Diplomarbeit

Analysis of Peripheral Lymphocytes in Patients with Sjögren's Syndrome

eingereicht von

Sabrina Theresa Hammerl

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Klinischen Abteilung für Rheumatologie und Immunologie

unter der Anleitung von

Ass.-Prof. Dr.med.univ. Martin Helmut Stradner und
Univ.-Prof. Dr.med.univ. Winfried Graninger

Graz, am 1.2.2017

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 1.2.2017 *Sabrina Hammerl eh*

Danksagungen

Diese Diplomarbeit möchte ich meinen Eltern Theresia und Werner Hammerl widmen, die einen Anker in meinem Leben darstellen, mich immer unterstützen und mir die Freiheit sowie das Vertrauen gegeben haben alle meine beruflichen und persönlichen Projekte zu realisieren.

Besonders bedanken möchte ich mich für die Unterstützung und Geduld meines Betreuers, Ass.-Prof. Dr.med.univ. Martin Helmut Stradner, der maßgeblich an der Entstehung dieser Arbeit beteiligt war.

Des Weiteren möchte ich noch Dipl.-Ing. Cristian Riccardi, Laura Promberger, Marina Berthold und Christoph Mödlhammer für ihre Unterstützung danken.

Zusammenfassung

Einleitung: Das Sjögren's syndrome (SS) ist eine langsam progressiv verlaufende Autoimmunerkrannung mit einer Prävalenz von 0,3%. Histologisches Kennzeichen ist eine lymphozytäre Infiltration des exokrinen Drüsenepithels, welche für die prädominierenden Symptome Xerostomie und Xerophthalmie verantworlich ist. Daneben können auch extraglanduläre Manifestationen auftreten. Eine schwerwiegende Komplikation stellt das Auftreten von Lymphomen da, welche in SS Patienten 20 mal häufiger als in der Normalbevölkerung auftreten.
In unserer Studie untersuchten wir die zirkulierende Lymphozyten im peripheren Blut von Patienten mit SS, um Hinweise auf die Pathogenese der Erkrankung und mögliche Biomarker für Prognose und Diagnose des SS zu identifizieren.

Material und Methoden: In dieser prospektiven Studie analysierten wir die Lymphozyten und deren Subpopulationen im peripheren Blut von 25 SS Patienten und von 129 gesunden Probanden mittels Durchflusszytometrie. Die absolute und relative Anzahl der unterschiedlichen Lymphozyten Populationen wurden zwischen beiden Gruppen verglichen und mit klinischen Daten korreliert.

Ergebnisse: Die Anzahl der T Lymphozyten war in SS Patienten erheblich reduziert. Dies war hauptsächlich auf einen Rückgang der T-Helferzellen zurückzuführen. Analysen dieser Population zeigten bemerkenswerte Veränderungen: Einen Rückgang der Th2, Th1/17, Effector-, Naïven und CD28-Zellen. Interessanterweise fanden wir mehr aktivierte CD4/CD8-doppel negative-, zytotoxische- und Th17-Zellen bei SS Patienten. Des Weiteren war die Anzahl der B Zellen aufgrund einer Reduktion der switched, CD21-negativen- und Marginalzonen B-Zellen erniedrigt.

Schlussfolgerung: Wir fanden einen Rückgang der T-Helfer Zellen in SS, welcher durch einen Rückgang der peripheren naïven und Effektor Zellen, Th2 und Th1/17 Zellen zustande gekommen ist. Als besonders interessant haben sich die aktivierten DN (doppelt negativen) Zellen herausgestellt, welche vermehrt bei Patienten mit SS vorzufinden sind und welche auch mit den Serum IgG korrelieren. DN Zellen sind

eine wichtige Quelle für IL-17, welches wiederum eine entscheidene Rolle in der Pathogenese des SS spielt.

Abstract

Introduction: Sjögren's syndrome (SS) is an autoimmune disease with lymphocytic infiltration of exocrine epithelia as a histological hallmark of disease. The resulting dysfunction of the exocrine glands leads to the predominant symptoms of dry eye and dry mouth summarized in the term Sicca syndrome.
SS can extend beyond the exocrine glands and affect other organ systems such as lungs, liver, kidneys, and the nervous system.
The Intention of this study was to analyze the distribution of lymphocyte populations in patients with SS to potentially gain insight into the pathogenesis and to identify biomarkers for the diagnosis or prognosis of SS.

Material and Methods: In this prospective study peripheral blood from 25 patients with Sjögren's syndrome (SS) diagnosed by AECG criteria and 129 healthy controls (HCs) was analyzed. We analyzed lymphocyte subsets in the peripheral blood of SS patients and HCs by flow cytometry. We compared the absolute and relative counts of lymphocyte subsets in both groups and correlated clinical data of our SS patients with the lymphocyte counts.

Results: We found a significantly decreased count of T lymphocytes in SS patients. This was mostly the result of a decline of the T-helper cell population. Analysis of this population showed remarkable changes in different subsets leading to this reduction: A decrease of Th2 cells, Th1/17, CD4+ effector-, naïve-, and CD28- cells. Interestingly, more activated CD4/CD8-double negative, CD8+, and Th17 cells were found in SS patients. The absolute count of B cells in SS patients was reduced due to a decrease of switched B cells, CD21-negative, and marginal zone B cells.

Discussion: We confirmed the reduction of T helper cells in SS. We found that this reduction is the result of a loss of peripheral naïve and effector cells, Th2 and Th1/17 cells. CD8+ cells in SS showed impaired proliferative capacity despite signs of activation Activated DN cells seem to be a promising lymphocyte subset as they are increased in SS and correlate with serum IgG. DN cells are a source of IL-17, which plays a pivotal role in the pathogenesis of SS.

Table of contents

DANKSAGUNGEN **3**

ZUSAMMENFASSUNG **4**

ABSTRACT **6**

TABLE OF CONTENTS **7**

LIST OF TABLES **9**

LIST OF FIGURES **10**

ABBREVIATIONS **12**

1 SJÖGREN'S SYNDROME **14**

1.1.1 Epidemiology 14

1.1.2 Pathophysiology 15

1.1.3 Clinical Manifestation 22

1.1.4 Diagnosis and Classification 26

1.1.5 Outcome Measures 31

1.1.6 Prognostic factors 32

1.1.7 Treatment 33

2 AIM OF THE STUDY **36**

3 MATERIAL AND METHODS **36**

3.1 PATIENTS 36

3.2 COLLECTION OF CLINICAL DATA 37

3.3 FLOW CYTOMETRY - FACS 38

3.4 MATCHING OF HEALTHY SUBJECTS 39

3.5 ANALYSIS OF FLOW CYTOMETRY DATA 41

3.6 ANTIBODY STAINING 41

3.6.1 T cell panel 1 41

3.6.2 T cell panel 2 43

3.6.3 B cell panel 44

3.6.4 B cell and T cell stimulation 45

3.6.5 NKT Panel 47

3.6.6 Cytokines 47

3.7 STATISTICS 48

4 RESULTS 48

4.1 PATIENTS CHARACTERISTICS 48
4.2 T CELL PANEL 1 51
4.3 T CELL PANEL 2 54
4.4 B CELL PANEL 57
4.5 B CELL STIMULATION PANEL 59
4.6 T CELL STIMULATION PANEL 60
4.7 NKT PANEL 62
4.8 COMPARING DIFFERENCES BETWEEN SS AND HC 64
4.8.1 T cells 64
4.8.2 B cells 72
4.8.3 T cell and B cell stimulation: 74
4.8.4 Cytokines 76
4.9 CORRELATION OF LYMPHOCYTE POPULATIONS WITH CLINICAL DATA 77
4.9.1 Correlation with the ESSDAI 77
4.9.2 Correlation with C3 80
4.9.3 Correlation with IgG 82
4.9.4 Correlation with BSG 83
4.9.5 Correlation with ANA 84

5 DISCUSSION 85

5.1 LIMITATIONS 89
5.2 CONCLUSION 89

6 LITERATURE 90

7 APPENDIX 104

List of tables

Table 1 Tools to objectify Sicca symptoms 26
Table 2 AECG Classification Criteria 27
Table 3 New ACR-EULAR Criteria 29
Table 4 Clinical association with autoantibodies 31
Table 5 Patients characteristics 37
Table 6 Patients for T1, T2 and B cell panel 40
Table 7 Patients for T cell stimulation panel 40
Table 8 Patients for T cell stimulation panel 40
Table 9 Patients for NKT panel 41
Table 10 Patients for cytokine release 41
Table 11 Antibodies of the T cell panel 1 42
Table 12 Antibodies of the T cell panel 2 43
Table 13 Antibodies of the B cell panel 45
Table 14 T cell antibodies 46
Table 15 B cell antibodies 46
Table 16 Antibodies for the NKT cell panel 47
Table 17 Collected clinical data from SS patients 49
Table 18 List of medication of SS patients 50
Table 19 Statistical results of the comparison of T cell populations in HCs to SS patients 66
Table 20 Statistical results of the comparison from B cell populations in HCs to SS patients 72
Table 21 Statistical results of the comparison stimulated T and B cell populations in HCs to SS patients 74
Table 22 Statistical results of the comparison from Cytokine levels in HCs to SS patients 76
Table 23 Correlation of lymphocyte populations with ESSDAI in SS patients 77
Table 24 Correlation of lymphocyte populations with C3 in SS patients 80
Table 25 Correlation of lymphocyte populations with BSG in SS patients 83
Table 26 Correlation of lymphocyte populations 84

List of figures

Figure 1 CD4+ Th cell subset differentiation under cytokine influence. APC: Antigen presenting cell (32) 19
Figure 2 Probability of NHL Development 33
Figure 3 The FACS machine detects individual cells by their cell surface antigens (55) 39
Figure 4 Density gradient centrifugation method (using Ficoll instead of Histopaque) WBC: White blood cells. RBC: Red blood cells (116) 44
Figure 5 CellTrace™ Violet method (120) 46
Figure 6 MAGPIX® Multiplexing System (122) 48
Figure 7 Classification Tree of Th lineage cells 51
Figure 8 Representative dot plots of the gating strategy for flow cytometric analysis of T cells lineages 53
Figure 9 Classification Tree for T cells 54
Figure 10 Representative dot plots of the gating strategy for flow cytometric analysis of T cells lineages 56
Figure 11 Classification tree for B cells 57
Figure 12 Representative dot plots of the gaiting strategy for cytometric analysis of B cells lineages 58
Figure 13 Proliferation rate of unstimulated B cells and B cells treated with ODN2395. ODN0125= 0,125 µM and ODN 025=0,25 µM. 59
Figure 14 Representative dot plots for the gating strategy for cytometric analysis of T cells lineages 60
Figure 15 Untreated and stimulated T cells with CON A 5mg, CON A 10mg and CD3/CD28 61
Figure 16 Classification Tree for NKT cells 62
Figure 17 Representative dot plots of the gating strategy for flow cytometric analysis of NKT cells 63
Figure 18 Overview about the major changes in the T cell population in SS 64
Figure 19 Simple box plot (124) 67
Figure 20 Absolute count (#) of CD3+ is reduced in SS patients with a p of 0,000097 68

Figure 21 Box plots showing differences in absolute and proportional count between HCs and SS 69
Figure 22 Overwiev about the major changes of the B cell population in SS 72
Figure 23 Box plots showing differences in B cells between HCs and SS 73
Figure 24 Box plots showing differences in proliferation of T cells between HCs and SS 75
Figure 25 Comparison of cytokine production in stimulated PBMCs between HCs to SS patients 76
Figure 26 Correlation between ESSDAI, absolute amount of CD3+ cells (left) and proportional amount of CD3+ cells (right) 77
Figure 27 Correlation of lymphocyte populations with ESSDAI 78
Figure 28 Correlation between lymphocyte populations and C3 81
Figure 29 Correlation between C3, absolute amount of CD3+ cells (left) and proportional amount of CD3+ cells 82
Figure 30 Correlation between C3 and proportional count of activated CD8+ cells (left) and between C3 and proportional count of activated DN cells (right) 83
Figure 31 Correlation between BSG and proportional count of activated Th2 cells (left) and proportional count of activated Th17 cells 84

Abbreviations

AECG	American European Consensus Group criteria
AIH	Autoimmune hepatitis
ANA	Antinuclear antibodies
Breg	Regulatory B cells
CD	Cluster of Differentiation
DMARD	Disease-modifying antirheumatic drugs
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EULAR	European league against rheumatism
FoxP3	Forkhead box protein P3
FSC	Forward scatter
GC	Germinal center
HCQ	Hydroxychloroquine
HCV	Hepatitis C Virus
HLA	Human Leukocyte Antigen
IL	Interleukin
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
MSG	Minor salivary gland
MTX	Methrothexat
NK	Natural Killer cells
NKT cells	Natural Killer T cells
PBC	Primary biliary cirrhosis
PBMC	Peripheral mononuclear blood cells
pEC	Preliminary European Classification
pSS	Primary Sjögren's syndrome
RA	Rheumatoid Arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RTX	Rituximab
SLE	Systemic lupus erythematous
SS	Sjögren's syndrome

SSC	Side scatter
sSS	Secondary Sjögren’s syndrome
TGF-β	Transforming growth factor β
Th cells	T helper cells
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cells

1 Sjögren's Syndrome

1.1.1 Epidemiology

Sjögren's Syndrome (SS) is a autoimmune disease with an estimated prevalence ranging from 0,01 to 0,3 % of the general population. (1) The epidemiology of SS is complicated by several factors. The symptoms are highly variable and range from mild manifestation with general dryness as a main feature to a more severe course of disease with systemic complications including the development of lymphoma. Above all, the mild manifestation can easily be mistaken for part of a normal aging process or for a side effect of medication and therefore not be taken seriously. (2) Although the AECG classification is the gold standard for SS, several other classifications still exists, complicating clinical research. (3) In November 2016 the new ACR-EULAR classification criteria were published with major changes.
Due to this variation, prevalence of pSS can vary even in the same epidemiological study depending on the classification criteria applied – In a Norwegian study the prevalence is 0,44 % using the European criteria and 0,22% using the AECG criteria. Consequently half of the patients diagnosed with SS by one set of criteria are not included in another one. (4)

SS can either appear as a single disease – defined as primary SS (pSS) or as a combination with other autoimmune diseases sSS (secondary SS). (5) Autoimmune disorders in which sSS occurs are: Rheumatoid arthritis (RA), Systemic lupus erythematosus (SLE), Systemic sclerosis, Primary biliary cirrhosis (PBC), Autoimmune hepatitis, Grave's disease, Hypothyroidism, Mixed connective disease, Poliomyositis and Sarcoidosis.
Like in most other autoimmune diseases a female propensity exists. The ratio male to female is reported to be from 1:9 (4) to 1:20. (6)
Besides propensity, also the type of manifestation and time for diagnosis is different in men than in women. The average male patient with pSS is 10 years older at the time of diagnosis than the average women with pSS and has already more extraglandular, systemic symptoms and complications. Additionally, men are more likely to be autoantibody negative. (7)
Mean onset of pSS is normally in the 4^{th} or 5^{th} decade, but also a later onset in the 6^{th} or 7^{th} decade is quite common.

1.1.2 Pathophysiology

The pathophysiology of SS and of autoimmunity in general still remains to be elucidated. It is believed to be multifactorial. (8) Multiple genetic, epigenetic, and environmental triggers like virus infection have been implicated. For the onset (9)of the disease several steps and the interaction between different mechanisms may be necessary. (10) Disturbance in the adaptive and in the innate immune system may play a part.

1.1.2.1 Genetic variants and epigenetics

Several family studies verified the genetic susceptibility to develop SS or other autoimmune diseases in relatives of pSS patients.
A Taiwanese research study including 105 patients with SS showed that in twins the probability to develop pSS is 19 times higher than the prevalence of a normal population if one of the twins suffer from SS. Children from pSS parents have a 11 times higher risk for pSS (11) and 4,4 % of first degree relatives with SS develop SS too. (12)

The strongest genetic association of SS exists with the Major Histocompatibility Complex (MHC) genes. In 2013, the completion of an international genome-wide association study shed more light into the understanding of HLA genetic variants in pSS. Patients and the control population were Europeans or European descendants from all over the word: The verified HLA loci were: HLA-DR3, B8, DQ2 and C4. (13, 14)
Other genes outside of the HLA region genes involved in the homeostasis of the immune system like IRF5, Stat4, TNIP1 and various cytokines seem to contribute in the in the pathophysiology of SS.

1.1.2.1.1 Epigenetic factors

The ancient Greek word επί/epi means 'upon', 'over'. Epigenetic therefore stands for mechanisms that change the DNA but don’t change the genetic code itself. These changes are determined by environmental conditions. The main mechanisms of epigenetics are DNA methylation, which represses or increases the expression

of genes, histone modification and micro RNAs which result in degradation or disruption of translation.

In SS a few pathological epigenetic mechanisms were identified: Reduced DNA methylation of salivary gland epithelial cells, abnormal autoantibody production associated with pathologic chromatin positioning and abnormal expression of micro RNA (9). In the closely related disease Systemic Lupus erythematosus pretreated hypomethylated CD4+ cells transferred into mice became autoreactive and produced anti DNA antibodies which led to a characteristic immune complex glomerulonephritis. (15)

1.1.2.1.2 Enviromental factors

Several studies showed that persistent or past viral infections play a part in the pathogenesis of pSS:

- Epstein Barr virus, which targets salivary glands cells, can lead to an asymptomatic infection or to infectious mononucleosis. In both cases the virus remains in the body lifelong with the possibility of reactivation.
  Virus-encoded micro RNA interact with the innate immune system and affects auto tolerance. (16)
- HIV, HTLV1 and HCV infections can cause SS like symptoms. Especially the HTLV1 virus was the focus of interest in some Japanese studies: HTLV1 infected patients showed a similar focus score to SS and had also an ANA positivity. This should be considered in diagnosis and suggests a partial role in pathogenesis of pSS. (17, 18)
- Coxsackie virus may be another virus with impact on pathogenesis of SS. Whereas Greek studies extracted (19, 20) Coxsackie RNA or proteins in infiltrated salivary glands of SS, other studies failed to prove these findings. (21)

A French case–control study with 170 cases and 350 gender and age matched controls evaluated the correlation between the occurrence of primary Sjögren's syndrome (pSS) and occupational risk factors.

In a questionnaire study, participants were asked about their socioeconomic and personal background, their complete medical and occupational histories. A significant association between pSS and white spirit, chlorinated or aromatic solvent exposure was identified. (22)

1.1.2.2 Epithelial Destruction

The initial step to autoimmunity in SS is thought to be epithelial dysfunction and epithelial damage. Activated immune pathways in the innate and then in the acquired immune system contribute to a perpetuation of the epithelialitis:

For a normal epithelial and secretory epithelial, cell polarity is an important feature. In pSS patients pro-inflammatory cytokines, like TNF alpha and IFN alpha (produced by the epithelia itself) change the cell to cell connection (tight junction). This results in a dysfunctional epithelial barrier function and leads to a misdirected exocytosis of salivia into the extracellular matrix. The altered mucin probably contributes to the development of an inflammatory process. (23)
Observations in mouse models (the nonobese diabetic mouse) support the idea that the first step is made in absence of the immune system, but in the second step the lymphocyte infiltration, which disturbs or even destroys the normal secretory function, is needed to develop the typical Sicca symptoms. (24)

1.1.2.3 Immune System in Sjögren Syndrom

The human immune system can formally be divided into two parts, the innate immune system with ancient origin and the adaptive immune system with highly specialized cells which are able to create immunologic memory.
Both systems collaborate closely to protect the human body against bacteria, viruses and all kinds of microorganisms. (25)
The adaptive immune system consists of Thymus derived cells (T cells) and bone marrow derived cells (B cells). Depending on their maturational stage different terms can be used for cells: Lymphocytes that still didn’t encounter any pathogen are called naïve and after activation they are named effector cells. Some effector cells develop into long-lived memory cells. If a reinfection occurs the memory cells can

rapidly recruit other cells and attack the invading pathogen more effetely than the first time.

T cells are main players of the cell mediated immune system and consist of several subsets with distinct function. The T cell receptor (TCR) of most T cells consists of an alpha and beta chain. Consequently they can be called alpha beta T cells. Major T cell subset are the CD4+ T helper cells, the CD8+ cytotoxic T cells. Besides, there exists a poorly understood T cell subpopulation with lack of CD4+ and CD8+ termed double negative (DN) T cells. Recent studies show lines of evidence of their importance in the pathogenesis of autoimmunity. (26)

CD4+ cells orchestrate the immunologic process by influencing the behavior and activity of other immune cells like the B cells. After activation, a naive CD4+ cell can differentiate depending on characteristic cytokines into different subsets (Figure 1). Classically, CD4+ cells have been divided into Th1 and Th2 cells, but in the last decade other Th cell lineages have been identified like Th17, regulatory,and follicular helper T cells. (27)

Th 17 cells are thought to be one of the main producers of IL-17. Their effect on autoimmunity became the focus of interest of immunological studies. (28) Studies with IL-17 knockout mice showed less susceptibility for autoimmunity disease. Blocking IL-17 signaling also stops the development of auto reactive germinal center (GC) formation, which is a histopathological sign of a more severe stage of epithelialitis. (29) Furthermore, permanent expression of IL-17 in minor salivary glands (MSG) infiltration and correlation with severity of inflammation was identified. (30, 31)

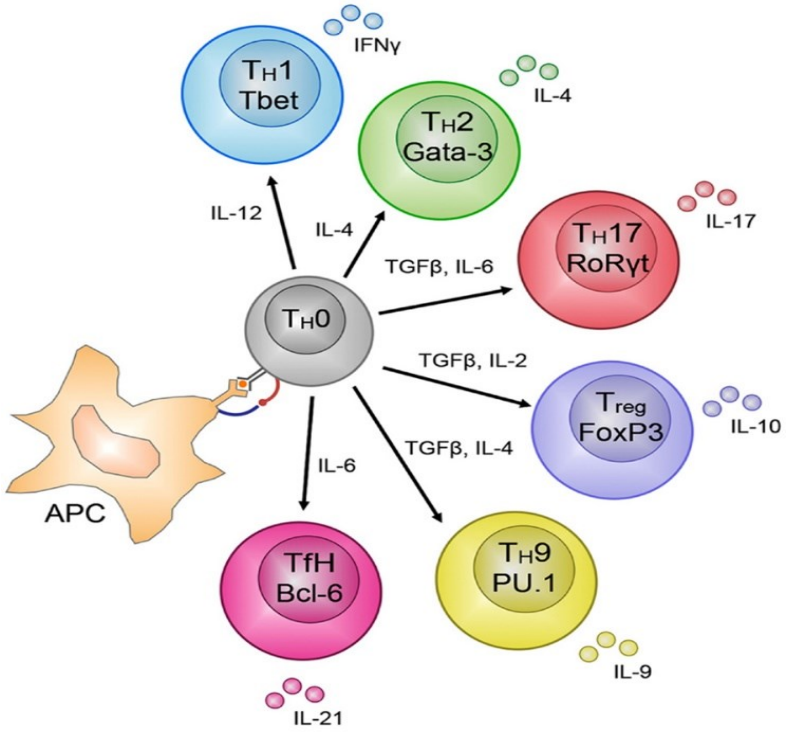


Figure 1 CD4+ Th cell subset differentiation under cytokine influence. APC: Antigen presenting cell (32)

CD8+ cytotoxic T-cells are responsible for the elimination of virus infected cells or altered cells in general. CD8+ T cells use two main pathways to kill target cells: either via exocrine granules containing toxic substance (perforin, granzyme B) or via Fas ligands promoting apoptosis of target cells. (33)

Antigen specific CD8+ cells are present in affected tissues and in peripheral blood of autoimmune diseases such as Multiple Sclerosis (MS) (34), RA (35) and autoimmune hepatitis (36). An increase of CD8+ cells correlates with disease severity not only in the Sjögren's syndrome (37) but also in other immune disorders like SLE (38) or MS (39).

