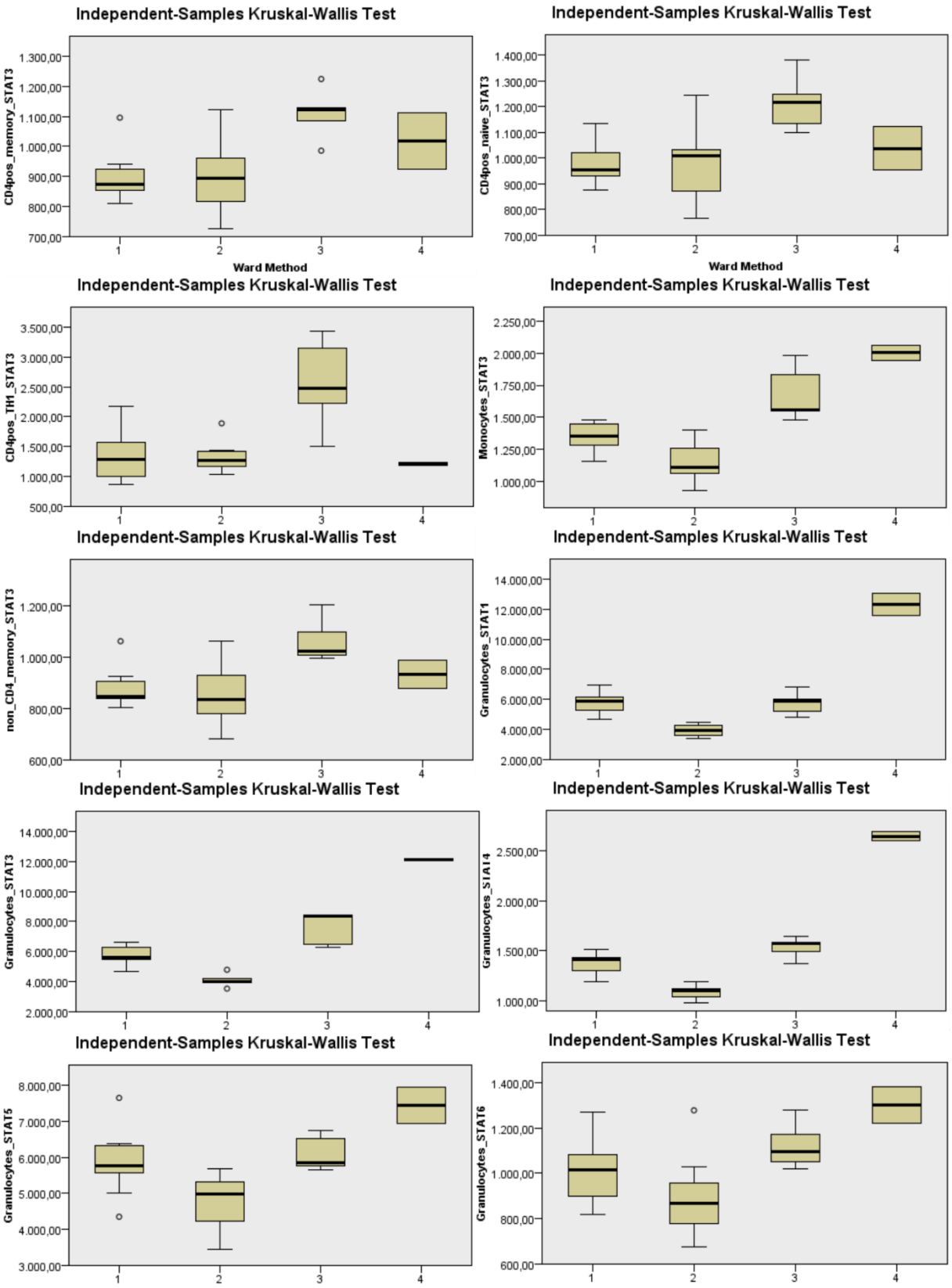

Figure 38 | ***Box-Plot received from Kruskal-Wallis test regarding unstimulated variables and their distribution onto cluster 1 to Cluster 4.***