In addition, the depletion of CD8+ cells in experimental studies delayed or reduced the severity of autoimmune disorders like MS (40) or Glomerulonephritis (41).

Hypotheses trying to explain auto reactive CD8+cells include underrepresentation of self-antigen in thymus, where auto-reactive cells won't be negatively selected. Also low avidity of auto-reactive CD8+ cells to tissue-restricted antigen allows them to escape central and peripheral tolerance. (42)

In addition to central tolerance, peripheral tolerance eliminates auto-reactive cells which escaped from the thymus. As main actors of peripheral tolerance, Regulatory

T cells (Treg) oppose auto-reactive cells. Tregs have been identified in an experimental animal model expressing the high affinity IL-2 receptor, CD25+ showing and inhibiting function on effector lymphocytes. (43, 44) For the differentiation of a naive T cell into a Treg cell, transforming growth factor beta (TGF-β) and the expression of the transcription factor forkhead box protein3 (FoxP3) are necessary. (45)
The role of Tregs in pSS has been evaluated in several studies with controversial results. Whereas the majority reported a decrease of Tregs in pSS compared to healthy controls, others found no difference or an increase of Tregs. (46)
An interesting aspect of similarity between Th17 and Treg cells has been observed. TGF-β is not only responsible for the differentiation from a naive T cell into a Treg cell but is also required for the differentiation from a naive T cell into a pathologic Th17 cell. Under the influence or absence of IL-6 the cell either turns into a Th17 cell, with potentially auto-reactive features or into a Treg cell, respectively. (44)

Natural killer T cells (NKT) are another unique population of lymphocytes which share properties of both T cells and natural killer cells. They have the ability to produce copious amounts of cytokines and are important players in regulating immune response. In comparison to the conventional T cells they recognize lipid antigens presented by CD1 molecules. (47) There is accumulating evidence that they have a protective role against the onset of autoimmunity similar to Tregs cells. In several autoimmune diseases a decreased amount of NKT cells has been identified including SS, (46) MS (48) and RA (49, 50).
Mouse models of diabetes and colitis confirmed the hypothesis by showing that a delayed onset of autoimmunity correlated with an overexpression of NKT cells. (51, 52)

Contrary to the long-standing opinion that SS is a T cell driven autoimmune disease, recent advances changed the view on B cells in the pathogenesis of pSS.
The B cell hyperactivity found in SS results in a hypergammaglobulinaemia, an increased level of rheumatoid factor and the appearance of SSA and SSB autoantibodies. Besides this, some efficacy of B depletion therapy with anti-CD20 reagents and the enhanced risk of developing a B cell lymphoma indicate that B cells play a role in pSS. (53, 54)

It has been suggested that B cell activation factor BAFF is responsible for B cell hyperactivity via up regulating of type 1 interferon. (55) BAFF is part of the tumor necrosis factor family and is induced under IFN- stimulation. Its pathological role was shown in pathogenesis of pSS. An increased expression of BAFF correlates with the autoantibody level. The BAFF-transgenic mice developed SS like symptoms in experimental studies. (24, 56-58)

Cytokines are a broad category of peptides used by the immune system for communication and coordination. Cytokines can be divided according to their function in to different families: the interferon family induces an antiviral response; the chemokine family directs cell migration, adhesion and activation; the tumor necrosis factor family direct inflammatory and immune response; the hematopoietins promote cell proliferation and differentiation and the transforming growth factor beta family is important for cell differentiation. (59, 60) In the interleukin family- the IL-17 and IL-17 producing cells have become the focus of interest regarding their impact on autoimmunity. IL-22 is produced by a large number of cells like TH17, y γδ T cells and NKT cells mainly influencing epithelial and non-hematopoietic cells. Increased levels of IL-22 are associated with epithelial inflammation but also regeneration of affected tissue. (61, 62) In MSG biopsies increased levels of IL-22, IL-23 and IL-17 were found in pSS patients compared to healthy controls. (63)

IFN γ plays a major part in the innate immune system and is produced by plasmacytoid dendritic cells. It activates natural killer cells and B cells autoantibody production, promotes the maturation and the development and maturation of macrophages. The so called IFN signature has been described in blood and in MSG tissue of pSS patients and stands for several changes in IFN inducible genes resulting in response to an over expression of IFN. The positivity of 5 certain IFN genes explained 95% of the total variance of the 11 IFN type I inducible genes and got summarized in the term ‘IFN type I signature’. (8, 64) Here, mainly IFN alpha and IFN gamma were involved Over 50 % of p SS patient display a positive IFN signature. (64, 65)

1.1.3 Clinical Manifestation

Sjögren's syndrome is a slowly progressive autoimmune disease with lymphocytic infiltration of exocrine and non-exocrine epithelia as histological hallmark of the disease. The predominant symptoms of the Sjögren's syndrome dry eye and dry mouth - Xerophtalmy and Xerostomy, respectively, are a consequence of lacrimal and salivary gland destruction by this pathological sialdenitis. Systemic symptoms can be fever, myalgia, fatigue and malaise.
In contrast to the usually benign clinical course, extraglandular manifestations can be found in a subset of patients such as interstitial nephritis, interstitial lung disease (ILD), arthritis or polyneuropathy. The most worrisome complication is the development of mucosa-associated lymphoid tissue (MALT) lymphoma. (2, 66)

1.1.3.1 Exocrinopathy - Sicca Syndrome

About 98 % of pSS patient suffer from either dryness of the mouth or dryness of the eye and about 89 % report to have both symptoms which is termed Sicca syndrome. (67)
Patients suffering from Xerostomia report a burning sensation in the mouth, difficulties to swallow dry food, changes in sense of taste and the inability to speak for a long time due to dryness. Absence of saliva leads to dental caries and to problems with wearing complete dentures. The physical examination shows an dry sticky mucosa and an atrophic tongue with lack of papillae.
Parotic gland enlargement or hypertrophy of other major exocrine glands is found in about in 60 % of pSS patients, but seem to be rare in secondary Sjögren's syndrome patients. (68)

Decreased tear production results in destruction of the bulbar conjunctive and the corneal epithelium leading to Keratoconjunctivitis sicca. Patients complain about sandy, scratchy or burning sensations under the lid. Other symptoms are redness, itchiness and an increased photosensitivity.

Besides, the Sicca symptoms can also affect other tissues, like the oropharynx or respiratory tract, causing hoarseness or recurrent bronchitis. Vaginal dryness and dermal dryness are other common features of pSS. Vaginal dryness impairs the

sexual life of pSS women significantly. They experience dyspareunia more often, are less sexually active and have less orgasms than healthy controls. (69)

1.1.3.2 Extraglandular manifestation

In pSS systemic manifestations and extraglandular organ involvement are reported in about one quarter of patients, more recent studies even suggest a higher percentage of up to 78 %. (70) The extraglandular organ involvement can be divided into two to groups:

- Inflammation of epithelial organs–autoimmune epithelitis: Manifestations include liver involvement, interstitial nephritis or bronchitis, which appear early and have a benign course.
- Effects of immune complex depositions due to the B cell hyperactivity can result in glomerulonephritis, palpable purpura or peripheral neuropathy. This complications correlate with an increased morbidity and the risk to develop lymphoma. (66)

In addition, over 50 % of SS patients experience an episode of joint involvement at least once during the course of disease. Reported symptoms are arthralgia, intermittent synovitis, morning stiffness and chronic polyarthritis.

The Raynaud syndrome is common in pSS and often precedes the sicca symptoms by several years. In contrast to scleroderma patients with Raynaud syndrome, digital ulcers or telangiectasias are absent in pSS patients.
Other skin features are purpura (often present in patients with hypergammaglobulinaemia), annular erythema located in the face or in the upper extremities, which fade without leaving scares within a few months, and pernio-like lesions.

Respiratory involvement can be found in many pSS patients. This includes dry cough due to lack of tracheobronchial mucus, bronchiectasis, obstructive airway disease and interstitial lung disease (ILD). Occurrence of ILD indicates a more severe course of disease The incidence of developing ILD increases with duration of disease: 10 % after one year, 20 % after 5 years and 43 % after 15 years after pSS onset. (71)

5% of pSS patients suffer from renal involvement, presented by either glomerulonephritis or interstitial nephritis. Subclinical involvement tested by abnormal urine acidification tests is observed in even one third of p SS patients and can cause complications as kidney stones, nephrocalcinosis and lead to a compromised renal function. Hypokalemia leading to life threatening situations is a severe complication of the renal tubular acidosis. (72)
Glomerulonephritis is a manifestation of pSS rarely seen and is mainly reported in patients with cryoglobulinemia and hypocomplementemia.

Gastrointestinal features are quite common in pSS. Patients complain often about dysphagia because of pharyngeal and esophageal dryness or abnormal esophageal motility. Gastric mucosa biopsy showed chronic atrophic gastritis with lymphocytic infiltration similar to the glandular infiltration and explaining epigastric pain and nausea.
The correlation between chronic liver disease and pSS is a well-established manifestation of pSS. 5 to 26 % of SS patients show at least mild abnormal biochemical hepatic tests. Primary biliary cholangitis and autoimmune hepatitis can be the cause of elevated liver enzymes. Besides this, hepatitis C virus infection, drug toxicity and nonalcoholic fatty liver should be considered. (73)
High prevalence of sSS is found in patients with primary biliary cholangitis.(74)
Interestingly enough is the special association of HCV an SS - a multicentric study reported that most cases of HCV related to SS are indistinguishable using the main sets of classification criteria to primary SS (HCV is part of the exclusion criteria for diagnosis) It is believed that in this case, HCV is the direct causer for SS.

The most common symptoms of either small or medium sized vessel vasculitis are purpura, skin ulcerations and mononeurits multiplex which appear in about 5% of SS patients.

Neuromuscular involvement in pSS is a well-documented feature of disease and the prevalence varies in between 10 up to 60 %. Peripheral sensorimotor polyneuropathy is the most common symptom but also cranial neuropathy of the optical or trigeminal nerve can be seen. Other rare CNS manifestation range from

hemiparesis, hemisensory deficits, diffuse brain injury up to aseptic meningitis and dementia. (75)
Hashimoto thyreoiditis is often described in SS patients. Some studies even find a prevalence of up to one third. Anti-thyreoidal antibodies and altered thyroid function are found in these patients.

The association between lymphoma (non Hodgkin lymphoma or lymphoproliferative disease) is has been known for many years and will be discussed in more detail in chapter 1.1.6 Prognostic factors. (66)

1.1.4 Diagnosis and Classification

1.1.4.1 American European Consensus Group Criteria

Symptoms of SS can vary over a wide range and can easily be mistaken for symptoms of another cause. Keeping in mind that up to 30 % of the elderly report Sicca symptoms without any correlation to SS, it seems to be difficult to select for evaluation of SS. (76) Consequently, a classification with high sensitivity and specificity is necessary for the diagnosis of SS.

Patients with following clinical manifestations should be tested for SS:

- Parotic gland enlargement
- Permanent symptoms of dry mouth or dry eyes
- Unexplained dental caries
- Lab abnormalities like the presence of anti-Ro, anti-La, RF and hyperglobulinemia

Table 1 Tools to objectify Sicca symptoms

MARKER FOR DRY EYES	• Schirmer's test • Ocular surface staining • Tear break up time
MARKER FOR DRY MOUTH	• Saxon test • Sialometry • Salivary gland scintigraphy

American European Consensus Group classification criteria (AECG) are still assumed by many clinicians to be gold standard. The AECG criteria, published in 2002, combine clinical subjective elements with laboratory abnormalities and has a focus on the presence of anti-Ro and anti-La autoantibodies. The AECG criteria are nowadays the most commonly used tool for diagnosis worldwide. (3)

Table 2 AECG Classification Criteria

I.OCULAR SYMPTOMS	• Dry eyes >3 months or • Use of artificial tears >3x per day or • Foreign body sensation or gravel in the eyes
II.ORAL SYMPTOMS	• Dry mouth >3 months or • Recurrent or persistently swollen salivary glands or • Need liquids to swallow dry foods
III. OCULAR SIGNS	• Schirmer's test, (without anesthesia) ≤5 mm/ 5 minutes or • Positive vital dye staining (van Bijsterveld ≥4)
IV. HISTOPATHOLOGY	• Minor salivary gland biopsy showing focal lymphocytic sialoadenitis and focus score ≥1 per 4 mm2 (more than 50 lymphocytes)
V. ORAL SIGNS	• Unstimulated whole salivary flow ≤1.5 mL in 15 minutes or • Abnormal parotid sialography showing presence of diffuse sialectasias or • Abnormal salivary scintigraphy presenting a delayed uptake, reduced concentration and/or delayed excretion of tracer
VI. AUTOANTIBODIES	• Anti-SSA (Ro) or • Anti-SSB (La) or • Both

Positivity is defined as:

a) The presence of any 4 of the 6 items, as long as either item IV or VI is positive
b) The presence of any 3 of the 4 objective criteria items

To be diagnosed with pSS, either a positive antibody or a positive lip biopsy is required

For secondary SS:

a) presence of item I or II plus any 2 of III, IV, V

Exclusion criteria:

- Past head and neck radiation treatment
- Pre-existing lymphoma
- Sarcoidosis
- Hepatitis C infection
- Aquired immunodeficiency disease, AIDS
- Graft versus host disease
- Use of anticholinergic drugs (since a time shorter than 4 fold the half-life of the drug) (77)

1.1.4.2 Are the AECG criteria too stringent?

The AECG criteria are more specific but maybe less sensitive compared to the preliminary European criteria (pEC) or the Copenhagen criteria. A Norwegian study showed that out of 116 patients diagnosed by pEC just 83 fulfilled the AECG criteria, which results in losing 30 % of the patients. Suchlike has been reported for the Copenhagen criteria, where in a study from Denmark just 205 patients out of the 321 diagnosed pSS were able to fulfill AECG criteria. (76)
When comparing patients fulfilling AECG criteria to patients excluded by AECG, but included by other criteria, AECG patients have a higher prevalence of parotid enlargement, arthralgia, skin involvement, hypergammaglobulinaemia and Raynaud's phenomenon. Yet, they have a similar frequency of sicca symptoms, abnormal ocular eye test, dry vagina and peripheral nervous system involvement and, most importantly, no differences in treatment strategies and outcome.

The AECG criteria on one side guarantee homogeneity of patients, making clinical or epidemiological studies more comparable, but on the other hand, they require invasive technique (lip biopsy), can't be used as a predictive tool and do not cover the broad clinical heterogeneity of pSS. (78)
Ramos Casals for example suggest to use the term "Sjögren's disease" for patients fulfilling the AECG criteria and "Sjögren's syndrome" for patients not fulfilling AECG criteria to keep their pathological background in mind. (79)

1.1.4.3 New ACR-EULAR Criteria

In November 2016 the new classification criteria were published by the American College of Rheumatology and the European League against Rheumatism. (80)
Major changes of the AECG criteria were made: From the seven main domains just five entered the new criteria. The subjective part (with a questionnaire regarding the Xerostomia and the Xerophtalmy) and the autoantibody anti-SSB/La were excluded. For ocular signs the Ocular Staining Score (OSS) using lissamine green and fluorescein and the Van Bijsterveld Score were added to Schirmer's Test.
Each domain is differently weighed and summed up for the total score. Persons with a total score ≥4 meet the criteria for primary SS. Specificity and Sensitivity are 95 % and 96 % respectively.

Table 3 New ACR-EULAR Criteria

Characteristic	Weight/ Score
Labial salivary gland with focal lymphocytic sialadenitis and focus score of ≥1 foci/4 mm^2	3
Anti-SSA/Ro-positive	3
Ocular Staining Score ≥5 (or van Bijsterveld score ≥4) in at least one eye	1
Schirmer's test ≤5 mm/5 min in at least one eye	1
Unstimulated whole saliva flow rate ≤0.1 mL/min	1
Total score: ≥4 meet the criteria for primary SS	

(80)

1.1.4.4 Autoantibodies

Autoantibodies play an important role in diagnosis and pathogenesis of autoimmune disorders. In 80 % of the pSS patients, antinuclear antibodies (ANA), are found. ANA is a generic term for various kinds of Antibodies, which bind to different parts of the cell nucleus. ANA can be found in various kinds of other autoimmune diseases like in Hashimoto thyroiditis. High levels of rheumatoid factor (RF) are associated with rheumatoid arthritis and Sjögren's syndrome. Anti SSA/Ro and anti SSB/La are known to be the hallmark auto antibodies of SS and are one of the cardinal features of the AECG criteria classification. They are targeting ribonculeonprotein complexes associated with three proteins:

- the Ro 52 kDa,
- the Ro 60 kDa and
- the La protein

Ro 60 is thought to function as a RNA chaperon and has anti-inflammatory potential decreasing the expression of type 1 Interferon. (81) Ro 52 has an E3 ligase activity which promotes the ubiquitation of proteins. La is a transcript termination factor of RNA polymerase III. Anti La/SSB is normally just positive in combination with anti SSA/Ro, whereas anti SSA/Ro can also be detected solely. (82) The Ro/La antibody profile usually stays unaltered during the course of disease. (83)

Anti- SSA/ Ro and anti- SSB/ La can be associated with other autoimmune diseases including SLE, systemic sclerosis, idiopathic inflammatory myopathies, PBC, and RA. (84)

A recently published Swedish study reported that autoantibodies (ANA, RF, Ro and La) could be found up to 20 years before the disease onset (median 4-5 years) and that anti Ro and anti La positivity was strongly associated with an early onset of pSS and a severe course of the disease (85). A Norwegian study showed differences in detection of these autoantibodies. Ro 52 differs depending on the immunoassay used from 48 to 79 % positivity. Ro 60 and La differs from 69 to 77 % and from 39 to 44 % respectively. (86)

Table 4 Clinical association with autoantibodies

Autoantibodies against	Clinical association
Ro 52	• Younger age at diagnose • Exocrine gland hypofunction
Ro 60	• Severe infiltration of salivary glands • Salivary gland enlargement • Extraglandular manifestation
La	• Hypergammaglobulinaemia • Cryoglobulinemia • Congenital heart block
RF	• Younger age at diagnose • Extraglandular manifestation
ANA	• Younger age • Salivary gland enlargement • Extraglandular manifestation

(82, 87)

1.1.5 Outcome Measures

Symptoms of pSS caused by epithelitis are normally slowly progressive, whereas extra epithelial symptoms are often a sign for a more severe course of disease and require a different management. The clinical evaluation should therefore be divided into two perspectives:

- The benign symptoms of dryness, general fatigue, muscular pain affecting almost all patients are measured by the EULAR SS patient reported index (ESSPRI) and
- The systemic manifestation like renal and lung involvement, neurological features or lymphoma assessed by the EULAR SS disease activity index (ESSDAI) (88)

1.1.5.1 Evaluation of Systemic manifestation of pSS/ ESSDAI

The ESSDAI was developed by a large expert group from several European and North American countries in 2009. It includes 12 domains where each domain consists of 3 to 4 levels according to their degree of activity. Each domain is rated from none to severe and points are scored depending on domain weight. (ESSDAI Score can be found in chapter 7 Appendix)

1.1.5.2 Evaluation of the SICCA symptoms

ESSPRI is a score based on previous scores (SSI and Profad) and was developed in 2010 by international experts. It's was created in a user friendly style and should be filled out by the patients themselves. In the three domains: dryness, fatigue and pain (articular and or muscular), the patient scales their actual status from 0 up to 10 points, where 10 point represent the maximum of dryness, fatigue and pain. The final score is the mean of the three domains.
A study proved that ESSDAI und ESSPRI are significantly correlated with health outcome values. (89)

The ESSDAI and ESSPRI are nowadays broadly used and seen as the gold standard for clinical evaluation of pSS. (90) Because they are complementary, it is recommended to use them together in clinical trials. (91)

1.1.6 Prognostic factors

The connection between SS and lymphoma has been known for many years. Patients with SS have a 20 times increased risk to develop a non-Hodgkin lymphoma compared to the general population and the highest risk among all other systemic autoimmune diseases. Most of the lymphoma are MALT, but also aggressive subtypes like the diffuse large B cell lymphoma can be found. (92, 93)
A systemic literature review compared 19 studies related to lymphoma prediction. Parotic gland enlargement was considered as a good predictive factor regarding not only lymphoma prediction, but also the appearance of extra glandular manifestations. (88)

Other biomarkers identified were: Low C4 levels and presence of cryoglobuline as strong markers. Lymphadenopathy, palpable purpura and a CD4/CD8 ratio under 0,8 are also suggested to predict the development of a Lymphoma. (94)

A recently published study by Moutsopoulus presents a new predictive tool to identify high risk patients. The following seven parameters were identified as independent adverse predictors for lymphoma in SS patients:

- Persistent salivary gland enlargement
- Lymphadenopathy
- Raynaud phenomenon
- RF positivity
- Anti-Ro/SSA, anti-Ia/SSB positivity
- Monoclonal gammopathy
- C4 hypocomplementemia (92)

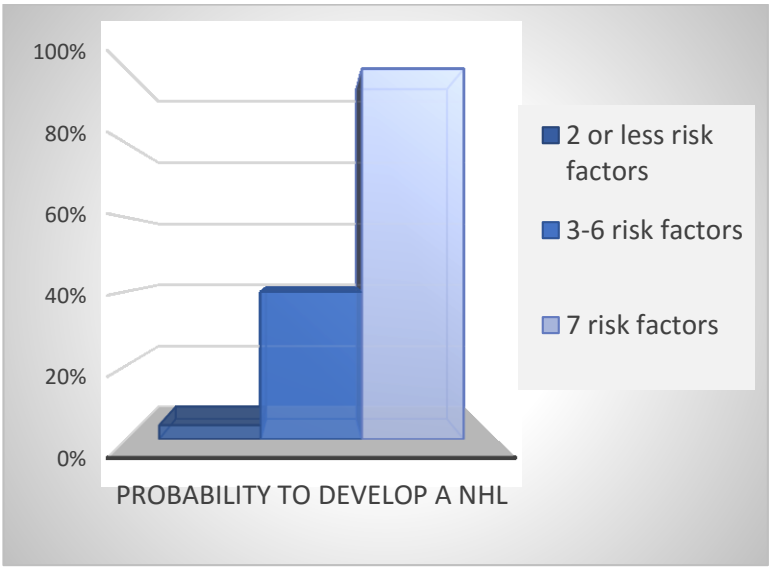


Figure 2 Probability of NHL Development

1.1.7 Treatment

The relief of the symptoms caused by exocrinopathy stands in the center of SS treatment. In case of secondary SS, the therapy of the underlying disease has priority. Just in a few patients with more severe extraglandular manifestation, a treatment with immunosuppressants or steroids is recommended. SS shows a wide range of symptoms and multidisciplinary care is therefore necessary. (66)

1.1.7.1 Treatment of Keratoconjunctivitis sicca

One of the most essential parts of management is educating the patient how to protect the eye against dryness and to prevent complications.The use of glasses against wind, using eye drops as often as needed and closing the eyes sometimes for a few minutes should be included.(95)

The use of glucocorticoid eyedrops, tacrolismus (a calcineurin inhibitor) eye drops and autolog serum in combination with a punctum plug (Plug inserted in the entrance to close the canaliculus lacrimalis) seem to decrease eye irritations.
(96)

The efficacy of fluoromethalon eyedrops and cyclosporine eyedrops were compared in a Chinese study, wherefluormethalon were found to be more potent. (97). The use of hydrogel contact lenses is recommendable in the prevention of Sicca symptoms. (98, 99)

1.1.7.2 Treatment of Xerostomia

The treatment of Xerostomia includes the relief of the dry mouth sensation and the prevention of dental caries, periodontal disease, dysphagia, halitosis, salivary gland calculi and oral candidiasis.