6.1.4 Essential parameters of clustering

In order to identify essential parameters for clustering, we took the variables responsible for clustering decisions which we received from the Kruskal-Wallis test and compared them to each cluster. According variable values from each patient were organised in a sheet depending on their associated cluster and highlighted in colour by height of value (MFI) to allow a better differentiation. This "heat map" can be seen in *Figure 39* for "unstimulated variables" and in *Figure 40* for "stimulated" variables. We then looked for defining variations of MFI in each cluster and for each pSTAT. This analysis is shown in *Table 24* and *25.*

		STAT1	STAT3							STAT4	STAT5	STAT6
Patients	Cluster	Granulocytes	CD3neg	CD4pos_mem	CD4pos_naiv	CD4pos_TH1	non_CD4_me	Monocytes	Granulocytes	Granulocytes	Granulocytes	Granulocytes
H1	1	5953	398	874	952	1002	845	1358	5625	1428	6059	1048
H2	1	5875	379	871	930	1579	877	1356	5481	1483	7655	1083
H3	1	6170	365	853	959	870	838	1472	4690	1301	5760	1015
H6	1	6962	391	925	1020	1077	905	1255	6337	1408	6384	1093
P7	1	5331	435	823	881	1484	803	1284	4897	1245	6313	872
P9	1	4655	429	942	1028	1285	923	1162	6648	1396	5573	1270
P13	1	5308	397	811	875	993	820	1295	6292	1515	5762	971
P15	1	6861	422	1096	1135	2168	1061	1481	5578	1414	4996	899
P16	1	4929	361	882	937	1724	844	1446	6149	1188	4348	817
H4	2	4319	325	745	766	1384	730	1097	4129	1097	5685	818
H5	2	3657	337	726	794	1048	683	1025	3525	1016	5261	889
P2	2	4448	471	1122	1244	1272	1061	1403	4785	1120	4862	1279
P3	2	4206	338	934	1045	1039	918	931	3934	1191	5363	1029
P4	2	3567	322	891	946	1890	830	1113	3901	1115	4990	869
P12	2	3404	349	893	1007	1439	836	1347	4002	974	3426	742
P17	2	3900	404	986	1017	1276	936	1169	4217	1056	3592	674
P1	3	5882	471	1122	1247	3155	1008	1483	6312	1368	5845	1022
P5	3	6022	514	1129	1216	2479	1098	1836	8416	1572	6743	1052
P6	3	6795	526	1225	1381	2228	1202	1980	8342	1494	6530	1278
P8	3	5185	430	986	1099	3445	994	1559	6511	1585	5641	1096
P10	3	4796	480	1087	1134	1498	1024	1556	8375	1648	5770	1171
P11	4	11563	540	924	952	1243	876	1947	12229	2697	6943	1381
P14	4	13064	464	1113	1123	1192	987	2064	12042	2597	7958	1221

Figure 39 | ***Unstimulated variables responsible for clustering decisions illustrated in colour by height of value (MFI).***

Cluster	Analysis	Summary
Cluster I	pSTAT1 average, pSTAT3 and pSTAT4 moderately decreased, pSTAT5 and pSTAT 6 moderately increased.	Average pSTATs without major variations
Cluster II	pSTAT1 and pSTAT4 decreased, pSTAT3 diffused, pSTAT5 moderately decreased. pSTAT6 diffused, mainly moderately decreased.	pSTATs mainly ↓, especially pSTAT1 and 4.
Cluster III	pSTAT1 and pSTAT4 average, pSTAT3 increased, pSTAT5 and pSTAT6 moderately increased.	pSTATs mainly ↑, especially pSTAT3 followed by pSTAT5 and 6
Cluster IV	Only two subjects. pSTAT1,4,5 and 6 increased, especially pSTAT1 and pSTAT 4. pSTAT3 diffused, partially decreased.	pSTATs ↑, most ↑ in all Clusters, especially pSTAT1 and 4, followed by pSTAT5 and 6.

Table 24 | ***Identification of essential parameters for clustering decisions in the "unstimulated" analysis.***

		STAT1			STAT2					STAT3			
Patients	Cluster	S_Monocyte	S_CD3_CD19	S_Granulocy	S_Monocyte	S_Granulocy	S_Tcells	S_CD3_CD19	S_Bcells	S_CD3neg	S_non_CD4_	S_non_CD4_	S_Granulocy
H1	1	3899	799	7295	1722	1201	571	568	696	580	1368	1143	6917
H6	1	3562	645	8273	1955	1068	576	468	639	539	1143	1010	7778
P1	1	4152	991	6490	1386	865	542	436	579	978	1599	1420	7685
P5	1	903	248	8058	1969	1239	732	489	752	751	1326	1138	7777
P6	1	903	228	7918	1722	1022	610	458	688	895	1603	1339	10447
P8	1	819	281	6239	3562	1747	939	779	984	913	1540	1325	8319
P9	1	514	177	5483	3916	1821	966	875	1068	998	1583	1490	9924
P10	1	697	264	3818	3050	1719	1135	805	1155	1074	1855	1451	6751
P11	1	867	298	8245	4542	2411	1198	1012	1418	959	1881	1355	9450
P16	1	3759	785	7123	1414	871	480	414	489	695	1424	1254	9433
H2	2	2747	508	5902	1400	1071	568	463	579	478	1130	831	5765
H4	2	1195	278	3966	1006	932	397	306	425	355	953	844	3978
H5	2	2594	560	5661	1495	942	594	458	577	487	1016	870	4826
P3	2	4684	867	5104	1429	764	425	393	455	636	1328	1057	5382
P4	2	1857	485	3122	1138	935	479	380	499	560	1150	1081	4917
P7	2	2399	735	3313	1138	814	495	385	492	618	1031	1017	7112
P12	2	4806	722	3750	1721	900	557	441	494	652	1214	1145	4287
P15	2	3850	680	6315	1723	824	593	404	554	634	1525	1153	5742
P17	2	4414	660	5243	1638	834	444	427	610	700	1220	1203	6322
H3	3	8041	831	6955	2550	1237	592	526	707	623	1207	932	6039
P2	3	5912	1207	5956	1888	889	540	486	675	951	1561	1472	5986
P13	3	6891	1133	5329	1812	1044	540	479	646	799	1267	1350	7886
P14	4	2032	454	15208	1201	1154	427	377	487	481	921	925	11832

		STAT4			STAT5							STAT6	
Patients	Cluster	S_Granulocy	S_CD4pos_n	S_Monocyte	S_non_CD4_	S_non_CD4_	S_naive_non	S_CD4pos_n	S_CD4pos_n	S_Monocyte	S_CD3neg	S_Granulocy	S_CD3_CD1
H1	1	1498	515	716	1685	870	2092	4063	3801	3892	961	2546	1451
H6	1	1553	378	556	1200	760	1715	2452	2797	2841	747	2159	1285
P1	1	1409	544	744	1999	1173	2318	3124	3968	4319	1297	2053	1370
P5	1	1480	303	586	1326	849	1569	1966	2505	3493	767	1782	1249
P6	1	1635	339	604	1883	945	1863	2205	3261	3587	950	2035	1241
P8	1	1784	511	823	1951	1219	2057	2851	3916	5570	1276	1736	1662
P9	1	1650	471	616	1783	1010	2033	3203	3886	3683	1313	2216	1584
P10	1	1260	646	747	2812	1396	2123	4518	5691	5150	1481	2021	2239
P11	1	1963	649	797	3093	1107	2826	4573	5537	4737	1188	1975	1902
P16	1	1569	436	668	1574	823	1803	2829	3346	3613	860	1693	1481
H2	2	1459	408	650	1267	813	1776	2562	2593	3256	846	2117	1315
H4	2	1067	223	536	569	463	653	984	1067	2224	521	1400	591
H5	2	1192	373	617	1151	690	1482	2188	2563	3098	740	1771	1140
P3	2	1273	365	521	2037	690	1383	1931	2511	2746	789	1297	886
P4	2	1178	370	577	999	707	1338	2201	2425	2565	734	1682	987
P7	2	1466	397	629	1317	880	1320	1757	2258	2653	914	1359	923
P12	2	881	376	587	1168	686	1963	2579	2947	4229	895	1449	1072
P15	2	1441	389	558	1400	728	1715	2599	2872	2760	741	1846	1219
P17	2	1248	355	566	963	693	1709	2789	2144	3249	941	1673	1174
H3	3	1380	461	725	1515	895	2039	3224	3164	4281	1020	2407	1667
P2	3	1089	567	680	2332	1183	2352	3225	4177	4489	1420	2151	1625
P13	3	1491	494	661	1499	969	2234	2831	3687	4509	1196	1862	1262
P14	4	2772	317	750	1137	633	859	1423	1915	2569	753	1820	1141