To relieve mucosal dryness, the maintenance of hydration includes sipping sugar free liquid regularly and avoiding aliments irritating the oral mucosa like alcohol, coffee, nicotine or acid drinks. Additionally, it should be mentioned that excessive sipping can worsen the symptoms and even destroy the oral mucosa. (68, 100, 101)
Regular visits to a dentist familiar with the treatment of SS and a good dental self-care by using dental floss and brushing frequently are basic tools in the treatment. Besides that, awareness for symptoms of candidiasis should be raised. (101)

More severe complaints can be treated pharmacologically with pilocarpine (M1, M2, M3 agonist), which has also a positive effect on nasal, vaginal and skin dryness.(102, 103) Cevimeline, a derivate of acetylcholine with high affinity to M1 and M3 receptors, proved its efficacy in several studies. (104)

1.1.7.3 Treatment of other symptoms

Vaginal dryness can be treated by lubricant jellies and dry skin with body lotions. In case of the persistence of symptoms, the treatment with pilocarpine or cevimeline should be considered. (101) Sensory neuropathies, quite common in pSS patients are treated symptomatically with gabalin or pregabalin. Systemic corticosteroids can be considered for the treatment of interstitial nephritis, glomerulonephritis, organ threatening vasculitis or motor neuropathies. (61)

1.1.7.4 Immunmodulatory therapy, options

Hydroxychloroquine (HCQ), an antimalarial substance, is the first line therapy for arthralgia, lymphadenopathy, myalgia, and skin manifestations. Based on its efficacy in SLE SS patients were treated with HCQ. (105) It's efficacy in the treatment of SS is controversial. Many studies suggest an improvement of symptoms. Yet a big two year trial failed to report any improvement. (106)Patients with inadequate response to HCQ may be treated with methotrexate (MTX) at weekly intervals either alone or in combination with HCQ. Reduction of symptoms were found by several studies. (107, 108)

Rituximab (RTX) targets the CD20 molecules which are mainly found on mature and pre-B cells, but not on pro B cells, stem cells, plasma cells or on other tissue. (109) The efficacy of rituximab is not clearly proven, but various studies showed promising results: An Italian study, including 40 patient in early stage Sjögren's syndrome with a follow up of 120 weeks, demonstrated that RTX treatment leads to a faster and more pronounced decrease of ESSDAI and other clinical parameters compared to DMARDs treatment. (110) Other studies have reported moderate evidence with an improvement in lacrimal gland function. (111)

Belimumab, a monoclonal antibody against BAFF, showed efficacy in over 60% of patients but further investigation should reproduce the effects. The efficacy of belimumab seemed to depend on the amount of NK cells found in peripheral blood. Patient with low NK cells benefit more from belimumab therapy. (112)

One important adverse effects of monoclonal antibody therapy is the so called serum sickness which is characterized by fever, arthralgia and rash. It's a type III hypersensitivity reaction with immune-complex depositing into parenchyma 1-2 weeks after application of the antigen leading to activation of complement cascade

and systemic response. (113) Serum sickness is a self-limiting disease and is reported to be associated with rituximab, but also with belimumab, bupropion and antibiotics (e.g.: metronidazole, cefazolin). (114)
Azathioprine may be used either in combination or as monotherapy to treat severe extraglandular manifestations.(95)

2 Aim of the study

The aim of the study was to characterize the lymphocyte populations in patients suffering from SS as compared to healthy controls. This information may hint towards new aspects of the pathogenesis of SS. Furthermore, we aimed to identify candidates for new biomarkers that could aid in diagnosis or prognosis of SS.

3 Material and Methods

3.1 Patients

In a prospective study 28 patients with the diagnosis of SS according the AECG criteria were recruited at the rheumatology outpatient clinic of the Medical University of Graz. A pool of 129 healthy persons participating in the Austrian health examination program at the Steirische Gebietskrankenkasse were used as healthy controls. Permission to perform this study was obtained from the Institutional Review Board of the Medical University of Graz. A written informed consent was obtained from all participants. Forty-five ml of venous peripheral blood was collected from all participants.

Table 5 Patients characteristics

	26 SS *	129 HC
Age	61,24 (±12)	39,4 (±15)
Sex Female Male	25 1	58 71
Inclusion Criteria	• Legal age • Fulfilling the American European Consensus Group classification AECG	• Legal age
Exclusion criteria	• Pregnancy • Neoplastic disease	• Pregnancy • Neoplastic disease • Acute or chronic infectious disease • Autoimmune disease • Acute or chronic disease with organ manifestation • Anemia (< 9 Hb mg/dl)

*During the course of investigation we had to exclude one pSS patient because of pregnancy and another one because of not full filling the AECG criteria

3.2 Collection of Clinical Data

From all healthy participants, sex, age and CRP were recorded. Subjects with elevated CRP were excluded. The following clinical data were collected from the SS patients:

- CRP
- BSG
- IgG
- C3
- C4
- ANA
- Sicca Score
- Focus Score
- ESSDAI
- Rheumatoid factor
- Ro (positive or negative)
- La (positive or negative)
- ESSPRI

3.3 Flow cytometry - FACS

FACS stands for fluorescence-activated cell sorter (FACS) and is a frequently used tool in immunology to define and enumerate cells by using fluorochrome labeled antibodies against cell surface or intracellular proteins (antibody staining). Lymphocytes, comprise many subpopulations, which can be distinguished either by the cytokines they produce or by the Cluster of differentiation (CD). The CD refers to a group of surfaces antigens which used for the identification of lymphocytes. Most of the surface antigens are glycoproteins with different function like receptor, signal function or have an enzyme activity. This surface properties are used as a target for antigen staining and consequently for the identification of lymphocyte population. (115)

In the FACS machine antibody stained cells are forced through a nozzle. In the resulting fine stream each cells passes at a time through a laser beam. Cells passing through the laser scatter the laser light and fluorochrome dye molecules bound to the cell will be excited and emit fluorescence.
The forward scatter detector detects the amount of light in forward direction as laser light strikes the cell and is proportional to the size of the cell. The side scatter detector obtains information on cell granularity or complexity which can be very characteristic for certain cell types. Multiple detectors in an arrangement with mirrors finally detect the fluorescence emission.
Therefor cells can be sorted by their size, granularity and fluorescence which makes it possible to identify many subtypes of lymphocytes.(60, 115)
Four our study we used the FACS Canto II from BD Bioscience.

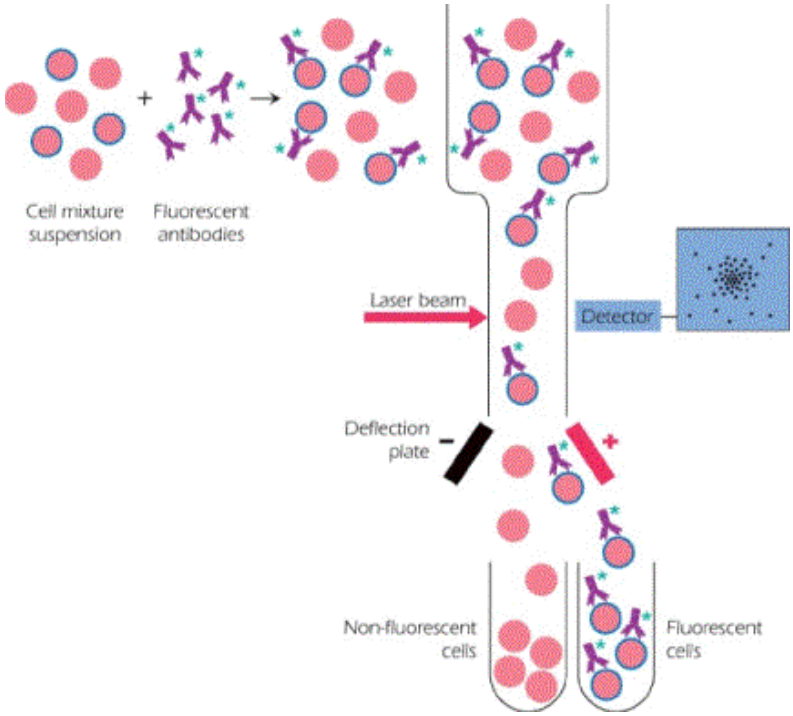


Figure 3 The FACS machine detects individual cells by their cell surface antigens (55)

3.4 Matching of healthy subjects

We performed 7 different sets of analyses:

- T cell subpopulation panel 1 (T1)
- T cell subpopulation panel 2 (T2)
- B cell panel
- B cell stimulation panel
- T cell stimulation panel
- NKT cell panel and
- Cytokine release

Due to the fact that we were not able to perform all panels for all SS patients we created 5 sex and age matched groups of HCs.

T1, T2 and B cell panel:

For the T1, T2 and B cell panel samples of 26 pSS patients were stained. From our pool of healthy patients, 30 were chosen to establish two age and sex matched groups. To verify the equality of both groups, a two sample T- test for unpaired samples was performed.

Table 6 Patients for T1, T2 and B cell panel

	SS	HC	T- Test p
N	26	30	
Female	25	29	
Male	1	1	
Age	61	59,5	0,49

T cell stimulation panel:

For the T cell stimulation panel, 42 samples were stained. T-Test with p = 0,87 confirmed no significant difference between both groups.

Table 7 Patients for T cell stimulation panel

	SS	HC	T- Test p
N	20	22	
Female	19	21	
Male	1	1	
Age	60,2	59	0,874

B cell stimulation panel:

For B-cell stimulation panel, 41 samples were stained. Equality regarding the age of both groups was proved.

Table 8 Patients for T cell stimulation panel

	SS	HC	T- Test p
N	20	21	
Female	19	20	
Male	1	1	
Age	60,9	59,6	0,587

NKT panel:

In comparison to our other staining's the NKT required a special, more complicated, procedure. Consequently, just 36 samples were stained and it was not possible to create a more equal group regarding sex.

Table 9 Patients for NKT panel

	SS	HC	T- Test p
N	18	18	
Female	17	9	
Male	1	9	
Age	59,8	57,2	0,92

Cytokine release:

Samples from 51 participants were used for cytokine analysis. The T- Test results in p = 0,54 and shows no significant difference in age between both groups

Table 10 Patients for cytokine release

	SS	HC	T- Test p
N	25	26	
Female	24	25	
Male	1	1	
Age	60,4	61,7	0,56

3.5 Analysis of flow cytometry data

Flow cytometer results were analyzed with FlowJo Version 10.0.7r2 (Ashland, OR, USA) and BD FACS Diva (BD).

3.6 Antibody staining

3.6.1 T cell panel 1

Surface staining was performed at room temperature for 15 min in darkness. Darkness is required because of the use of fluorescence colors. Then, cells were fixed and permeabilized using the FoxP3 staining buffer reagents (e-Bioscience, San Diego, USA) for 30 min by 4ºC. Fixation immobilizes antigens and retains cellular and subcellular structure giving antibodies access to all subcellular

compartments. Permeabilization creates holes in the membrane of the lymphocytes which is necessary to reach intracellular compartments.

Furthermore, cells were washed twice with permeabilization buffer and then incubated for further 30 min in darkness with an antibody against the intracellular antigen Ki67. Afterwards, cells were washed again twice with permeabilization buffer. Thus all unnecessary content like erythrocytes, thrombocytes and granulocytes are washed out. In the end the sample was resuspended with staining buffer and finally analyzed in the FACS machine.

Protocol:

1. 200 µl blood from EDTA coated tube
2. + 10 µl T-cell Cocktail 1 (customer cocktail, Miltenyi Biotec, Bergisch Gladbach, NRW, Germany) containing:

Table 11 Antibodies of the T cell panel 1

ANTIBODIES	ANTIBODY DYE
CD3	APC Vio 770
CD4	VIT4 Vio Blue
CD8	VioGreen
CD183 CXCR3	PE
CD196 CCR6	PE Vio 770
CD194 CCR4	APC
CD38	FITC

3. 15 min incubation in darkness
4. Prepare Fixation/Permeabilizationsolution1:4 (1ml concentrate + 3ml diluent of Foxp3 Staining Buffer Set, e-Bioscience, San Diego, USA)
5. Add 1 ml Fixation/ Permeabilization solution
6. 30 min incubation by 4ºC
7. Washing 2x with permeabilization buffer
   Centrifugation 300-400 g 5 min
   (1 ml buffer Flow Cytometry Staining Buffer Solution, e-Bioscience)
8. Add 100 µl 1x Permeabilization Buffer
9. Add 10 µl Ki67 PercP-Vio700 (Becton Dickinson, Franklin Lakes, New Jersey, USA)
10. 30 min incubation in darkness
11. Washing 2x with 2 ml Permeabilization buffers
    300-400g 5 min
12. Add 100µl Staining buffer (e-Bioscience)

3.6.2 T cell panel 2

Surface staining was performed at room temperature in darkness for 15 min. Then red blood was lysed by incubation with FACS Lyse. Leukocytes were centrifuged and then washed two times with Cellwash and resuspended with Cellwash. Finally, tubes were counted in FACS machine.

Protocol:

1. 200 µl blood from EDTA coated tube
2. + 10 µl T-cell Cocktail 2 (Miltenyi), containing:

Table 12 Antibodies of the T cell panel 2

ANTIBODIES	ANTIBODY DYE
CD3	APC-Vio770
CD4 VIT4	PerCP Vio 700
CD8	Vio Green
CD197 CCR7	APC
CD127	PE Vio 770
CD 28	PE
CD25	VioBright FITC
CD45RA	VioBlue

3. 15 min incubation in darkness
4. Add 2 ml FACS Lyse (BD)
5. Washing 2x with 2 ml Cellwash (BD)
   300-400g 5 min
6. Add 150 µl Cellwash

3.6.3 B cell panel

For purifying peripheral mononuclear blood cells (PBMCs) the density gradient centrifugation method using Histopaque (H1077 Hybri-Max; Sigma-Aldrich, St. Louis, Missouri, US) was used. Histopaque has a density in between the density of PBMCs/Plasma and red blood cells. Due to this difference a layer of white blood cells -the so called buffy coat - appears after centrifugation (1870 RPM, 30 minutes,) above the Histopaque and can easily be taken out.

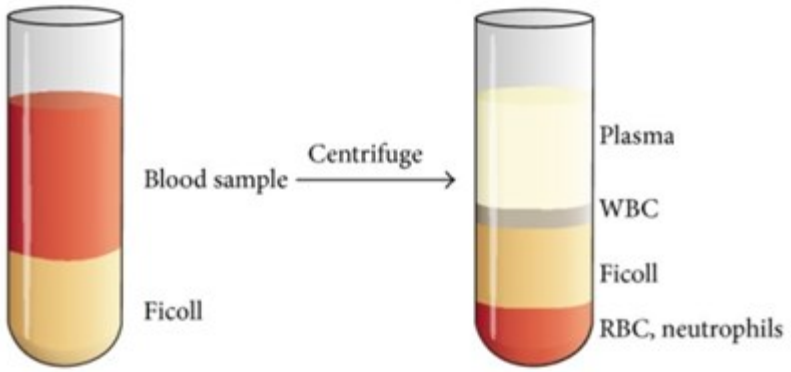


Figure 4 Density gradient centrifugation method (using Ficoll instead of Histopaque) WBC: White blood cells, RBC: Red blood cells (116)

Subsequently cells were counted with the help of a cell counter (Beckmann Coulter, Brea, California, USA). One million PBMCs were diluted into 100µl PBS followed by surface staining at room temperature in darkness for 15 min. Afterwards cells were washed twice with Cellwash, resuspended with 150 µl Cellwash and were analyzed in the FACS machine.

Protocol:

1. Dilute two Lithium Heparin tubes with PBS 1: 2,5 (phosphate buffered saline Puffer, pH 7,2-7,3, pharmacy of LKH Graz)
2. Add Histopaque to a 50 ml conical tube Histopaque (for 1ml blood 1 ml Histopaque)
3. Gently overlay Histopaque with diluted blood and avoid mixing the two phases
4. Centrifuge tube by 400g (400: 1400 U/min) 30 min without break
5. Take buffy coat and add it to a 12 ml tube
6. Fill tube with 12 ml PBS
7. Centrifuge tube by 250g (400: 1200 U/min) 10 min
8. Decent supernatant and refill with 12 ml PBS
9. Centrifuge tube by 250g (400: 1200 U/min) 10 min

10. Decent supernatant and resuspend with 300 µl PBS
11. Count cells with the help of a cell counter (Beckmann Coulter, California, USA)
12. 10^6 PBMCs diluted in 100µl PBS
13. + 10 µl b cell cocktail, containing:

Table 13 Antibodies of the B cell panel

ANTIBODIES	ANTIBODY DYE
CD 19	Vio Green
IGD	Vio Blue
CD 24	PerCP-Vio 700
CD 27	APC
CD38	Fitc
CD86	PE-Vio 770
CD 21	APC-Vio 770
IGM	PE

14. 15 min incubation in darkness
15. Washing 2x with 2 ml Cellwash
    300-400g 5 min
16. Add 150 µl Cellwash

3.6.4 B cell and T cell stimulation

PBMCs gained by the density gradient centrifugation method were cultured in 96 well plates (Greiner, Kremsmünster, Austria) with RPMI 1640 (RPMI stands for Roswell Park Memorial Institute where the medium was invented, Invitrogen/Gibco Carlsbad, California, USA) containing 10% FBS (fetal bovine serum, Invitrogen/Gibco) and Penicillin-Streptomycin and L-glutamine (Invitrogen/Gibco). Then T Cells were stimulated with CD3/CD28 (Invitrogen/Gibco), Concanavalin A (ConA) (Sigma-Aldrich) 10 mg and ConA 5 mg for 72h at 37°C and 5% CO2. CD3/CD28 is a T cell stimulator mimicking the in vivo stimulation by Antigen presenting cells. (117) It contains a mixture of antibodies against the CD3 and CD28 molecules bound to magnetic beads. ConA is a carbohydrate binding protein extracted from jack beans stimulating T cells. (118) B cells were stimulated with ODN2395 (Miltenyi) 0,25 µM and 0,125 µM for 7 days at 37°C and 5% CO2. ODN2395 is an oligonucleotide stimulating human/murine TLR9 on B cells. (119)

Before cultivation CellTrace™ Violet (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing non fluorescent ester, was added to B and T cells. This ester is able to enter the cell where it is converted into a fluorescent molecule binding covalently to amine groups in proteins. After cell divisions, daughter cells receive about half of the fluorescent label of their parent cells. The fluorescence intensities allow then the analysis of cells labeled and grown in vivo.

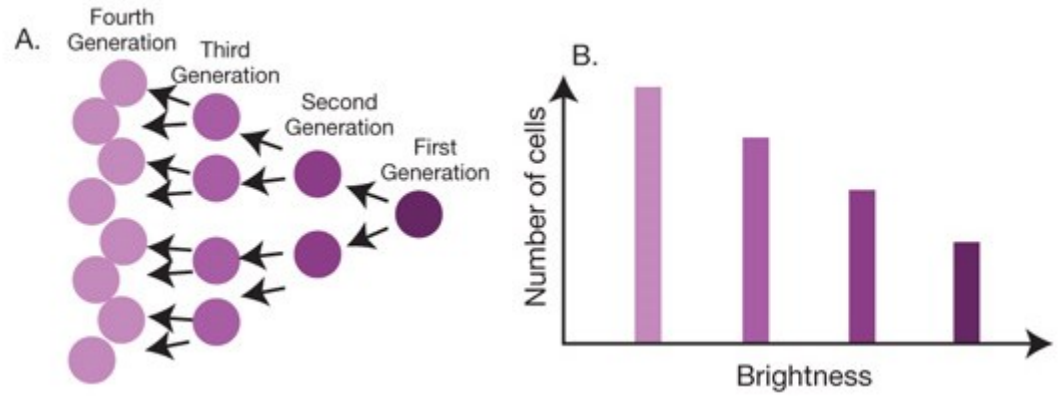


Figure 5 CellTrace™ Violet method (120)

A: Each daughter cells receives approximately the half of the fluorescent label from their parent cell.
B: Through cell divisions daughter cells reveals the half of the intensity of fluorescence of its parent cell.

After cultivation T cells were stained with premade antibodies (Miltenyi, Table 14) for 15 min in darkness, once washed with 1ml Cellwash, resuspended with 150µl Cellwash and analyzed in the FACS machine.
B cells were treated with the same procedure using premade antibodies (Miltenyi, Table 15).

Table 14 T cell antibodies

Antibodies	Antibody dye
CD 3	APC-Vio 770
CD 4	Vio Bright FITC
CD 8	PerCP-Vio 700

Table 15 B cell antibodies

Antibodies	Antibody dye
CD 19	APC-Vio 770
CD 38	FITC
IGD	PE-Vio 770
CD 27	APC
IgM	PE

3.6.5 NKT Panel

After gaining PMBCs, surface staining was performed with following protocol:

Protocol:

1. 10^6 PBMCs diluted in 100µl PBS
2. + antibodies:

Table 16 Antibodies for the NKT cell panel

ANTIBODIES	ANTIBODY DYE	VOLUME	COMPANY
CD3	APC-Cy7	5 µl	BD
CD4	V500	5 µl	BD
CD8A	FITC	5 µl	E-bioscience
CD19	V450	5 µl	BD
CD159A	PE	10 µl	Miltenyi
CD25	APC	10 µl	Miltenyi
VALPHA24	PE-Cy7	2,5 µl	Beck PN

3. 15 min incubation in darkness
4. Washing 2x with Cellwash
   300-400g 5 min
5. Add 150 µl Cellwash

3.6.6 Cytokines

In our study we evaluated levels of IL2, IL4, IL5, IL 9, IL 10, IL13, IL17A, IL 22, IL 23, IFN γ, GMCSF, TNFα, Granozyme B in the supernatant of stimulated T- cells of pSS patients and healthy controls with the multiplex protein analysis MAGPIX® System. This system uses magnetic beads/microspheres with specific antibodies on their surface, which are are added to the samples. Every bead is color coded with a unique spectral signature. A fluorochrome-labeled detection antibody is added to indicate the number of molecules bound to each bead. Light-emitting diodes excite the fluorochromes. A camera detects the signals, corresponding to each kind of beat and the number of bound molecules to it. (121)

For stimulation two different mitogens were used for stimulation: CD3/CD28 and ConA 10 mg.