Figure 40 | ***Stimulated variables responsible for clustering decisions illustrated in colour by height of value.***

Cluster	***Analysis***	***Summary***
Cluster I	pSTAT1 partially notable decreased, partially average, pSTAT2 and pSTAT3 partially increased, partially average, pSTAT4-6 diffused, partially increased.	pSTATs very inhomogeneous, pSTAT1 partially ↓, pSTAT2-6 diffused, partially ↑.
Cluster II	pSTAT1 diffused, pSTAT2-6 in general decreased.	pSTATs generally ↓, except for pSTAT1. Only 3 subjects
Cluster III	Only three subjects. pSTAT1 increased, pSTAT2 and pSTAT4 diffused, pSTAT3 diffused, partially increased, pSTAT5 and pSTAT6 average, slightly increased.	pSTAT1 notably ↑, other pSTATs diffused, partially ↑, partially ↓. Only 3 subjects.

Cluster IV	Only one subject. Granulocytes in pSTAT1 and pSTAT3 increased, pSTAT2 and pSTAT5 decreased, Granulocytes and Monocytes in STAT4 increased, pSTAT6 average.	Only one subject! Granulocytes mainly responsible and ↑ in pSTAT1,3 and 4. pSTAT2 and 5 ↓.

*Table 25 | **Identification of essential parameters for clustering decisions in the "stimulated" analysis.***

As shown, most clusters are defined through a significantly increased or decreased number of MFI, regarding certain pSTATs. Thus, it can be assumed, that there are subjects with differing expressions of certain STAT molecules, indicating different subgroups or endotypes within the subjects collective.

6.1.5 Cytokines compared to subjects

The following *Table 27*shows evaluated cytokine levels, compared to subjects' clusters, received from the "unstimulated variables" clustering. For a better overall view, cytokine values were highlighted depending on their elevation. *Table 26* shows cytokines mean, compared to their according cluster. Certain mean cytokine levels seem to differ greatly in each cluster, for example IL1RA, yet we accepted those as falsified by outliers.
Performed Kruskal-Wallis test found no significant influence of cytokine levels to clustering decisions, in both RA subjects and the overall collective.
In this case, elevation of cytokines seems not to be correlated to the clustering decision, indicating no causal connection to specific STAT phosphorylations.

Cytokines mean compared to according cluster										
Cluster	***IL8***	***IL1RA***	***TNFα***	***INFα***	***IL18***	***IL5***	***IP10***	***IL10***	***IL17A***	***IFNγ***
1	0	6,4	0,14	0	15,3	0,3	11,5	0,16	0	0,07
2	0	36,9	0,17	0	4,1	0,5	16,2	0,23	0,05	0,18
3	0	220,9	0,03	0	4,2	1,1	7,7	0,64	1,3	0,79
4	0	0	0,25	0	1,5	2,3	20,8	0,11	0	0

*Table 26 | **Cytokines mean compared to according cluster.** Cytokine values in pg/ml*

Cytokines compared to unstimulated subject clusters											
	Cluster	***IL8***	***IL1RA***	***TNFα***	***INFα***	***IL18***	***IL5***	***IP10***	***IL10***	***IL17A***	***IFNγ***
H1	1	0,0	0,0	0,0	0,0	0,0	0,0	4,5	0,0	0,0	0,0
H2	1	0,0	0,0	0,0	0,0	4,0	0,0	5,0	0,0	0,0	0,0
H3	1						0,7	25,8	0,2	0,0	0,6
H6	1						0,0	3,1	0,0	0,0	0,0
P7	1	0,0	0,0	0,0	0,0	6,1	0,0	10,0	0,0	0,0	0,0
P9	1	0,0	0,0	0,0	0,0	17,1	0,0	19,7	0,0	0,0	0,0
P13	1	0,0	0,0	0,5	0,0	78,3	2,3	28,1	1,2	0,0	0,0
P15	1	0,0	0,0	0,0	0,0	0,0	0,0	7,4	0,0	0,0	0,0
P16	1	0,0	44,7	0,5	0,0	1,7	0,0	0,0	0,0	0,0	0,0
H4	2	0,0	0,0	0,0	0,0	0,0	0,0	4,2	0,0	0,0	0,0
H5	2						0,0	6,7	0,2	0,0	0,0
P2	2	0,0	0,0	0,5	0,0	6,2	0,7	6,3	0,2	0,0	0,0
P3	2	0,0	0,0	0,0	0,0	5,1	0,7	40,1	0,2	0,4	0,0
P4	2	0,0	0,0	0,5	0,0	5,1	0,7	12,7	0,2	0,0	0,0
P12	2	0,0	0,0	0,0	0,0	0,0	0,7	11,8	0,6	0,0	1,0
P17	2	0,0	221,1	0,0	0,0	8,3	0,7	31,2	0,2	0,0	0,2
P1	3	0,0	0,0	1,0	0,0	3,2	3,4	10,6	2,8	4,7	2,2
P5	3	0,0	0,0	0,5	0,0	2,6	0,7	9,7	0,2	1,8	1,8
P6	3	0,0	0,0	0,0	0,0	5,6	0,7	6,2	0,0	0,0	0,0
P8	3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
P10	3	0,0	1104,4	0,0	0,0	9,4	0,7	12,1	0,2	0,0	0,0
P11	4	0,0	0,0	0,0	0,0	2,9	2,3	34,6	0,2	0,0	0,0
P14	4	0,0	0,0	0,5	0,0	0,0	0,0	6,9	0,0	0,0	0,0