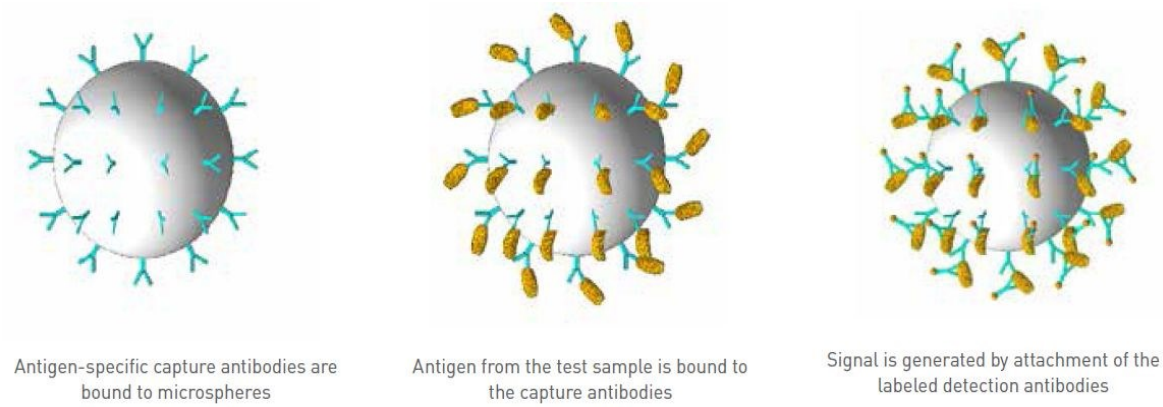


Figure 6 MAGPIX® Multiplexing System (122)

3.7 Statistics

Statistics were performed using SPSS (Chicago, IL, USA).

One way ANOVA was performed for comparing differences in absolute and proportional amount of lymphocyte populations between HCs and SS patients. Spearman‘s rank correlation coefficient was used to evaluate the correlation between clinical data and the absolute and proportional number of cells of lymphocyte populations. The results are presented as range and median deviation. Significance was defined by a P value ≤ 0,05.

4 Results

4.1 Patients characteristics

Following clinical data were collected from the SS patients. The data were taken from documents of the rheumatology outpatient clinic of the Medical University of Graz.

Table 17 Collected clinical data from SS patients

Patient number	Age	CRP mg/dl	BSG mm/h	igG g/dl	C3 g/l	C4 g/l	ANA IU/ml	Sicca Score	Focus Score	ESSDAI	Rheumafaktor IU/ml	Ro	La	ESSPRI
1	66	1.7	23				5120	15,8	3	4		1	1	4,3
2	69	1.9	23	10,3	0,909	0,223	0	55,6	1	0	9	0	0	
3	56	2.9	25	34,6	0,961	0,128	5120	0		15		1	1	
4	49	8.2	4	9,84	1,41	0,193	0	17,3		0	5	1	0	
5	57		27	1,264			2560	18,6	4	2	59	1	1	
6	57	1.5	45	16,6	1,01	1,6	40	19,8	3	1	123	0	0	
9	68	0.7			0,874	0,192	1280	53,4	0	3	7	0	0	
10	75	3.1	10		0,816	0,142	1280	30,4		3	7	1	0	
11	47	4.0	3	17,5	0,767	0,129	5120	18,6	4	5	33	1	1	
12	63	3.0	10	7,72	1,6	0,25	5120	57,2	3		11	1	0	
13	82	1.3	7	7,97	1,02	0,289	0	36,3	3	5	7	0	0	4
14	62	4.8	6	14,3	1,22	0,219	1560	82,2	3	0	16	1	1	6
15	49	4.1	15	17,4	1,39	0,142	5120	44,1	4	1	89	1	1	5,6
16	66	4.8	21	14,41	1,28	0,162	320	35,4	0	2	12	1	1	
17	68	0.7	6	10,5	0,94	0,196	160	59		1	17	1	0	
18	63	3.4	11	20	0,985	0,194	640	35,3	4	2	194	1	1	2,3
19	74	12.9	77	34,5			5120	58,4		15	889	1	1	6,6
20	80	4.3	14	10,7	1,19	0,25	1280	2,1	4	0	26	1	1	1
21	60	3.2	7	12,4	0,838	0,067	2560	0		4	5	1	1	
22	32	1.0	70	30,1			5120	0	3	8	500	1	1	2
23	61	4.4	55	25,7	1,12	0,198	640	36,8	4	2	79	1	1	
24	50	3.5	2	12,7	1,09	0,201	5120	33,4	4	5	4,6	1	0	
25	50	0,5	0,6	9,29	0,837	0,125	0	36,2	3	0	0	0	0	5,6
26	57	5.4	68	34,4	0,923	0,059	320	85,7	3	13	21	1	1	9,3
27	59	1.0	2	6,97	1,03	0,068	1280	46,5	4	4	16	1	1	
28	85	23.1	59	19,5	1,08	0,179	1280	58,5	4	5	500	1	1	

Table 18 List of medication of SS patients

Patient number	Medication
1	No medication
2	Pantoloc 40 mg
3	Prograf 1 mg, Salagen 5mg, Voltaren 50mg on demand, Hylocomod eye drops on demand, Euthyrox 75µg, Dilzem ret 90 mg
4	Mexalen 500mg on demand up to 4x/d, Noax 100mg, Cymbalta 60mg, Hyabak eyedrops on demand
5	Seractil on demand, Oculotect eye drops on demand
6	Voltaren 50 mg on demand, Hylo-comod eye drops on demand
9	Dilatrend 12,5 mg, ramipril 5mg, Calciduran Vit. D3 500mg/800IE, Oleovit D3 drops once a week, Amlodipin 10mg, Crestor 10 mg
10	Resochin 250 mg, Hydal ret 2 mg, Pantoloc 40 mg, Hypren plus, Magnosolv, Kombi Kaz KT, Fosamax 70 mg once a week, Euthyrox 75µg, Trittico 150mg, Paspertin gtt on demand, Ramipril 5mg, Aquatears eyedrops on demand, Aristocor 100mg
11	Quensyl 200mg, Salagen 5mg, Artelac eyedrops 2x on demand
12	Salagen 5mg, L Thyroxin 100µg und every second day, Sevikar 40/10/25mg, Sevikar 40/10mg, Nomexor 5mg ½-0-1/2, Urosin 300mg,Torasemid, Xarelto 20mg, Trittico 150mg, Berodual on demand, Novalgin on demand, Oculotect eye drops on demand
13	No medication
14	Cyclosporin, Salagen 5mg, Tramal, Thealoz eye drops, Monodex eye drops
15	Cyclosporin, Ambroxol
16	Seropram 10mg, Protagent eye drops on demand, Glandosane Spray on demand
17	Deflamat 50mg on demand, Restasis eyedrops, Euthyrox 75µg, Adjuvin 50 mg, Trittico 50mg, Sialin- Sigma Spray on demand
18	Euthyrox 88µg, Profenid 50mg on demand, Protagent eye drops 1-2 daily,
19	Cortison 5mg, Macumar, Concor, Deanixt
20	Macumar, Euthyrox 75µg, Cyprostol, Salagen 5mg
21	Pantoloc 40mg, Euthyrox 75µg, Hyabak eye drops on demand
22	No medication
23	Aprednisolon, Euthyrox 75µg, Blopress, Protagent eyedrops on demand
24	Benzobromaron, Salagen 5mg, Opthiolen eye drops
25	-
26	Hydroxychloroquin, Celebrex, Salagen 5mg, Marcumar, Olevit, Bisolvon, Floxal, Hyabak eye drops on demand
27	Resochin 250mg, Seractil, Euthyrox 75µg, Immunoprim 50mg, Salagen 5mg
28	Euthyrox 88µg

4.2 T cell panel 1

As general marker for T cells we used CD3+ and as marker for B cells we used CD19+. After gating CD3+ cells T helper cells (CD4+CD8-), cytotoxic T cells (CD4-CD8+), double negative (DN, CD4-CD8-) and double positive (DP, CD4+CD8+) were separated. Then the T helper cells got divided in CXCR3+ cells (pre TH1 cells) and in the CXCR3- CD194+ (pre TH2 cells).

The CD194+ subpopulation got thought divided in the TH2, CD194+CD196-, and in the Th17, CD196+. The CD183+ population got divided into the Th1 ,CD196+, and the TH1/TH17 ,CD196+CD194- subpopulation.

From CD+, DN, DP and each T helper subpopulation a gating for activation CD38+ and a gating for activation and proliferation CD38+Ki67+ was performed:

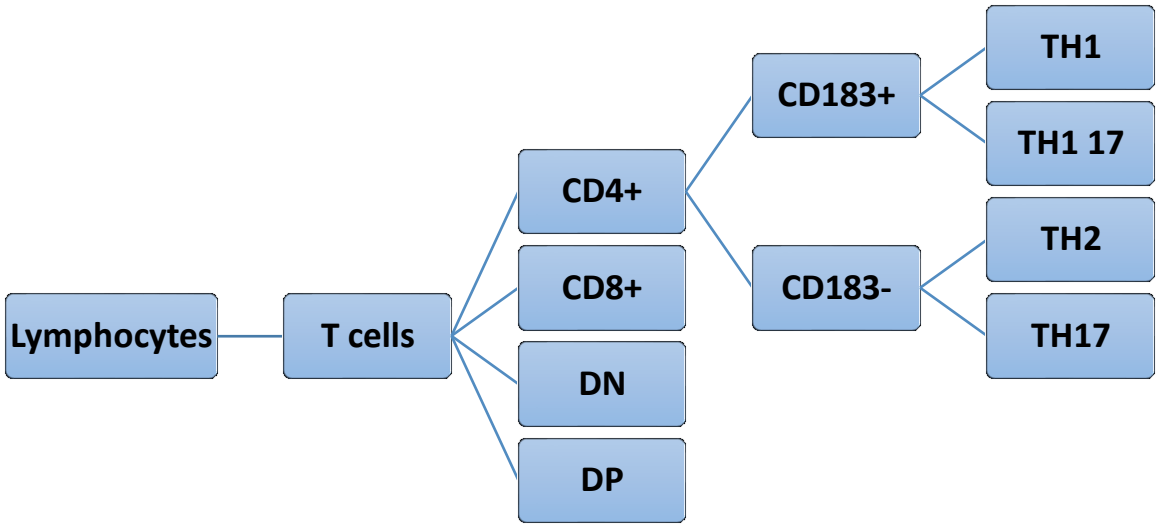
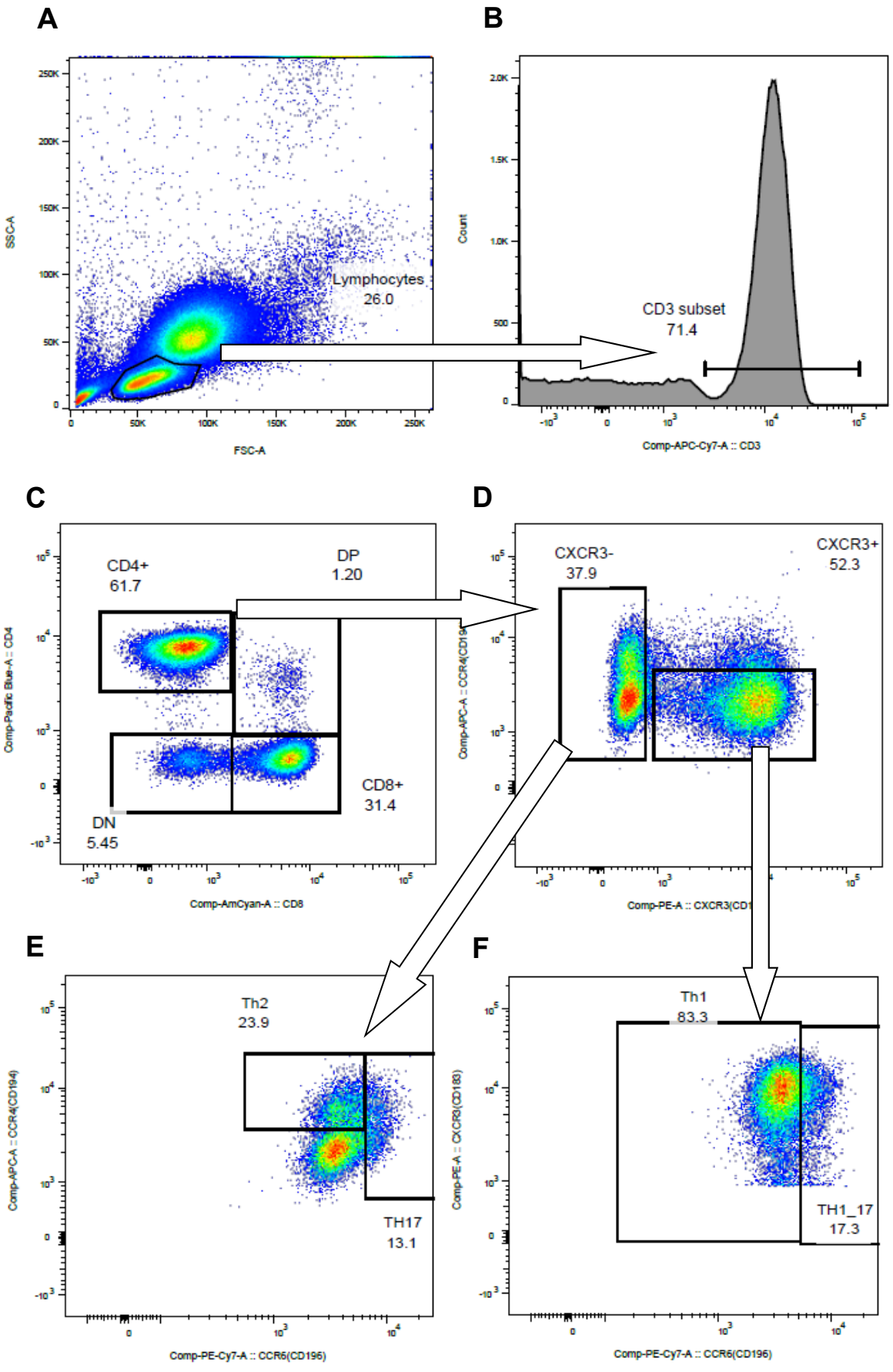


Figure 7 Classification Tree of Th lineage cells


A
B
SSC-A
FSC-A
Lymphocytes
26.0
Count
CD3 subset
71.4
Comp-APC-Cy7-A :: CD3
C
D
CD4+
61.7
DP
1.20
CD8+
31.4
DN
5.45
Comp-Pacific Blue-A :: CD4
Comp-AmCyan-A :: CD8
CXCR3-
37.9
CXCR3+
52.3
Comp-APC-A :: CCR4(CD19
Comp-PE-A :: CXCR3(CD1
E
F
Th2
23.9
TH17
13.1
Comp-APC-A :: CCR4(CD194)
Comp-PE-Cy7-A :: CCR6(CD196)
Th1
83.3
TH1_17
17.3
Comp-PE-A :: CXCR3(CD183)
Comp-PE-Cy7-A :: CCR6(CD196)

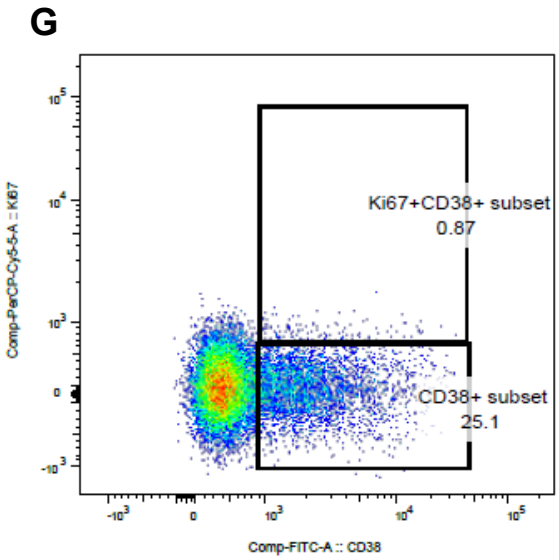


Figure 8 Representative dot plots of the gating strategy for flow cytometric analysis of T cells lineages

A: First, Lymphocytes were determined on the basis of cell size (FSC) vs. granularity (SSC).
B: From the lymphocyte gate T cell were gated via anti-CD3 staining.
C: Furthermore T cell were separated into CD4+ CD8-, CD4+ CD8+ (DP), CD4- CD8- (DN) and CD4-CD8+ cells.
D-F: CD4+ population was divided into the CXCR3+ and CXCR3- population whereas TH1+ TH1_17 and Th2 +Th17 respectively were gated.
G: From each population activated cells were determined on the base of CD38+ positivity and activated proliferating cells using CD38+ and the intracellular stain ki67

4.3 T cell panel 2

Again CD3+ cells were gated into T helper cells, cytotoxic T cells, DN, and DPThe cytotoxic and the T helper cells got divided into effector cells (CD197+CD45RA-), central memory (CD197- CD45RA+), effector memory (CD197- CD45RA-), naive (CD197+CD45RA+) cells. CD28- cells were also gated from cytotoxic and T helper cells.Treg cells were defined as CD25+ CD127- cells.

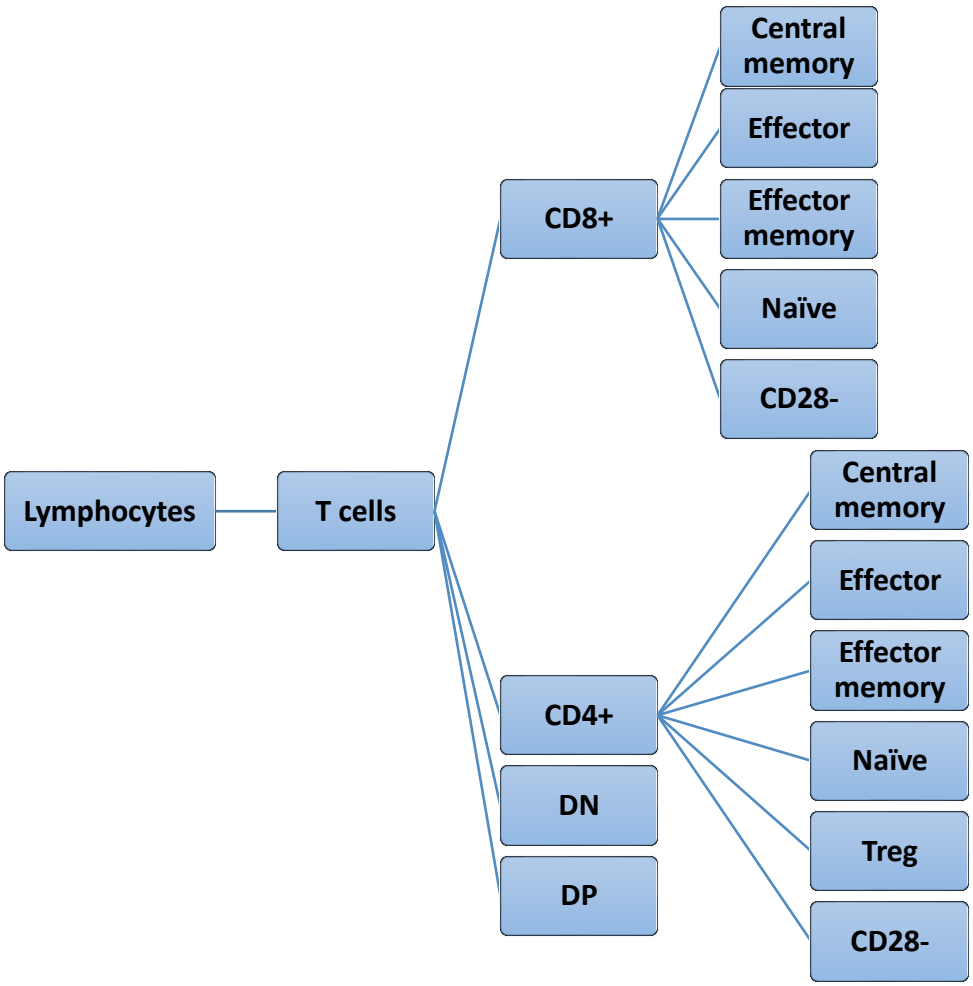
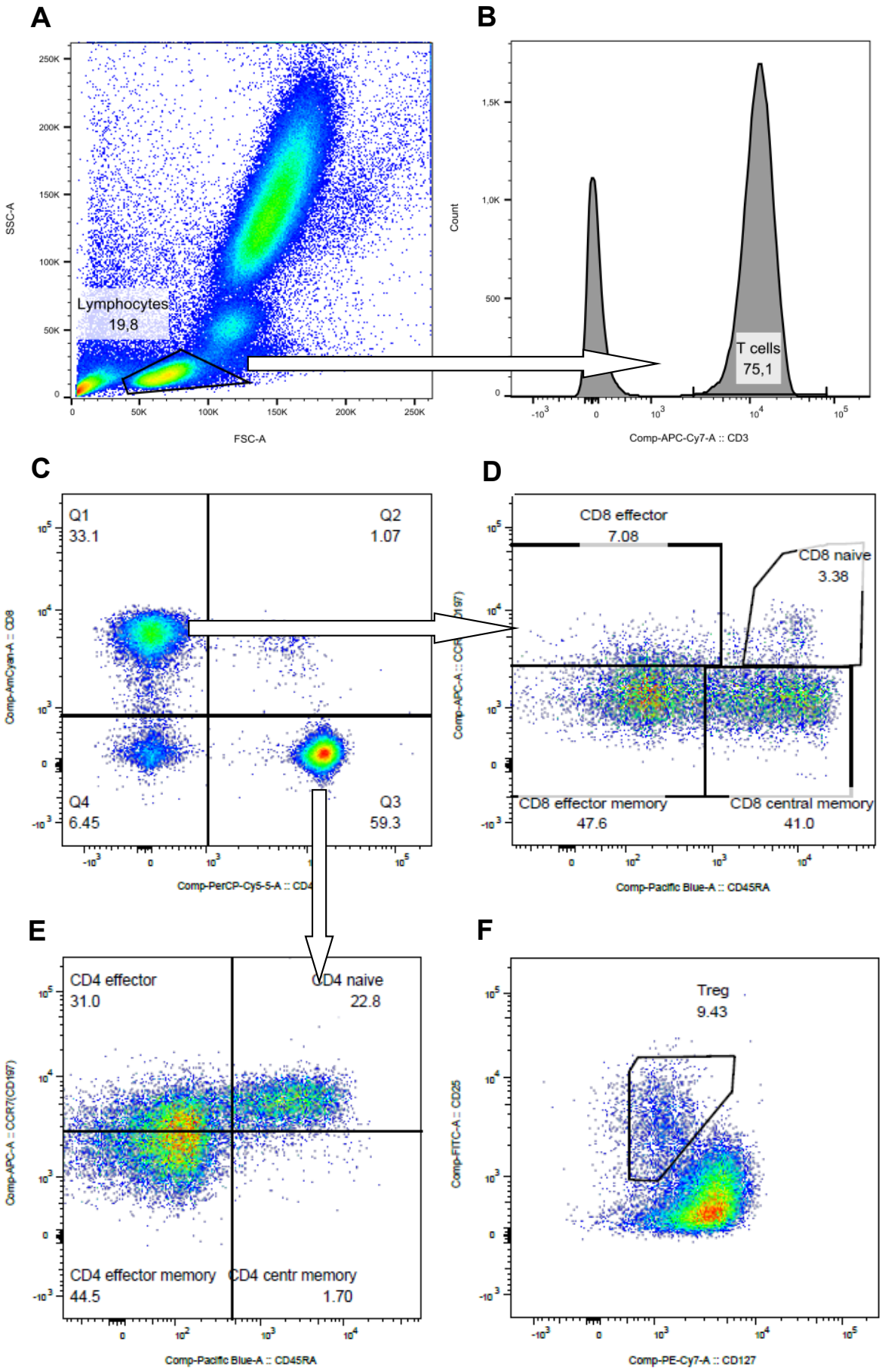


Figure 9 Classification Tree for T cells


A
SSC-A
FSC-A
Lymphocytes
19,8
B
Count
T cells
75,1
Comp-APC-Cy7-A :: CD3
C
Q1
33.1
Q2
1.07
Q4
6.45
Q3
59.3
Comp-AmCyan-A :: CD8
Comp-PerCP-Cy5-5-A :: CD4
D
CD8 effector
7.08
CD8 naive
3.38
CD8 effector memory
47.6
CD8 central memory
41.0
Comp-Pacific Blue-A :: CD45RA
E
CD4 effector
31.0
CD4 naive
22.8
CD4 effector memory
44.5
CD4 centr memory
1.70
Comp-APC-A :: CCR7(CD197)
Comp-Pacific Blue-A :: CD45RA
F
Treg
9.43
Comp-FITC-A :: CD25
Comp-PE-Cy7-A :: CD127

G

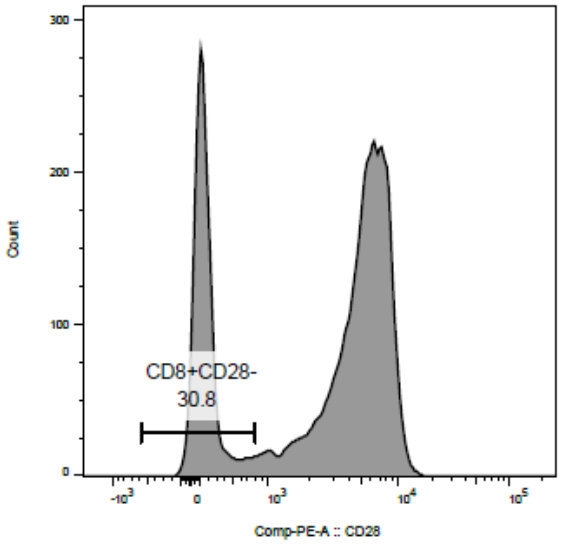


Figure 10 Representative dot plots of the gating strategy for flow cytometric analysis of T cells lineages.