Table 27 | ***Cytokines compared to unstimulated subject clusters.*** *Cytokine values in pg/ml*

7 Discussion

The aim of this study was to investigate the phosphorylation of STAT-molecules in leucocytes and identify potential variations between patients with rheumatoid arthritis and healthy subjects. The study also aimed to analyse whether certain endotypes exist within the subjects collective. Additionally, we planned to establish a laboratory protocol and procedure to facilitate further research for these particular issues.

Laboratory protocol procedure

To obtain the required data to analyse the phosphorylation of STAT-molecules in leucocytes, we established a laboratory protocol capable of depicting the JAK-STAT signaling pathway. Phosphorylation of STAT-molecules was measured as mean fluorescent intensity (MFI) in a flow cytometer, thereby showing the activity of the JAK-STAT pathway in certain cells. Based on the "BD Phosflow Staining Protocols", we designed an own protocol.

In difference to previous studies regarding the activity of the JAK-STAT pathway, such as from Jerzy Świerkot et al [86] who used immunocytochemistry to show the activation STATs indirectly through stained nuclei, we chose to analyse the activation of the JAK-STAT pathway directly through the measurement of phosphorylation on STAT-molecules.

Pia Isomäki et al [87] showed a similar approach to our protocol, yet different time intervals were used as well as different staining antibodies. We also chose to analyse both stimulated and unstimulated blood samples. 9

In the procedure of establishing this protocol, several issues were to be addressed. We had to analyse which stimulants we were to use for the "stimulated group", in which concentration these stimulants should be applied and which stimulants could be excluded. Another issue was the question, whether cells would be stable enough to keep the on low temperature for several days, in order to apply the protocol onto several subjects' probes at the same time. These issues distinguished our protocol from the initial staining protocols.

The resulting laboratory protocol was also designed to be easily replicated to enable other students to continue enlarging the patients collective up to a significant number.

Variations between subjects with RA and healthy subjects

In 2016, Jerzy Świerkot et al [86] suggested a differing activity of the JAK-STAT pathway, compared in RA patients, compared to healthy individuals. They found a significantly higher expression of STAT1, STAT3, STAT4, STAT5 and STAT6 in patients with RA, compared to control groups. Pia Isomäki et al [87] suggested elevated expression and phosphorylation of STAT3 in T cells and monocytes in patients with RA, compared to healthy controls, as well as