A: Lymphocytes were gated on the basis of cell size (FSC) vs. granularity (SSC).
B: From the lymphocyte gate T cell were gated via anti-CD3 staining.
C: Furthermore T cell were separated into CD4+ CD8-, CD4+ CD8+ (DP), CD4- CD8- (DN) and CD4-CD8+ cells.
D, E: Origin from the CD4+ population and CD8 + population cells were gated in the flow cytometric Pacific Blue/ APC A dot plot into effector cells, effector memory cells, central memory cells and naïve cells.
F: Treg cells were defined as CD25+ CD127- cells.
G: Finally CD8+ CD28- cells were gated

4.4 B cell panel

B cells were gated into naive (IgD+CD27-), switched B cells (IgD- CD27+), transitional stage B cells (CD38+IgM+), plasmablasts (CD38+ IgM-), marginal zone B cells (CD27+IgD+), regulatory B cells (Breg, CD24+CD38+) and CD21-negative B cells (CD21-CD38-).

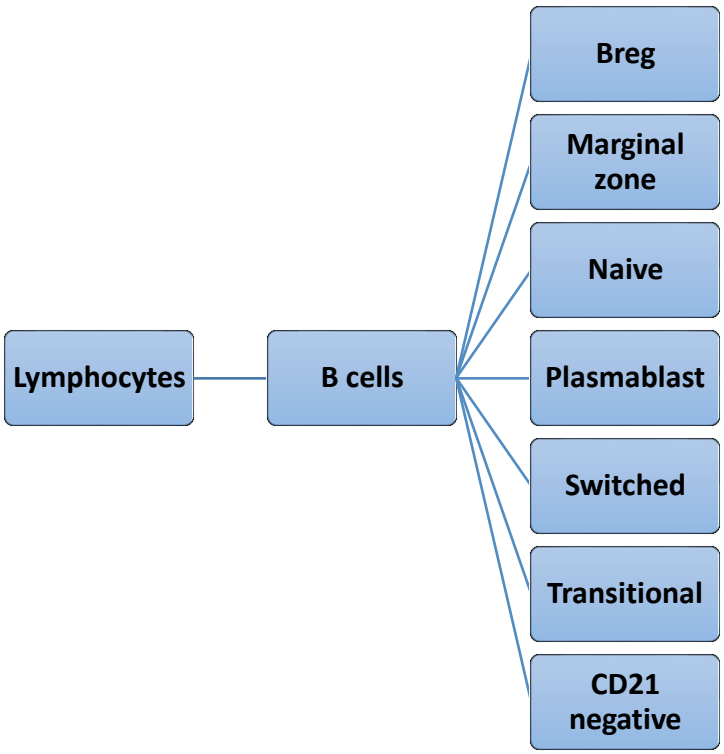
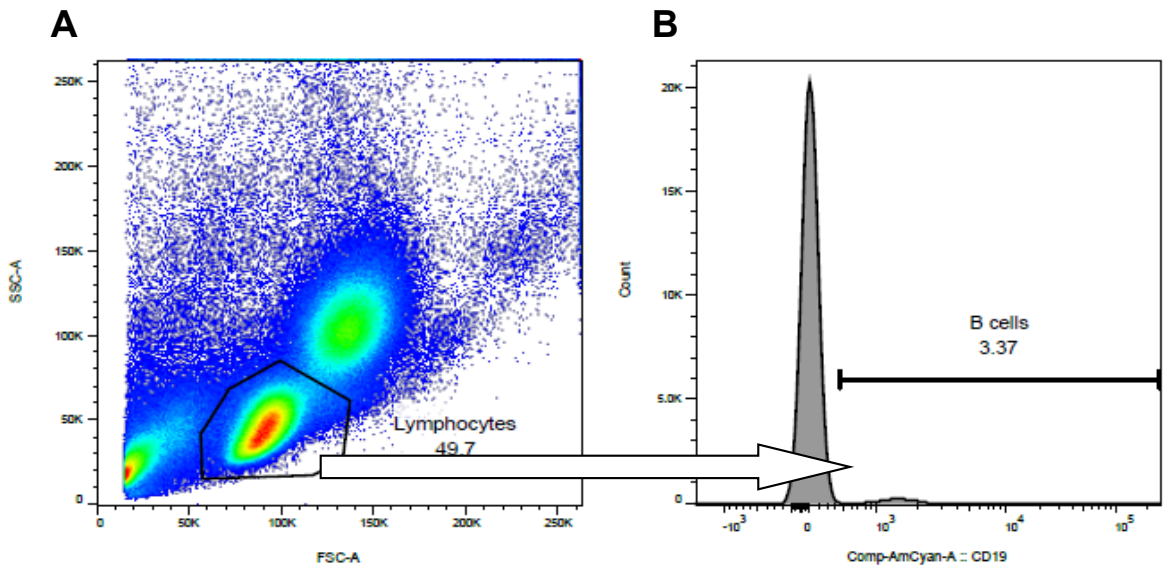


Figure 11 Classification tree for B cells



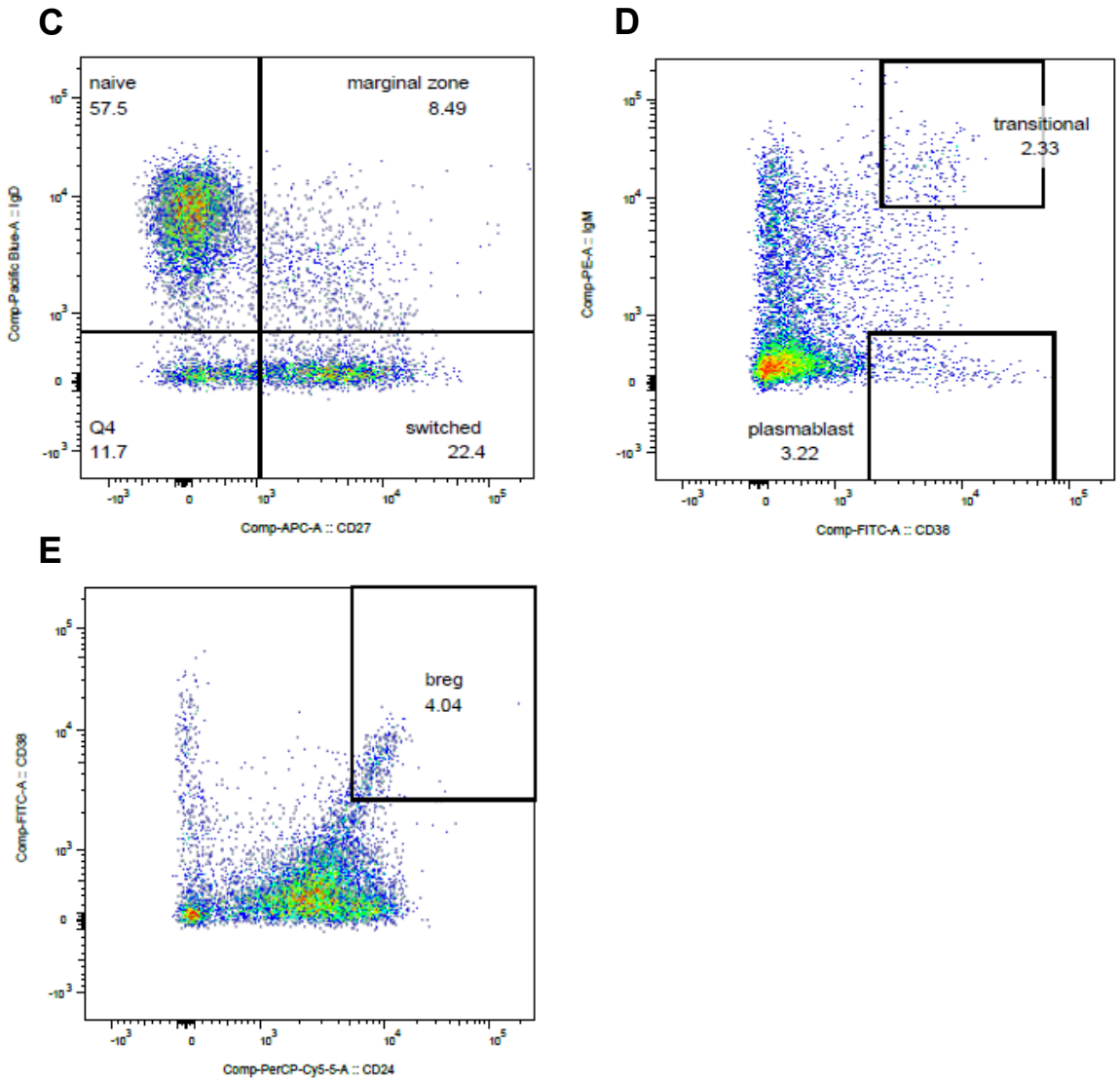


Figure 12 Representative dot plots of the gaiting strategy for cytometric analysis of B cells lineages

A: First, Lymphocytes were determined on the basis of cell size (FSC) vs. granularity (SSC).
B: Subsequently B cells were gated with anti-CD19 staining from the lymphocyte gate.
C: Origin from the B cell gate switched, naive and marginal zone cells were determined in the flow cytometric APC-A/Pacific Blue a dot plot.
D: Transitional and plasmablast B cells were gated in the CD38+ vs. PE-A plot.
E: Breg cells were defined as CD24+ CD38+ cells.

4.5 B cell stimulation panel

After gating B cells like in the B cell Panel, proliferating rates were analysed using CellTrace™ Violet.

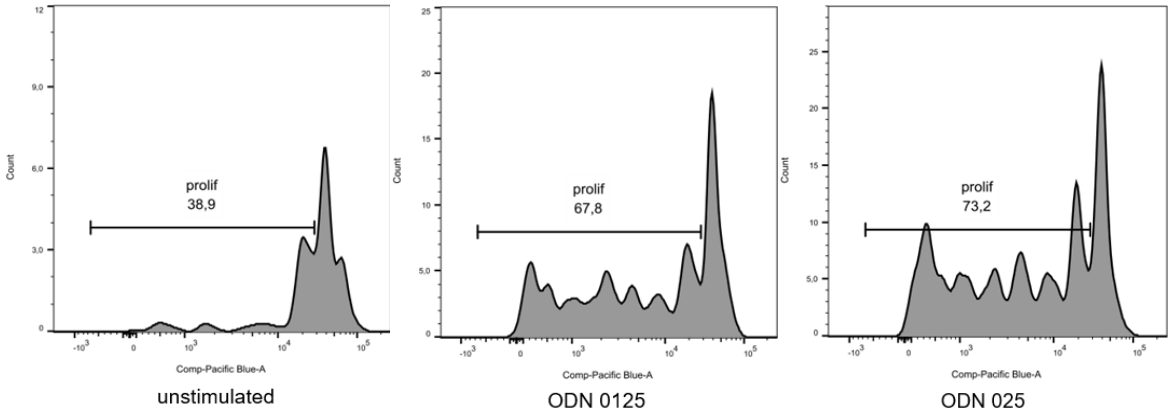


Figure 13 Proliferation rate of unstimulated B cells and B cells treated with ODN2395. ODN0125= 0,125 µM and ODN 025=0,25 µM.

4.6 T cell stimulation panel

As in the T cell panels T cells were gated until the CD4+ and CD8+ populations. Then the proliferation rate was measured with CellTrace™ Violet.

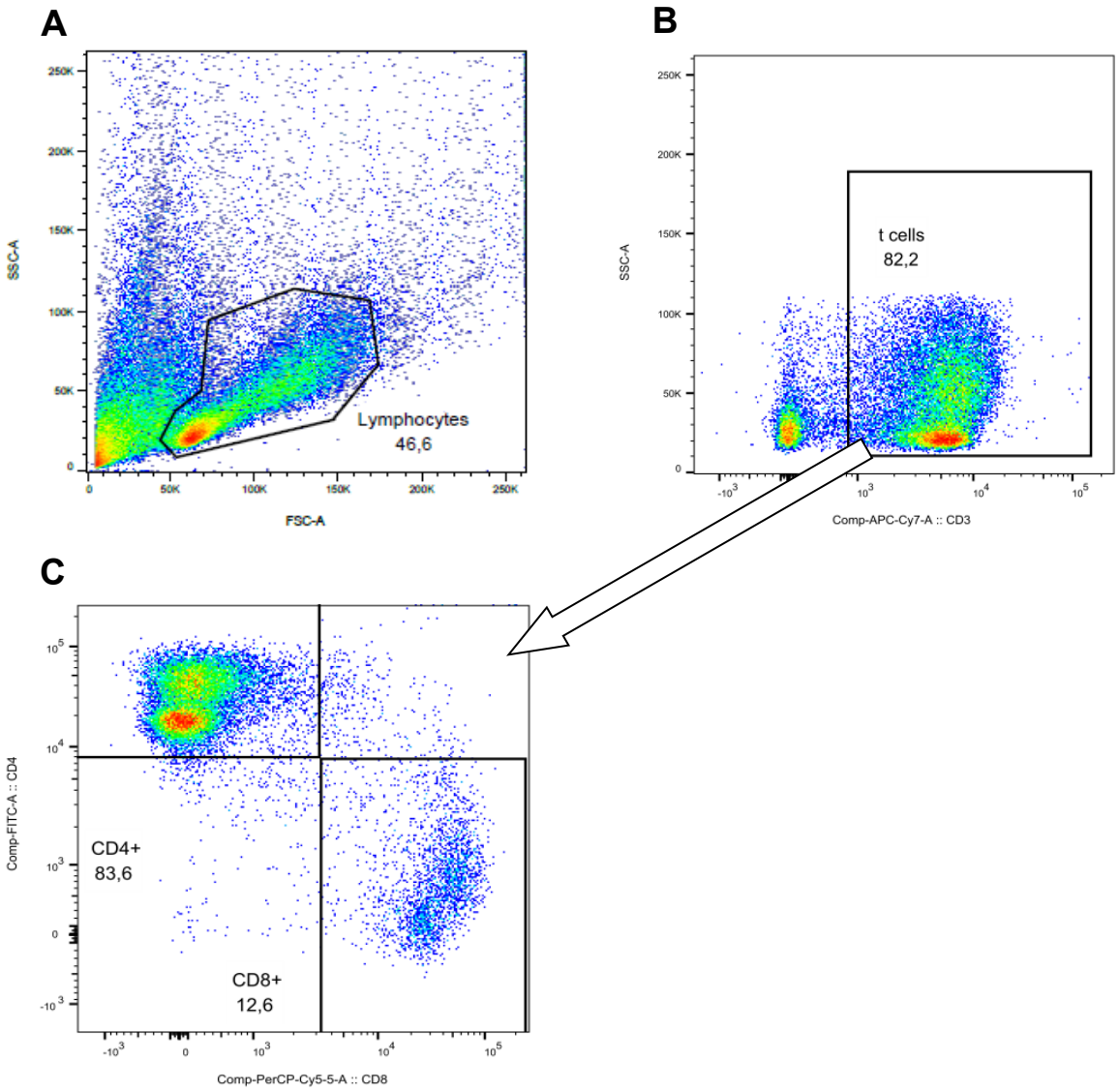


Figure 14 Representative dot plots for the gating strategy for cytometric analysis of T cells lineages

A: Lymphocytes were gated on the basis of cell size (FSC) vs. granularity (SSC).
B: From the lymphocyte gate T cell were gated via anti-CD3 staining.
C: Furthermore T cell were separated into CD4+ CD8-, CD4+ CD8+ (DP), CD4- CD8- (DN) and CD4-CD8+ cells Gaiting strategy for flowjo cytometric analysis of T cells

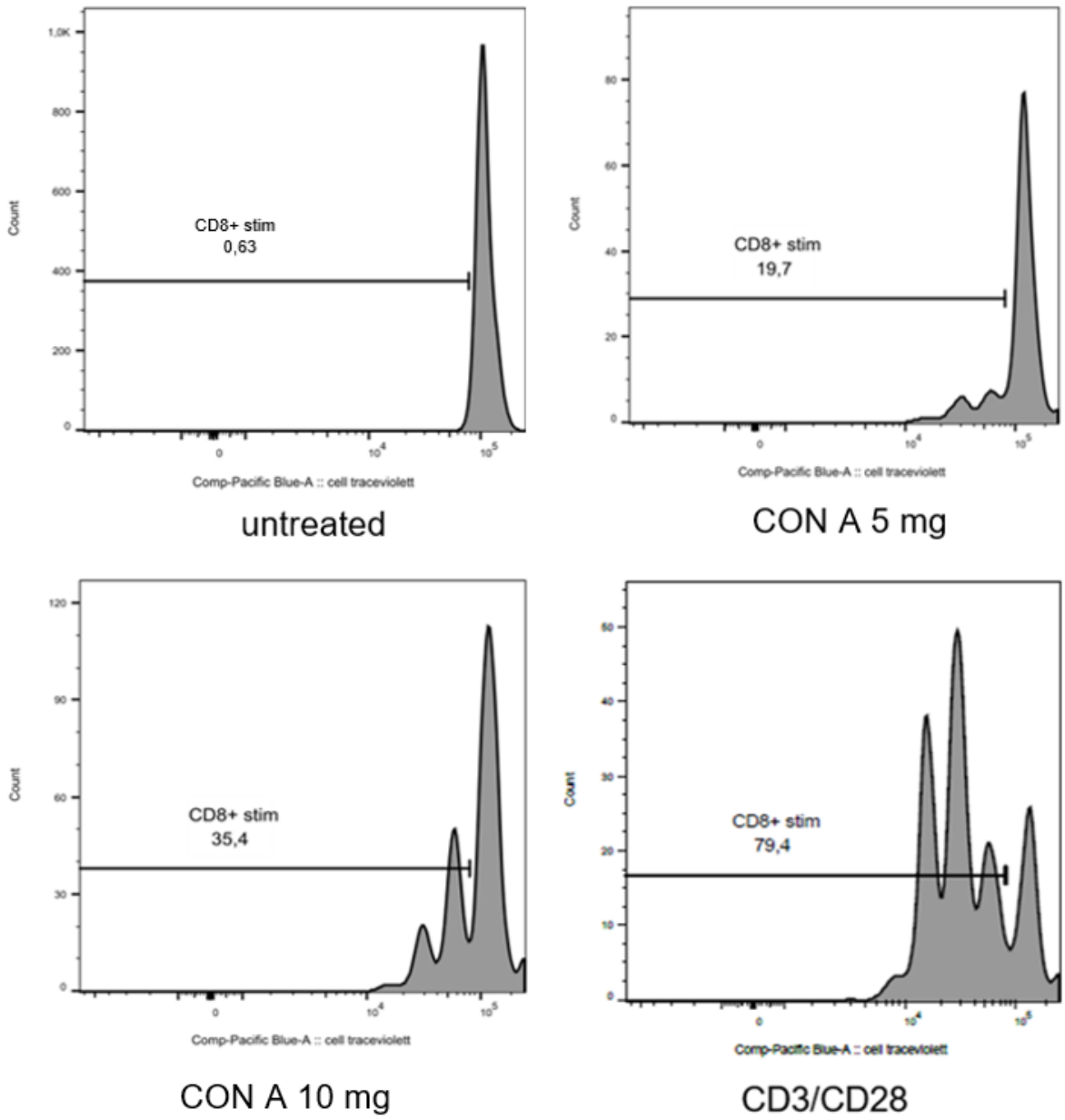


Figure 15 Untreated and stimulated T cells with CON A 5mg, CON A 10mg and CD3/CD28

4.7 NKT Panel

Lymphocytes were gated twice to exclude doublets. Then T cells were gated for CD3+CD19- and T cells defined as NKT-TCR antibody+,Vα24+ cells. Subsequently NKT cells were gated into CD4+, CD8+, DP, DN, CD159+ and CD161+.

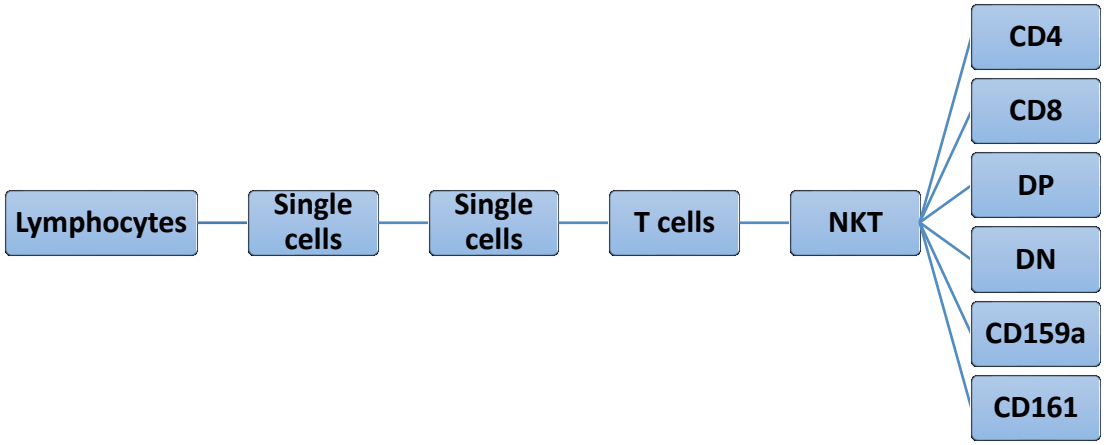
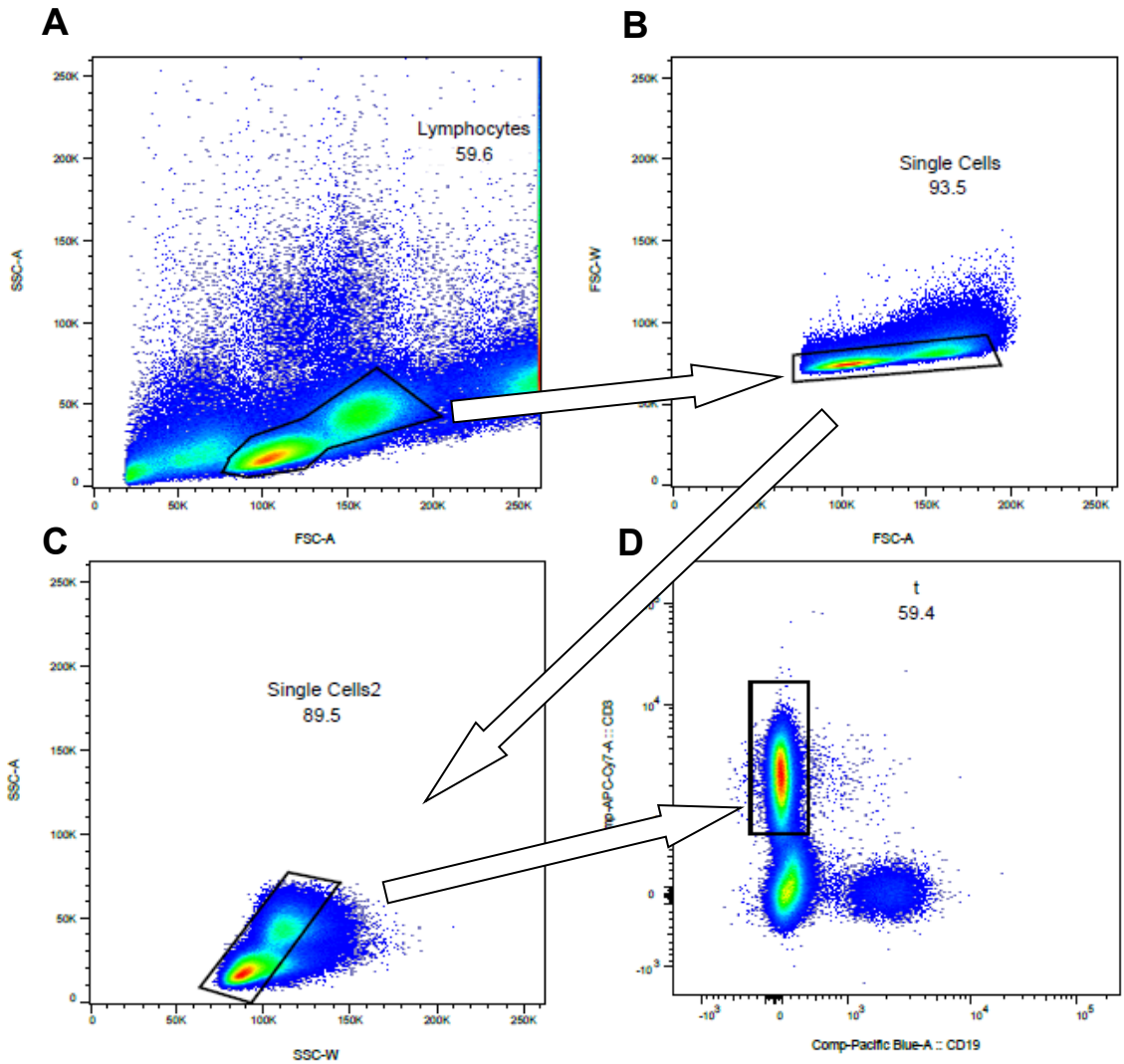


Figure 16 Classification Tree for NKT cells



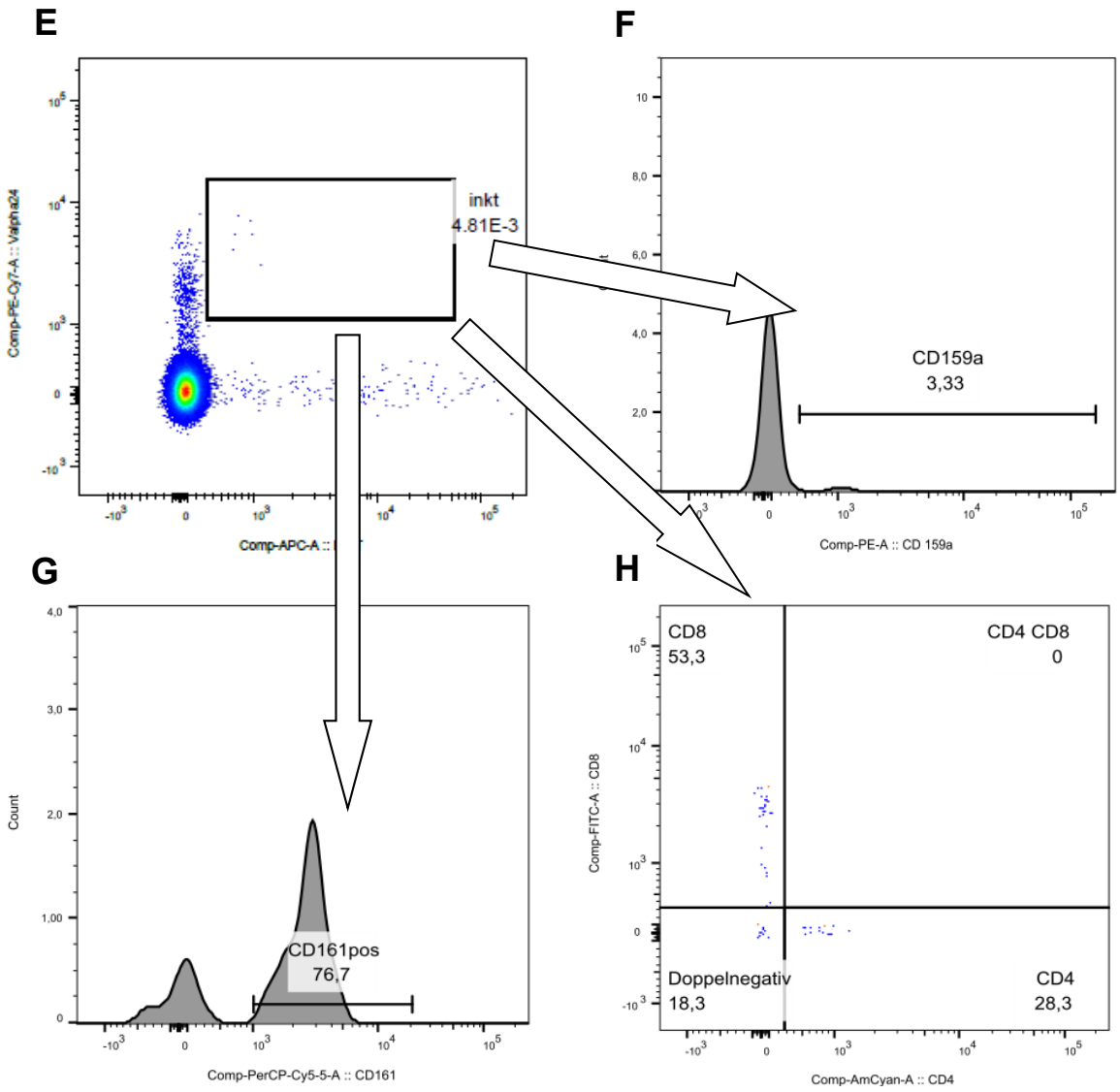


Figure 17 Representative dot plots of the gating strategy for flow cytometric analysis of NKT cells

A: Lymphocytes were gated on the basis of cell size (FSC) vs. granularity (SSC).
B, C: Then lymphocytes were gated twice to exclude doublets.
D: B cells were gated out and
E: NKT cells were determined in CD25+ vs. CD3+ plotting. Additionally NKT population were gated into F: CD159+,
G: CD161+ and
H: Based on CD4+ vs. CD8+ into NKT Cd4+. NKT CD8+, DP NKT and DP NKT.

4.8 Comparing Differences between SS and HC

4.8.1 T cells

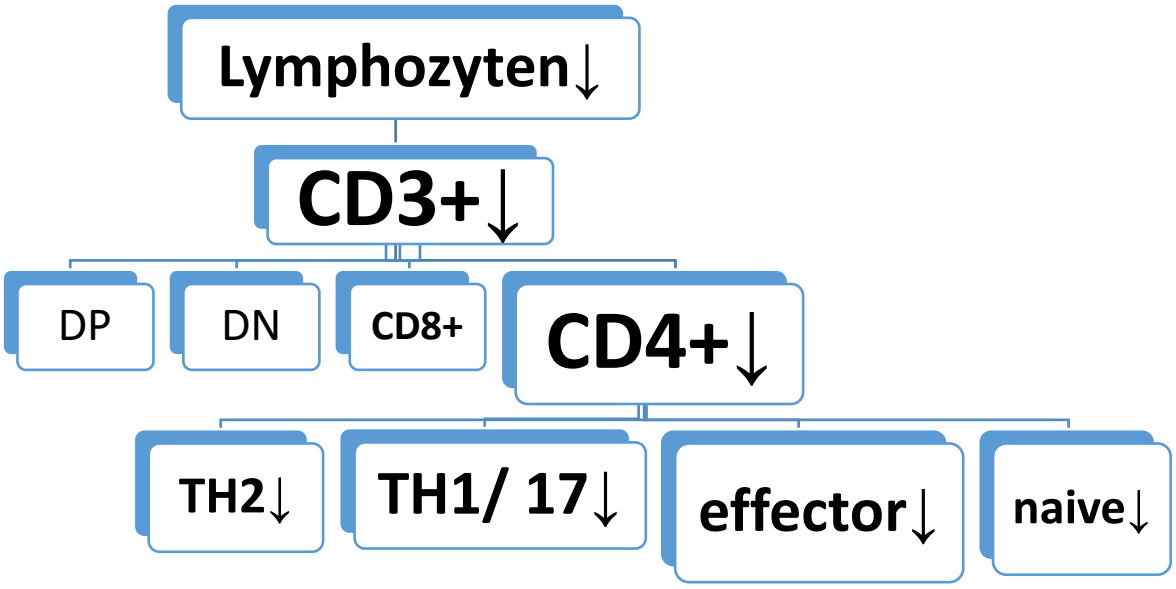


Figure 18 Overview about the major changes in the T cell population in SS

The absolute count of CD3+ cells was significantly decreased in SS patients compared to HCs (Table 19, Figure 20); whereas, no difference was found in frequency of total lymphoctes. Both, frequency and absolute numbers of CD4+ cells were decreased in SS patients (Table 19, Figure 21) In the CD4+ population the percentage of Th1 cells was elevated in patients with SS, but no difference was observed in absolute numbers between both groups (Table 19). The Th1/17 population showed significant differences in proportional and absolute count; both were decreased in SS patients (Table 19, Figure 21). Furthermore, SS patients had fewer activated Th1/17 than HCs (Table 19, Figure 21).

The absolute count of Th2 cells was reduced in SS patients compared to HCs (Table 19,Figure 21). In addition, a decrease in the absolute count of activated Th2 cells was found in SS patients (Table 19).

No difference between both groups was found in Th17 cells; but activated Th17 were increased in SS patients (Table 19). Less CD4+ CD28- cells were observed in SS patients (Table 19, Figure 23). The frequency of Treg cells showed a highly

significant increase in SS patients without difference in absolute cell count (Table 19). The percentage of CD4+ effector memory cells was increased in SS compared to HCs with no difference in absolute count (Table 19). Less CD4+ effectors cells were found in SS with no difference in proportional count (Table 19, Figure 21). A decrease of CD4+ naïve cells was found in proportional and absolute count in SS patients (Table 19, Figure 23). Activated DN cells were significantly increased in both, relative and absolute numbers in SS patients (Table 19, Figure 23).

The frequency of CD8+ cells was increased in SS, but no difference in absolute numbers between both groups was found. Additionally, SS patients had more activated CD8+ cells (Table 19, Figure 23). More CD8+ central memory cells were found in proportional and absolute count of SS patients compared to HCs. The naïve CD8+ cells showed exactly the opposite effect with less cells in relative and absolute cell numbers in SS patients (Table 19).

CD8+ CD28- cells were decreased in relative and absolute count in HC patients compared to SS patients (Table 19).

Table 19 Statistical results of the comparison of T cell populations in HCs to SS patients

	SS		HCs		P
	Average	**St. deviation**	**Average**	**St. deviation**	
CD3 +					
% of lymphocytes	27,5	13,1	27,7	5,9	NS
Absolute	904,6	361,8	1331,5	392,1	0,000097
CD4 +					
% of lymphocytes	62,3	10,2	70,4	9,2	0,003
Absolute	560,8	230,2	943,03	326,8	0,00000679
Th1					
% of CD4+	39,	10,8	28,5	10,3	0,00040
Absolute	219,4	101,5	267,4	121,0	NS
Th1/17					
% of CD4+	1,9	3,7	5,5	7,5	0,030
Absolute	11,5	19,4	46,0	64,5	0,011
Th1/17/CD38+					
% of TH1 17	16,2	11,7	15,4	9,6	NS
Absolute	2	4,2	10,1	15,8	0,015
Th2					
% of CD4+	10,5	7,8	9,1	4,6	NS
Absolute	26,1	13,9	49,4	25,1	0,00010
Th2/CD38+					
% of TH2	16,8	7,3	19,9	17,3	NS
Absolute	4	2	8,9	7,3	0,002
CD4+ effector					
% of CD4+	25,7	8,8	27	10,3	NS
Absolute	150,2	85,3	238,7	89,3	0,0004
CD4+ effector memory					
% of D4+	33,3	15,5	24,5	11,2	0,018
Absolute	180,2	110,8	219,8	112,4	NS
CD4+naive					
%of CD4+	35,6	13,1	42,6	12,4	0,047
Absolute	203	117,2	429,4	260	0,00014
CD4+ CD28-					
% of CD4+	1,6	2,1	2,8	2,5	NS
Absolute	10,4	14	28,3	29,5	0,007
Tregs					
% of CD4+	10,2	2,2	7,2	2,4	0,00001
Absolute	54,4	19,9	66,4	26,8	NS
DN/CD38+					
% of DN	34,8	16,3	15,6	4,9	0,000000090
Absolute	16	13,1	9,9	6,4	0,032
CD8+					
% of lymphocytes	29	8,3	20,8	8,1	0,00042
Absolute	268	142	268	128,9	NS
CD8+/CD38+					
% of CD8+	33,6	16,6	20,3	10	0,00054
Absolute	82,5	45,7	57	44,2	0,045

	SS		HCs		P
CD8+ central memory					
% of CD8+	48,6	19,8	31,3	19,2	0,002
Absolute	146,3	106,6	91,4	60,2	0,019
CD8+ naive					
% of CD8+	17,8	13,8	27,8	15,9	0,016
Absolute	39,5	29,5	82,9	63,1	0,002
CD8+ /CD28-					
% of CD8+	42,2	16,7	26,5	15,4	0,001
Absolute	136	108,2	81,8	60,2	0,022

4.8.1.1 The box plot

The box plot is a convenient graphical method to gain a quick overview about the distrubtion of a dataset.

The blue box represents the area where 50% of the values can be found. The line in the box is the median. The upper quartile and the lower quartile mark the point where 25 % of the the data are either greater or smaller than the median, respectively. The two lines reaching out from the box are called whiskers and represent the highest and the lowest values.(123)

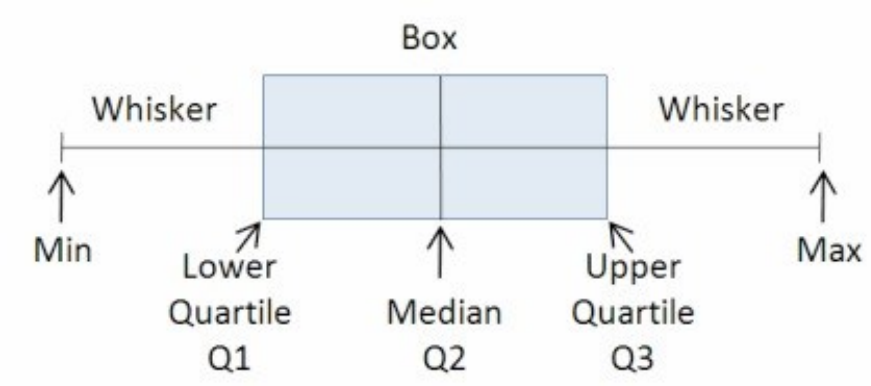


Figure 19 Simple box plot (124)

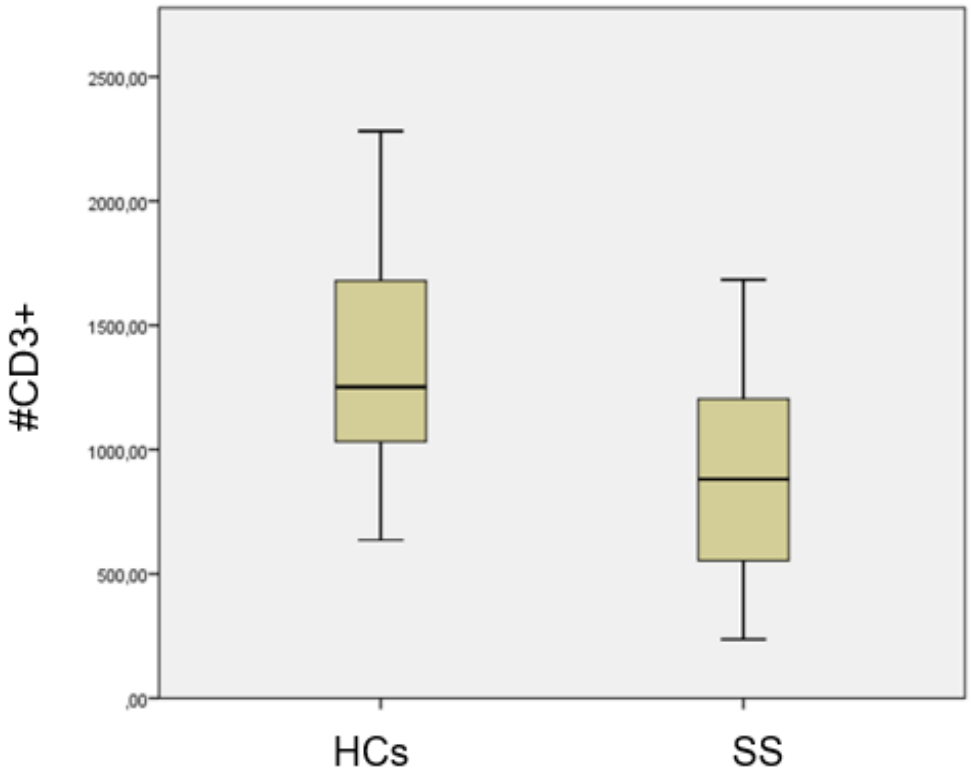


Figure 20 Absolute count (#) of CD3+ is reduced in SS patients with a p of 0,000097

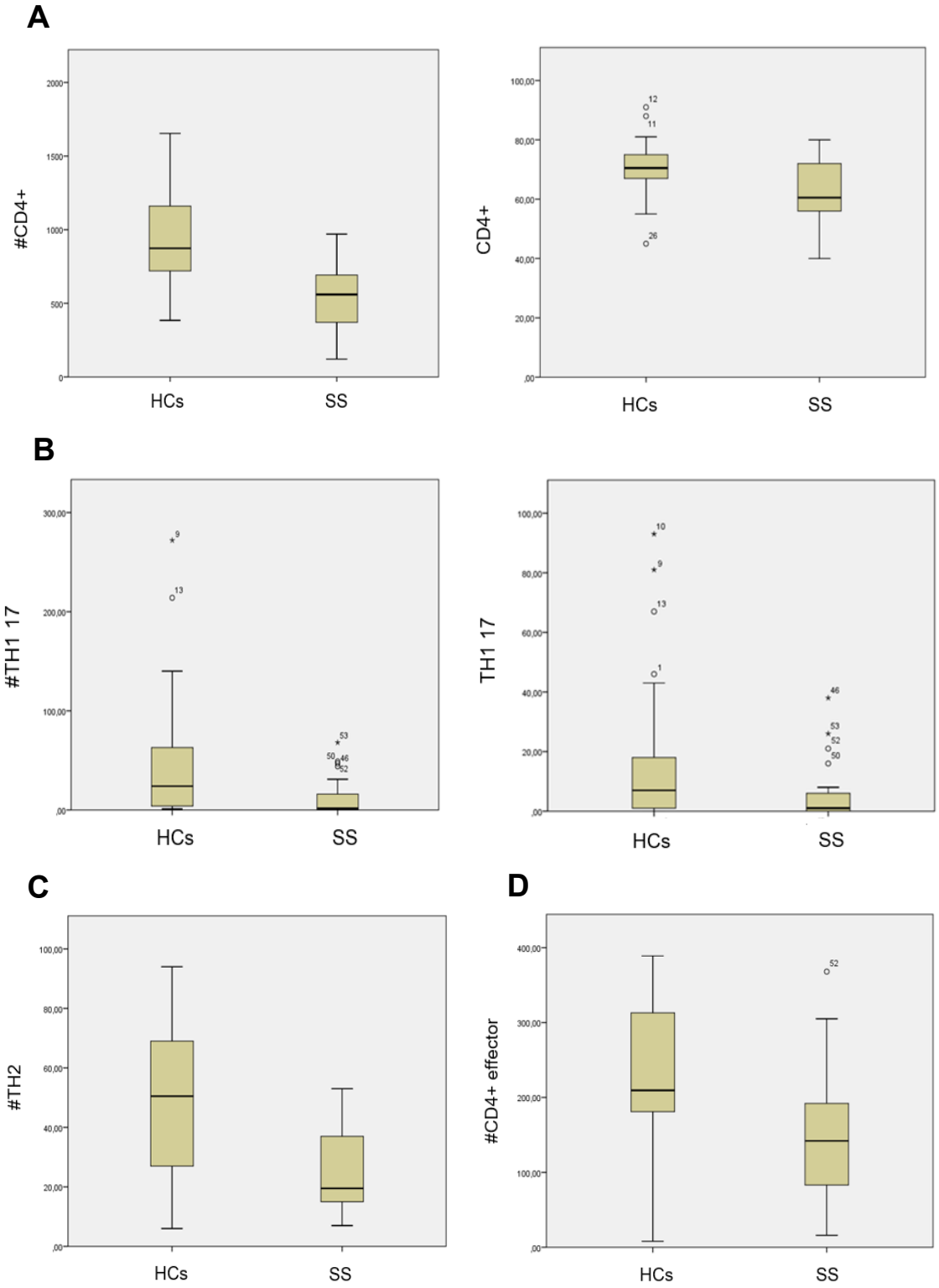


Figure 21 Box plots showing differences in absolute and proportional count between HCs and SS