Takatsugu Oike et al [64], who found STAT3 to be a main promoter of inflammation and joint erosion in mice and suggested STAT3-inhibitors as promising drugs for RA therapy.
We compared the mean (MFI) of 84 variables (stimulated and unstimulated) describing phosphorylation on STAT-molecules in healthy subjects and subjects with RA using the independent samples t-test (Significance level 0,05.). Mean in the patient's group seems to be statistically significantly higher in 5 of 9 unstimulated STAT3 variables ($CD3^-$ cells, $CD4^+$ memory cells, CD4+ naïve cells, nonCD4 memory cells, nonCD4 tBet cells) and in Four stimulated STAT3 variables ($CD3^-$ cells, $CD4^+$ TH1 cells, nonCD4 memory cells, nonCD4 tBet cells). 2 unstimulated non-STAT3 variables (STAT5 monocytes and STAT4 CD4+TH1 cells) and one stimulated non-STAT3 variable (STAT4 $CD4^+$TH1 cells) were statistically significantly higher in RA patients.
These findings suggest increased phosphorylation on STAT-molecules in subjects with RA, compared to healthy controls.
Hierarchical clustering was performed for both stimulated and unstimulated variables, only unstimulated variables and only stimulated variables. Therein, healthy subjects were distributed onto most clusters, therefor it was not possible to separate healthy subjects and subjects with RA from each other. Clustering for certain STATs only also did not show significant differences between healthy and RA-subjects. The overall STAT-phosphorylation between healthy and RA subjects seemed not to differ enough to allow for a significant discrimination in hierarchical clustering.
In synopsis, we found significantly increased phosphorylation of STAT3,4 and 5 in leucocyte subtypes, with STAT3 being notably dominant. Results seem to agree with the findings of Świerkot et al[86] , Isomäki et al[87] and Takatsugu Oike et al [64]. Overall STAT-phosphorylation did not allow for a clear discrimination of healthy subjects and RA subjects in hierarchical clustering. As the collective, with 17 subjects suffering from RA and 6 healthy subjects, is relatively small, a larger sample might be necessary to achieve meaningful results.

Parameters essential for clustering

Variables were analysed upon their distribution to the classification of subjects into clusters in order to identify parameters which might play an important part in the pathogenesis of RA. The analysis was performed for both stimulated and unstimulated variables.
Within the "unstimulated variables", Granulocytes from pSTAT1,3,4,5 and 6 were noticeably responsible for clustering decisions. Yet apart from Granulocytes, pSTAT3 seemed to make up every other variable of interest for clustering (Monocytes, $CD3^-$cells, $CD4^+$memory cells, $CD4^+$naive cells, $CD4^+$TH1-cells, NonCD4memory-cells). pSTAT2 was not essential in

clustering decisions.

In the ex vivo analysis (unstimulated subjects), Kruskal-Wallis test found Granulocytes and STAT3 phosphorylating cells significantly (<0,05) responsible for clustering decisions. This important role of STAT3 is postulated by different studies [58,62,63], wherein STAT3 is engaged in multiple biological roles with transcriptional functions of growth factors, pro-inflammatory IL-6 cytokines and G-CSFs and also anti-inflammatory responses induced by IL-10[58,62,63].

Stimulated variables responsible for clustering were distributed more evenly, with more variables of interest. Kruskal-Wallis test found following subpopulations to be significantly (<0,05) responsible: *pSTAT1* (CD3/CD19-cells, Granulocytes, Monocytes), *pSTAT2* (B-cells, CD3/CD19-cells, T-cells, Granulocytes, Monocytes), *pSTAT3* ($CD3^-$cells, nonCD4 memory-cells, nonCD4-tBet-cells, Granulocytes) *pSTAT4* ($CD4^+$memory-cells, Granulocytes, Monocytes), *pSTAT5* ($CD4^+$ naive-cells, naïve nonCD4-cells, nonCD4 memory-cells, $CD3^-$ cells, nonCD4-tBet-cells, $CD4^+$ memory-cells, Monocytes), *pSTAT6* (CD3/CD19-cells, Granulocytes).

This balanced influence of every STAT onto the "stimulated group", especially in comparison to the "unstimulated group", might be explained by the broad stimulation of subject's blood with cytokines before flow cytometric analysis. Due to the potent stimulation, STATs should be as actively phosphorylated as possible. The specific distribution of STATs, and thereby its influence onto the clustering is yet interesting. It might again suggest the presence of certain subgroups, or endotypes, in which certain STATs might be predominant.

When comparing the distribution of cell types in each STAT1-6, certain cell populations tend to differ greatly from other cell lines and also from itself, when comparing each subject. Especially Granulocytes show striking differences in each STAT1-6. This agrees with above stated influence of Granulocytes onto clustering decisions.

Endotypes within the collective

A 2019 study from Barbara Dreo et al[109] suggested the existence of yet undefined subtypes of RA. They defined three distinct subtypes of RA, based on the phosphorylation in STATs. MFI regarding phosphorylation of STATs allowed for the classification of subjects into clusters. When comparing both stimulated and unstimulated variables responsible for clustering decisions to their actual clusters, certain STATs were notably elevated or decreased in particular clusters, while some clusters were determined by an average distribution of STAT phosphorylation.