A: Absolute (#)(P=0,00000679) and proportional count (p=0,003) of CD4+ is decreased in SS patients
B: Less Th1 17 cells are found in absolute (p=0,011) and proportional (p =0,030) count of SS patients
C: Absolute amount of Th2 cells is reduced in SS patients (p=0,00010)
D: Absolute count of CD4+ effectors cells is decreased in SS patients (p=0,0004)

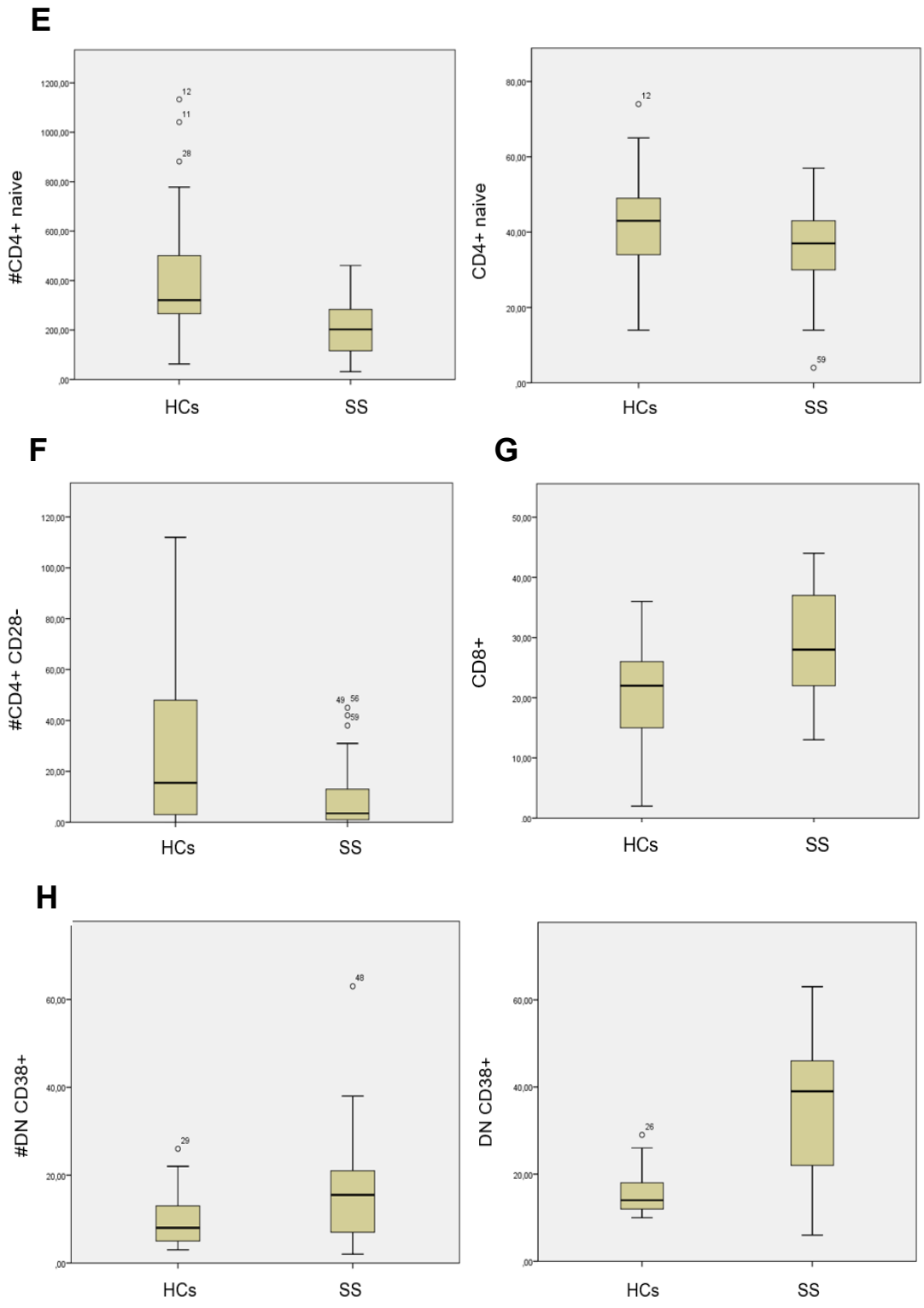


Figure 20 (continued) Box plots showing differences in absolute and proportional count between HCs and SS

E: Less CD4+ naive cells were found in SS patients compared to HCs with a p of 0,00014 in absolute and a p of 0,047 in proportional count
F: The CD4+ Cd28- populations was decreased in SS patients (p=0,007)
G: Percentage of CD8+ cells was elevated in SS patients (p=0,00042)
H: Activated DN cells were raised in SS patients with a p of 0,000000090 in proportional and a p of 0,032 in absolute count

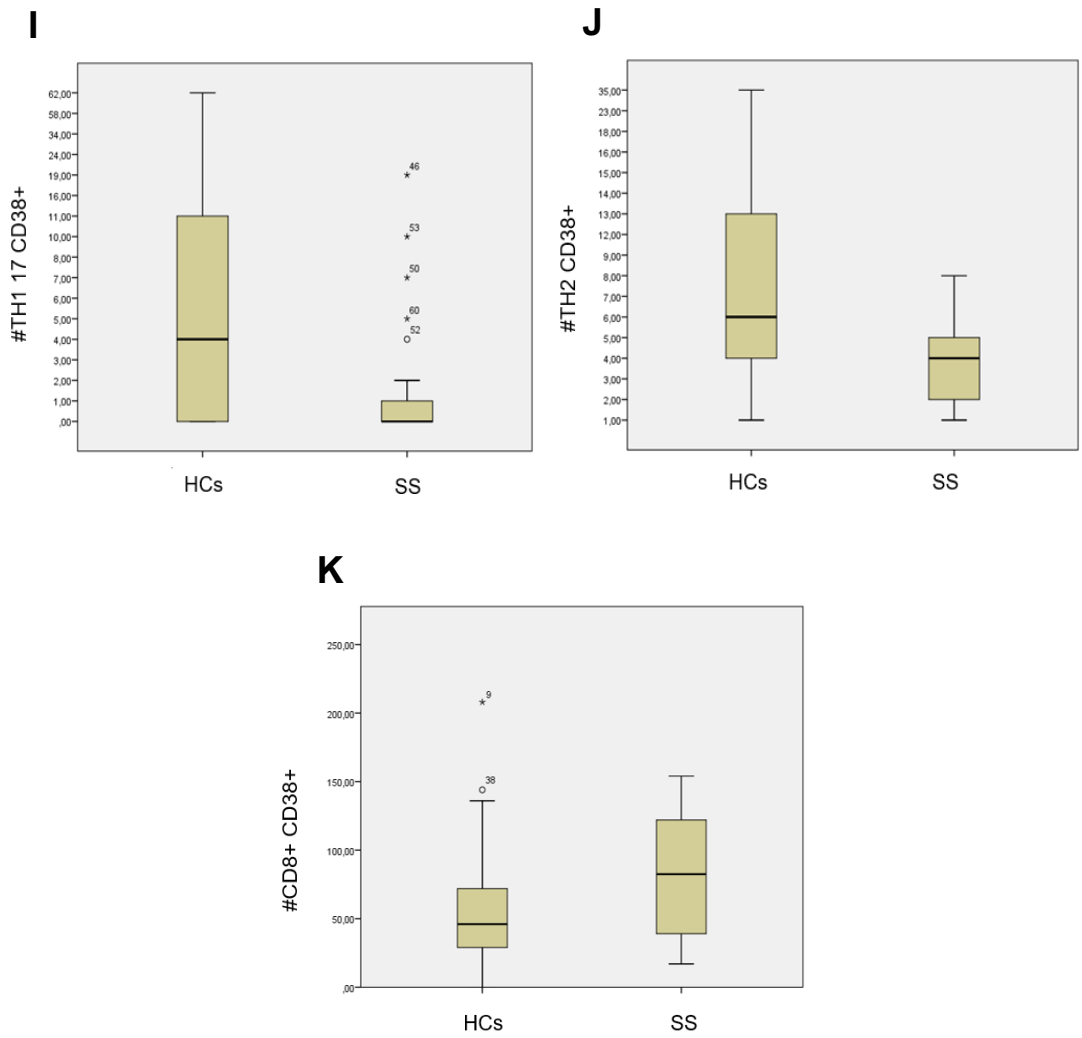


Figure 20 (continued) Box plots showing differences in absolute and proportional count between HCs and SS

I: HCs have more activated Th1 17 cells than SS patients (p=0,015)
J: Activated Th2 cells were reduced in SS patients (p=0,002)
K: Activated CD8+ cells are increased in SS patients (p=0,045)

4.8.2 B cells

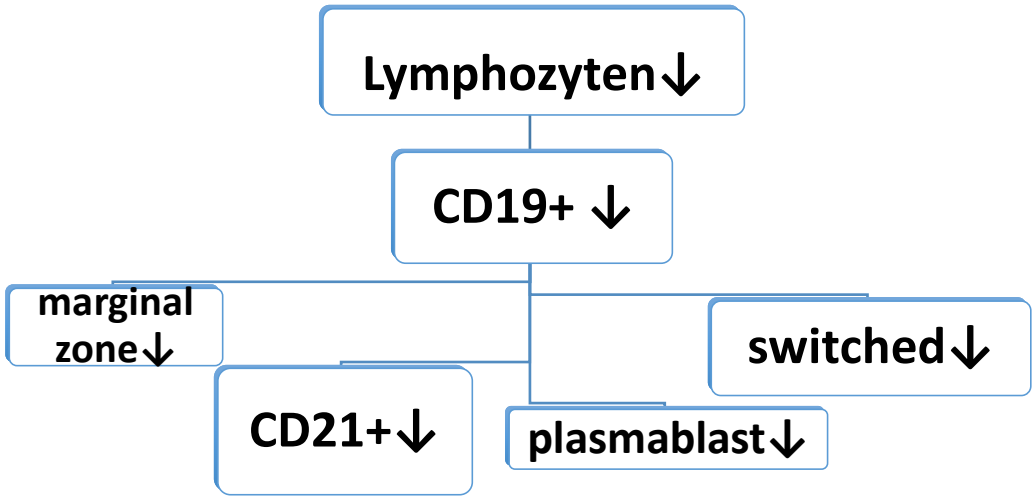


Figure 22 Overwiev about the major changes of the B cell population in SS

We found more B cells in HCs than in SS patients. B cells negative for CD21 were significantly increased in HCs compared to SS patients.

The marginal zone lymphocyte population was decreased in proportional and absolute count in SS patients, whereas the percentage of plasmablast cells were elevated in SS patients. Furthermore, SS patients had less switched B cells in absolute and proportional count (Table 20, Figure 23).

Table 20 Statistical results of the comparison from B cell populations in HCs to SS patients

	SS		HCs		P
	Average	**St. deviation**	**Average**	**St. deviation**	
CD19+					
% of lymphocytes	4,6	2,2	4,8	1,4	NS
Absolute	144	84,6	214,3	106,1	0,009
CD19+/CD21-					
% of CD19+	23	10,2	24,1	8,9	NS
Absolute	30,2	22,8	50,1	26,6	0,004
Marginal zone					
% of CD19+	6,8	6,1	14,3	9,2	0,001
Absolute	10	11,9	29,5	24,8	0,001
Plasmablast					
% of CD19+	5,1	4	3	2,1	0,016
Absolute	7,2	6,7	6,3	4,3	NS
Switched					
% of CD19+	15,5	9,2	20,9	9,7	0,04
Absolute	17,7	9,1	43,6	31,4	0,00016

A

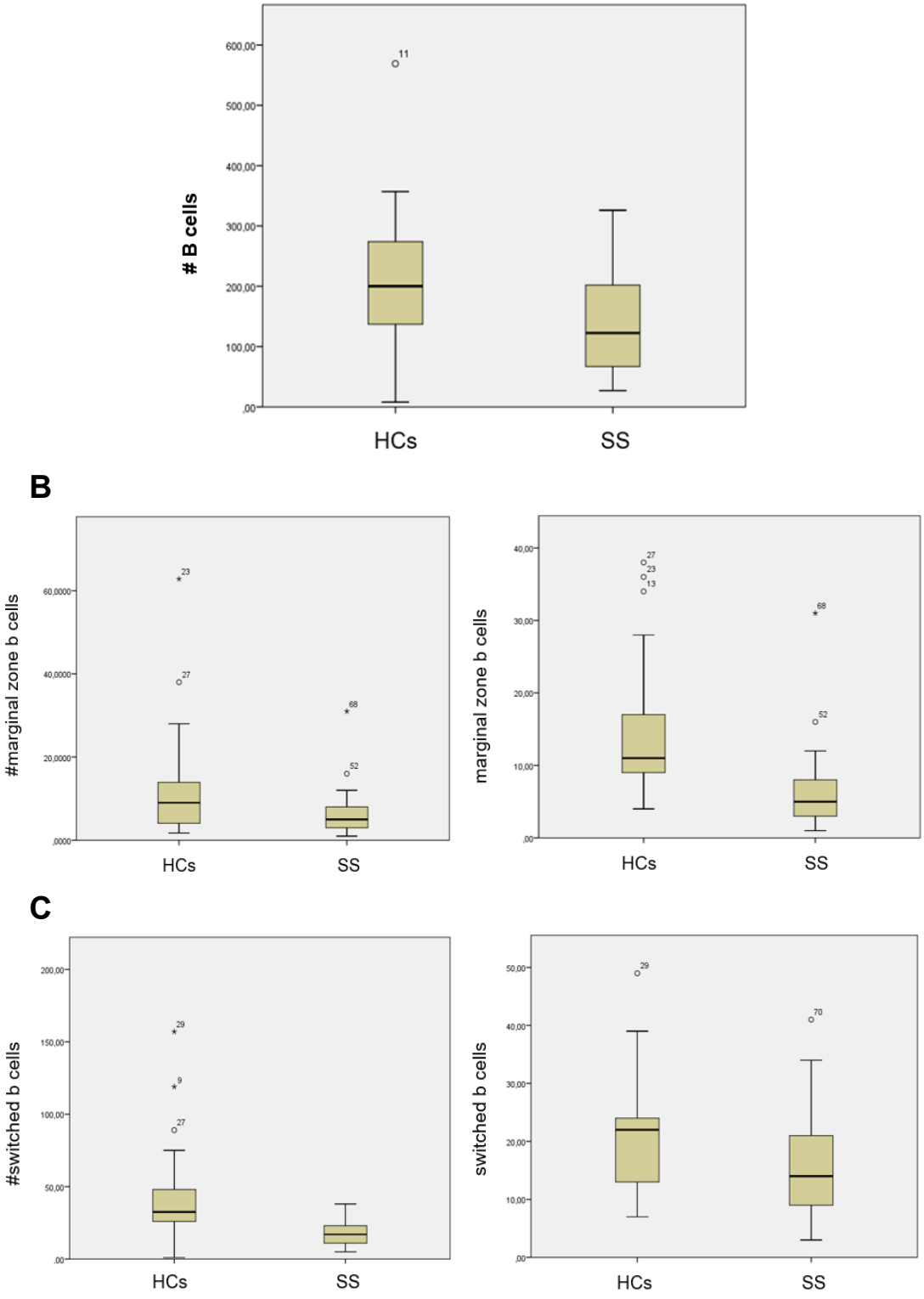


Figure 23 Box plots showing differences in B cells between HCs and SS

A: B cell population showed a decline in SS patients (p=0,009)
B: Less marginal zone cells were found in absolute (p=0,001) and proportional count (p=0,001) in SS patients
C: Switched cells are decreased in absolute (p=0,04) and proportional count (p=0,00016) of SS

D **E**

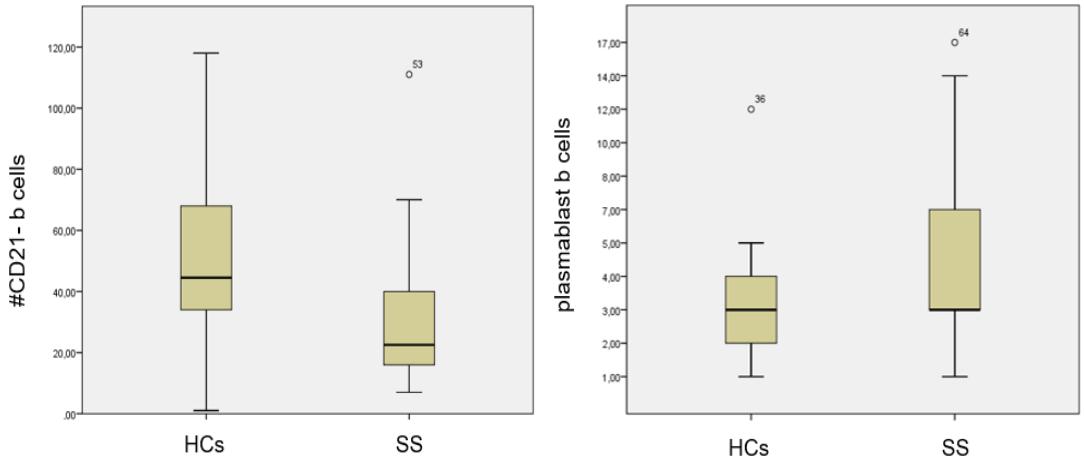


Figure 23 (continued)

D: Absolute count of CD21- was decreased in SS patients (p=0,004)
E: Percentage of plasmablast was elevated in SS patients (p=0,0016)

4.8.3 T cell and B cell stimulation:

In the simulation panel we analyzed the proportion of proliferating cells in each population (Table 21).

Table 21 Statistical results of the comparison stimulated T and B cell populations in HCs to SS patients

	SS		HCs		P
	Average	**St. deviation**	**Average**	**St. deviation**	
CD3/CD28 stim %proliferating of CD4+	80	13,7	87,6	6,4	0,025
CD3 /CD28 stim %proliferating of T cells	72,3	15,5	81	8,5	0,029
CD3/CD28 stim %proliferating of CD8+	58,1	18	73,5	13,8	0,0036
CON A 10mg stim %proliferating of CD8+	25,6	12,2	43,2	24,3	0,007
CON A 5mg stim %proliferating of CD8+	17,6	12,8	27,8	18,3	0,048

A **B**

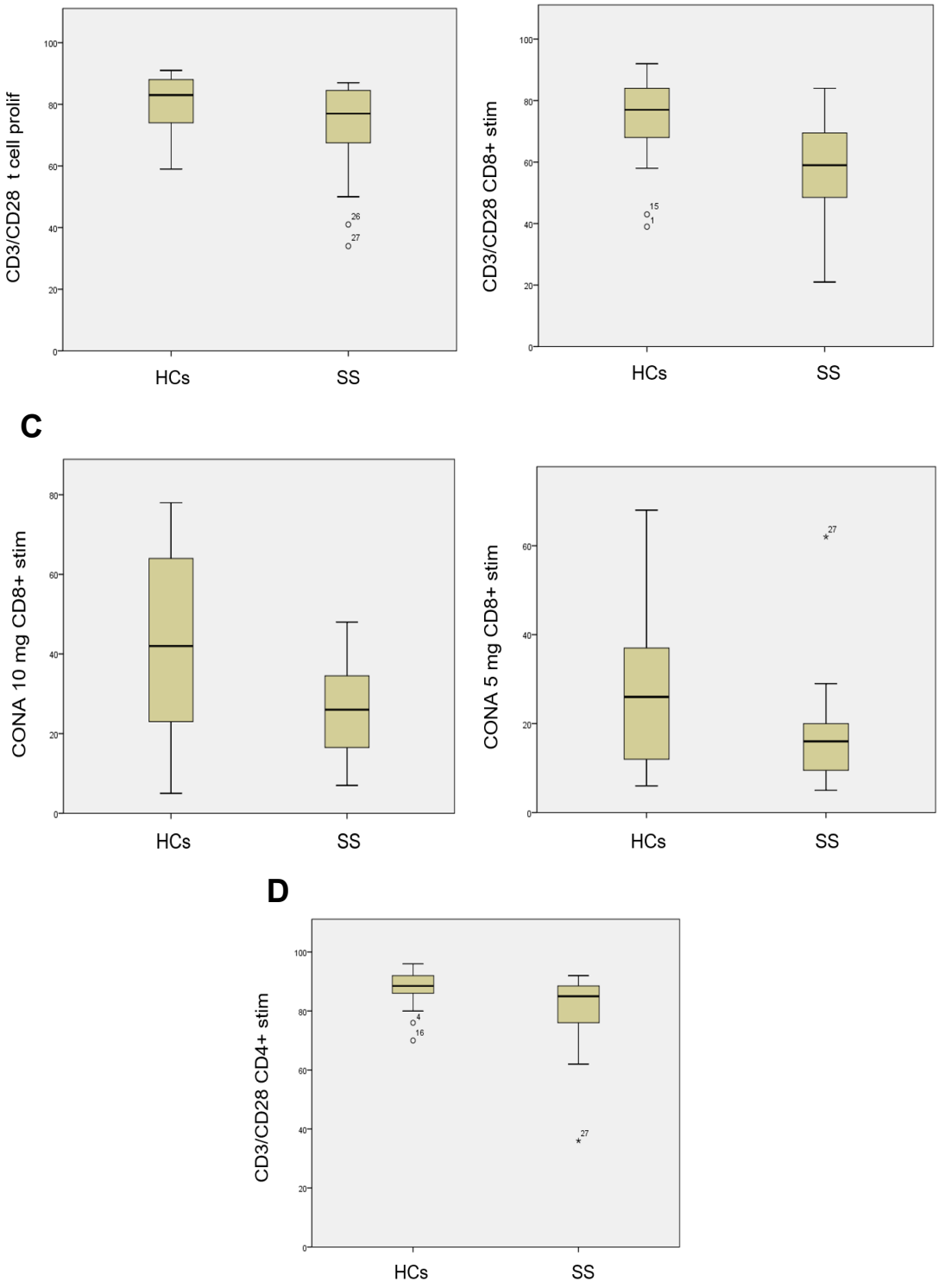


Figure 24 Box plots showing differences in proliferation of T cells between HCs and SS

A: Proliferating T cells stimulated with CD3/CD28 were decreased in SS patients (p= 0,029)
B: CD8+ cells stimulated with CD3/CD28 were reduced in SS patients (p= 0,0036)
C: CD8+ treated with CON A 10 mg (p=0,007) and 5 mg (p=0,048) were lower in SS patients than in HCs
D: Less CD4+ cells stimulated with CD3/CD28 were found in SS patients than in HCs (p=0,025)

4.8.4 Cytokines

In the cytokine panel IL-10 levels produced by PBMCs when stimulated with CD3/CD28 and IL-9 levels when stimulated with CON A 10mg was decreased in SS patients compared to HCs (Table 22, Figure 25).