Differences in the expression of STAT-phosphorylation between clusters was found

statistically significant by Kruskal-Wallis test. While these findings might suggest the existence of subgroups within the collective, where certain endotypes would have differing levels of particular STAT-types (STAT1-6), clustering showed no significant difference between the overall collective of RA patients and healthy subjects. Thus, it remains unclear whether endotypes within RA exist or if differences between subjects are caused by differing expressions of STAT-molecules in the human immune system of healthy subjects and patients suffering from RA alike.

Cytokines influence on phosphorylation

Several studies [51,52,55,53,56] confirm the selective or unselective association of at least 50 different cytokines with certain JAKs, with different cytokine receptors signalling through different JAKs. While certain cytokines are transmitted selectively through one JAK, others can be transmitted through different JAKs. Distinct JAKs are again considered to react with particular STAT-molecules. However, the degree of a cytokine receptor relying on a JAK seems to depend on the different subunits the cytokine receptor consists. Also, certain cytokines seem to affect one and the same signalling pathway in a different way by initiating different effects. We therefore assumed possible correlations of cytokine levels with phosphorylation of STATs.

We aimed to compare our classification of subjects in clusters to previously determined cytokine values. Our comparison showed no significant correlation between cytokine values and clusters. While this might indicate no causal connection of cytokine elevations to specific STAT phosphorylations, several limitations should be named. Only 10 cytokines were determined, also, those cytokines were only tested once. Cytokine values were too low to determine in several subjects, also, some subjects were missing data. For example, subject H3, H5 and H6 could only be analysed partially due to lack of data.

Limitations

The main limitation arose from a low number of subjects in our collective. Staining protocol was protracted, with a duration of several hours, also, only few samples could be applied to the protocol at the same time. Also, we did not reach the planed amount of 40 subjects (30 subjects with RA, 10 healthy subjects). With only 23 subjects participating (17 subjects suffering from RA, 6 healthy subjects) compared to a total of 84 variables, statistical analysis was prone to error. This should be resolved by creating a dataset with a notably larger collective.

Clustering decisions were subjective and depending on the author, resulting in possible alterations, which would extend onto other statistical analysis, such as the identification of

parameters and variables responsible for clustering decisions. We therefor set the maximum number of clusters to four, regarding the small number of subjects.
Subjects suffering from RA were, at the time of blood sampling in different states of RA, also they were treated with different medication, possibly influencing the phosphorylation of STATs or reducing certain cell types.

Conclusion

We established a laboratory protocol capable of depicting the JAK-STAT signaling pathway, with the intention to continue enlarging the patients collective up to a significant number.
As described by Świerkot et al [86], Isomäki et al [87] and Oike et al [64], We found significantly increased phosphorylation of STAT3 (unstimulated: $CD3^-$ cells, $CD4^+$ memory cells, CD4+ naïve cells, nonCD4 memory cells, nonCD4 tBet cells; stimulated: $CD3^-$ cells, $CD4^+$ TH1 cells, nonCD4 memory cells, nonCD4 tBet cells) in RA subjects, compared to healthy subjects. Increase also concerned phosphorylation of STAT4 and STAT5 to a lesser extent (unstimulated STAT5 monocytes and STAT4 CD4+TH1 cells; stimulated STAT4 $CD4^+$TH1 cells). Yet, overall STAT-phosphorylation did not allow for a clear separation of healthy subjects and subjects with RA from each other in hierarchical cluster analysis.
Distinct distribution in the expression of STAT-phosphorylation in subjects allowed for their classification into subgroups. This might suggest the existence of endotypes within the collective, which would be determined by differing levels of particular STAT-molecules. Yet, as clustering showed no significant difference between the overall collective of RA patients and healthy subjects, it remains unclear whether endotypes within RA exist or if differences between subjects are caused by differing expressions in the human immune system.
Still, results might be of further interest, as patients with differing expression of STAT-molecules would be affected differently either by certain cytokines, but also by selective medication (targeted DMARDs). Therefore, testing patients onto their expression level of certain STATs might enable to target an overexpressed JAK-STAT pathway directly.
As our subjects' collective was relatively small (23 subjects) compared to the number of analysed variables (84 variables), further expansion of the collective might be reasonable.

8 Literature

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