Table 22 Statistical results of the comparison from Cytokine levels in HCs to SS patients

	SS		HCs		P
	Average	**St. deviation**	**Average**	**St. deviation**	
CD3/CD28 stim IL-10	815,2	535	1262,7	813,3	0,036
CON A10mg stim IL-9	290,9	174,7	656,6	722,5	0,029

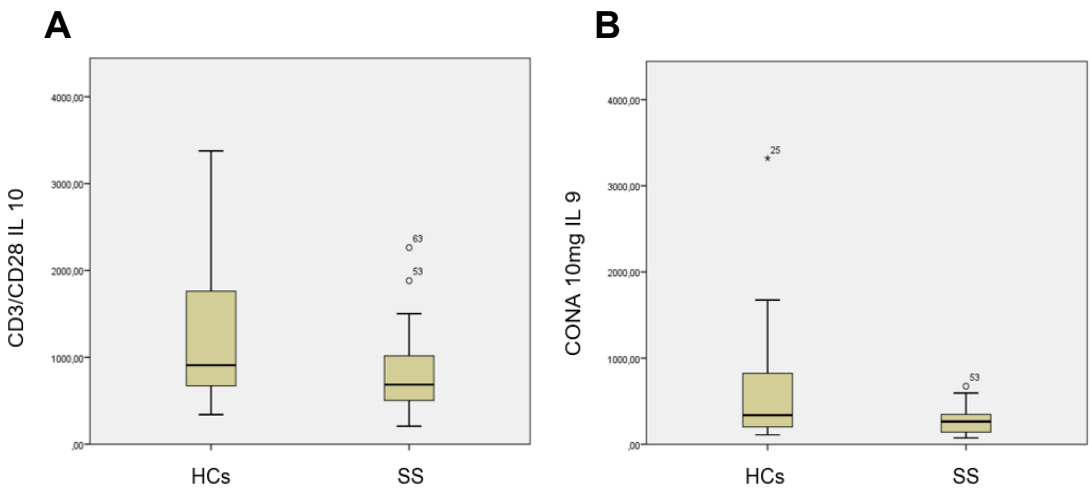


Figure 25 Comparison of cytokine production in stimulated PBMCs between HCs to SS patients

A: Levels of IL-10 were reduced in SS patients (p= 0,036)
B: Levels of IL-9 was decreased in SS patients (p= 0,029)

4.9 Correlation of lymphocyte populations with clinical data

4.9.1 Correlation with the ESSDAI

Correlation of lymphocyte populations with the ESSDAI showed mostly negative correlations. Just the level of TNF alpha after stimulation with CON A correlated positively with the ESSDAI. The correlation between ESSDAI and the absolute amount of CD4+ was strong, with a p of 0,00021 (Table 23). A strong correlation exists also between ESSDAI and the absolute count of activated Th2 cells. The other significant results are displayed in table 23.

Table 23 Correlation of lymphocyte populations with ESSDAI in SS patients

	Correlation	P
#CD3	-,573**	0,003
CD3 rel	-,407*	0,048
#CD4	-,686**	0,00021
#Th1	-,483*	0,017
# Th1 CD38+	-,515*	0,01
#Th1/17	-,439*	0,032
#Th2	-,636**	0,001
#Th2 CD38+	-,678**	0,00027
#Th17	-,515*	0,01
#Th17 CD38+	-,419*	0,042
#CD4 effector	-,510*	0,011
#CD4 effector memory	-,498*	0,013
CD4 naive rel	-,468*	0,021
#Tregs	-,600**	0,002
#DN	-,406*	0,049
#marginal zone	-,434*	0,034
#switched	-,469*	0,021
CON A10 TNFα	,464*	0,045

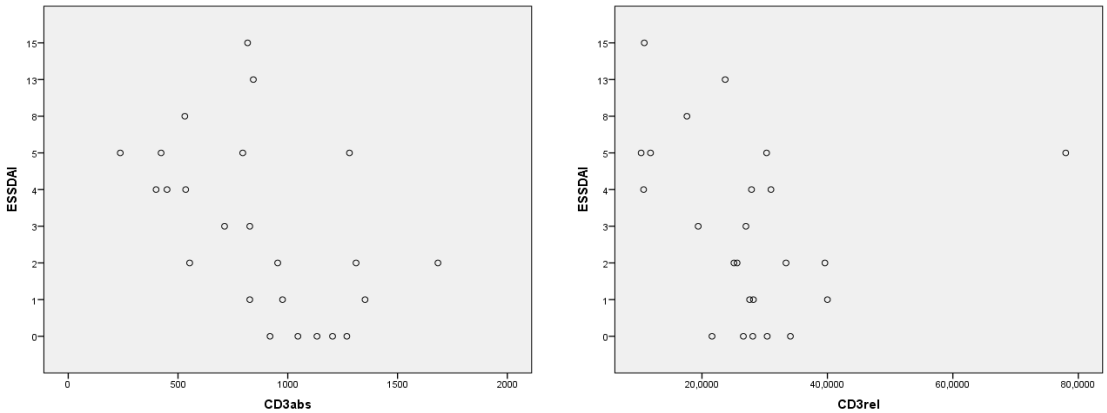


Figure 26 Correlation between ESSDAI, absolute amount of CD3+ cells (left) and proportional amount of CD3+ cells (right)

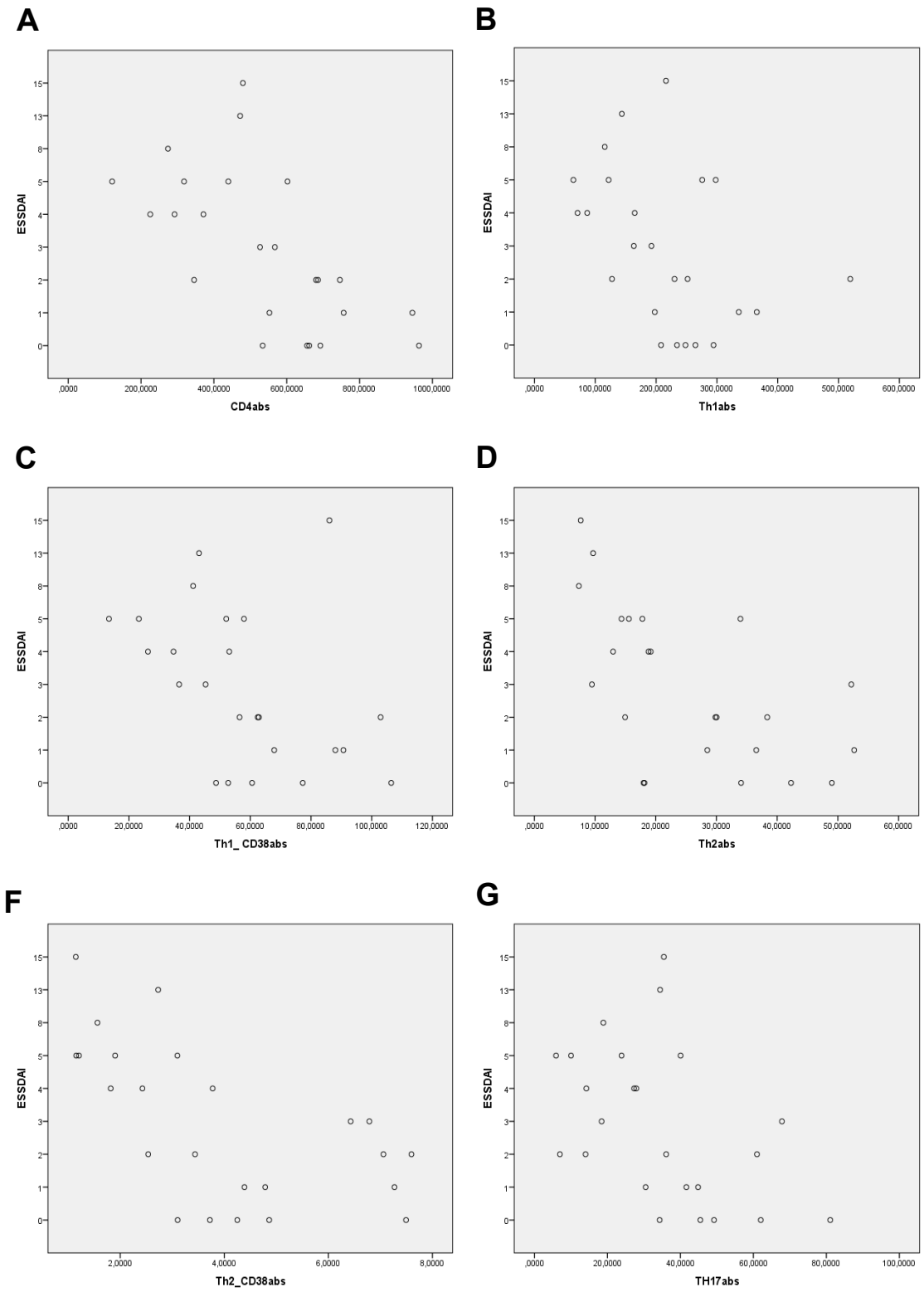


Figure 27 Correlation of lymphocyte populations with ESSDAI

A: Correlation between absolute count of CD4+ cells and ESSDAI
B: Correlation between absolute amount of Th1 cells and ESSDAI
C: Correlation between absolute count of activated Th1 cells and ESSDAI
D: Correlation between ESSDAI and absolute amount of Th2 cells
F: Correlation between absolute count of activated Th2 cells and ESSDAI
G: Correlation between ESSDAI and absolute amount of Th17 cells

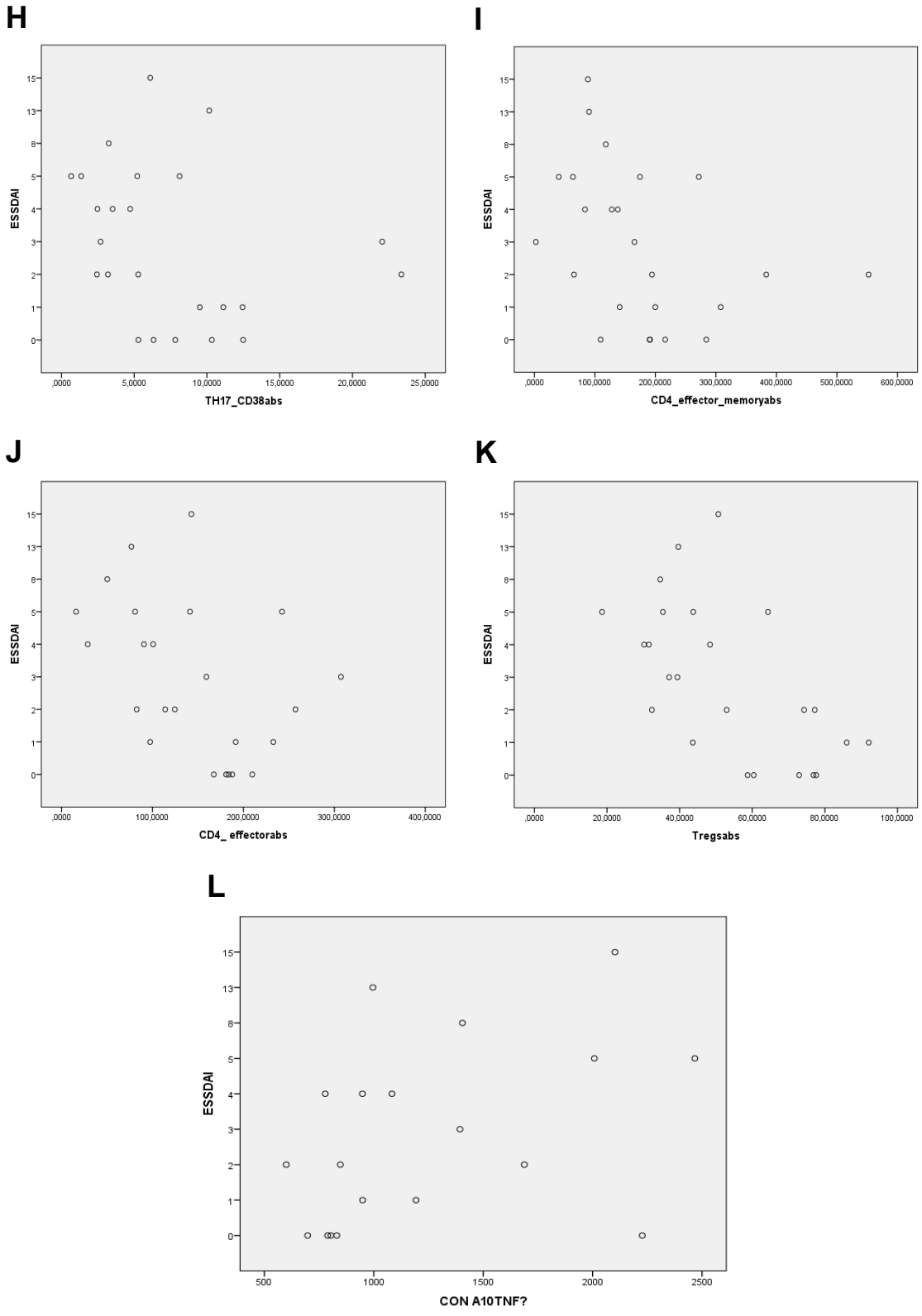


Figure 28 (continued)

H: Correlation between ESSDAI and absolute count of Th17 cells
I: Correlation between ESSDAI and absolute count of CD4+ effector memory cells
J: Correlation between absolute amount of CD4+ effector cells and ESSDAI
K: Correlation between absolute count of Tregs and ESSDAI
L: Invers correlation between TNFα stimulated with CON A 10 mg and ESSDAI

4.9.2 Correlation with C3

Correlation between C3 and lymphocyte populations showed mostly positive correlations. We found negative correlations between B cells, stimulated marginal zone B cells and transitional B cells.

Table 24 Correlation of lymphocyte populations with C3 in SS patients

	Correlation	P
CD3 rel	,427*	0,047
#CD3	,504*	0,017
#Th1	,466*	0,029
#Th1 CD38+	,531*	0,011
CD8 rel	,477*	0,025
#CD8	,600**	0,003
#CD8 CD38+	,633**	0,02
#CD8 central memory	,554**	0,007
CD8 CD28- rel	,571**	0,006
#CD8+CD28-	,584**	0,004
CD4+CD28- rel	640**	0,001
# CD4+ CD28-	,679**	0,001
B cells rel	-,518*	0,014
ODN 025 marginal zone prolif rel	-,565	0,023
ODN 025 transitionel prolif rel	-,562	0,024
ODN 012 transitionel prolif rel	-,626	0,013

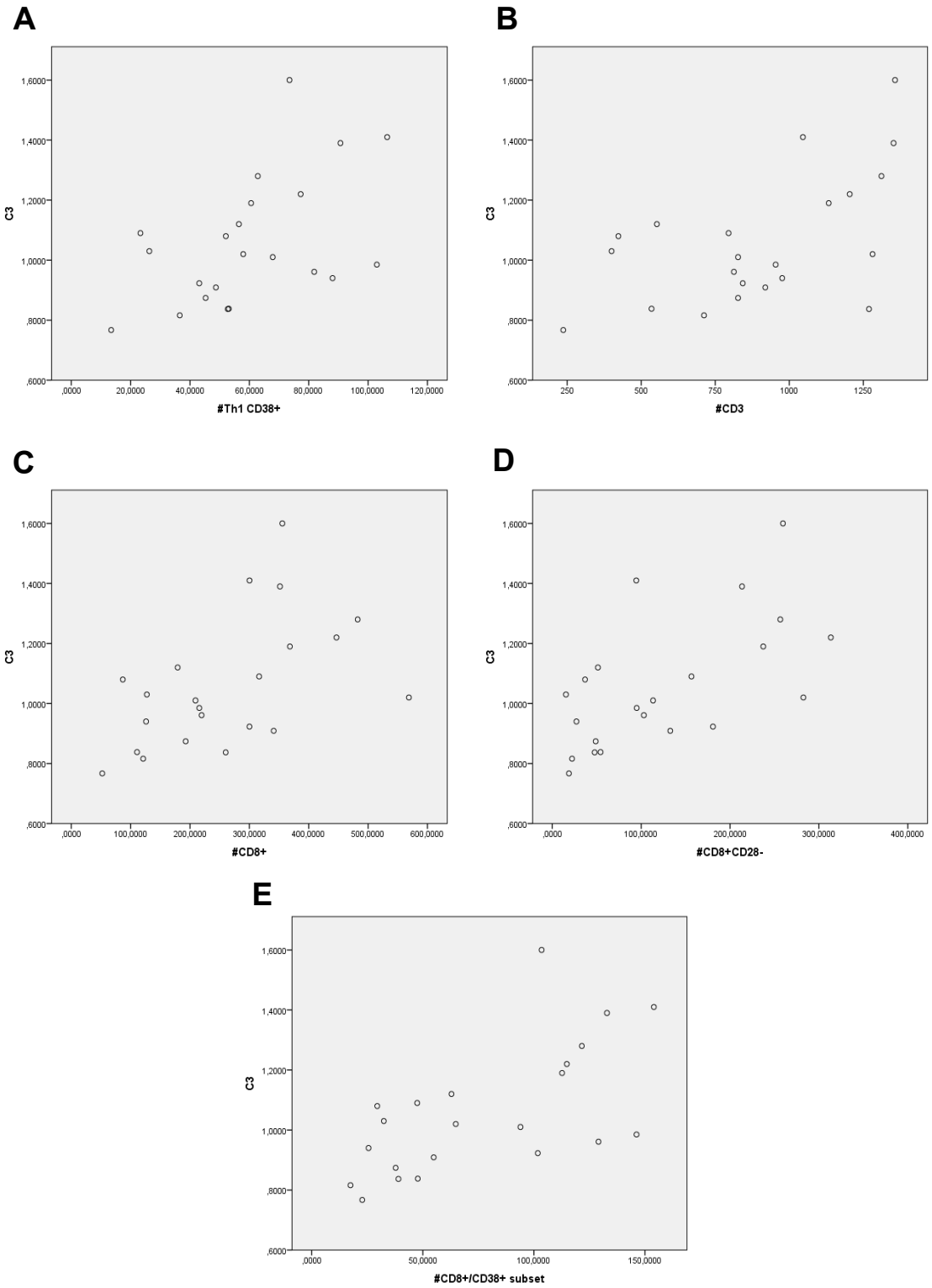


Figure 28 Correlation between lymphocyte populations and C3

A: Correlation between absolute amount of activated Th1 and C3
B: Correlation between absolute count of CD3+ cells and C3
C: Correlation between absolute amount of CD8+ cells and C3
D: Correlation between absolute count of CD8+ cells and C3
E: Correlation between absolute count of activated CD8+ cells and C3

4.9.3 Correlation with IgG

Correlation between levels of IgG and lymphocyte population showed positive and negative correlations. We found a highly significant correlation between DN cells and IgG.

Table 23 Correlation of lymphocyte populations with igG in SS patients

	Correlation	P
#CD3	-,463*	0,026
CD3	-,559**	0,006
#Th1/17 Ki67+ CD38+	-,429*	0,041
Th17 Ki67 +CD38+ rel	,434*	0,038
DN CD38+ rel	,703**	0,00018
DP CD38 rel	,447*	0,033
CD8+ CD38+ rel	,665**	0,001
# CD4 effector	-,589**	0,003
# CD4 effector memory	-,435*	0,038
#marginal zone	-,560**	0,005
# switched	-,531**	0,009
T/U IL-2	-,463*	0,046
CD3/CD28 IL-9	-,623**	0,004
CD3/CD28TNFα	-,516*	0,024

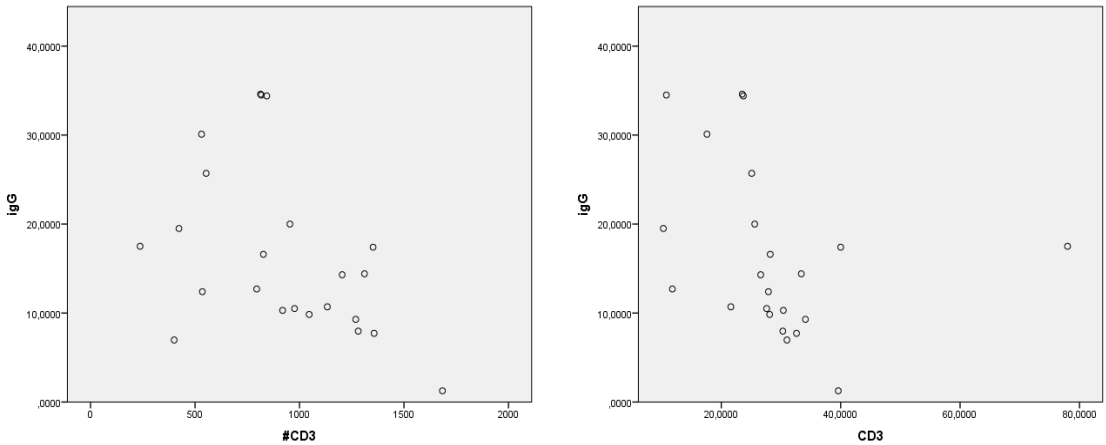


Figure 29 Correlation between C3, absolute amount of CD3+ cells (left) and proportional amount of CD3+ cells

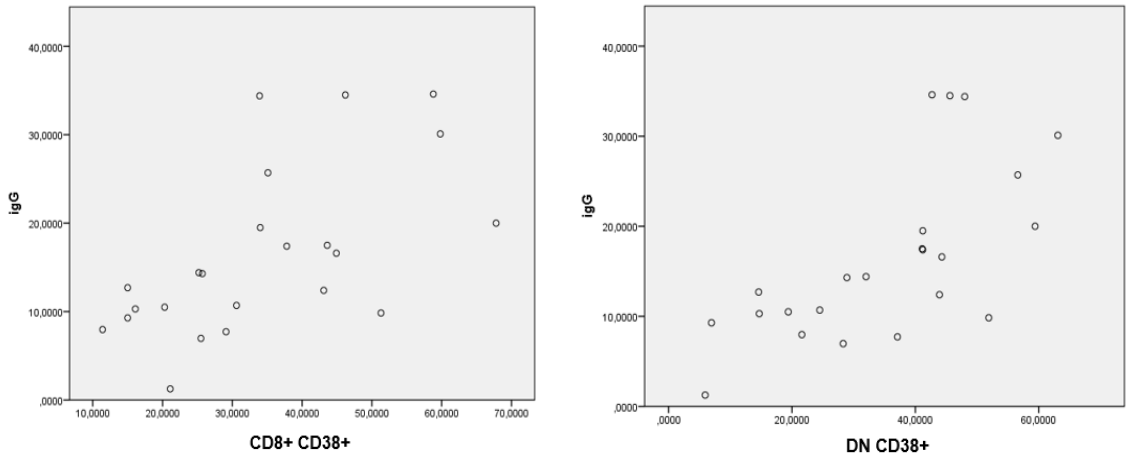


Figure 30 Correlation between C3 and proportional count of activated CD8+ cells (left) and between C3 and proportional count of activated DN cells (right)

4.9.4 Correlation with BSG

Correlation between lymphocyte subsets and BSG showed positive and negative correlations.

Table 25 Correlation of lymphocyte populations with BSG in SS patients

	Correlation	P
#CD3	-,433*	0,03
Th1 CD38+ rel	,457*	0,022
Th2 CD38+ rel	,514**	0,009
Th17 CD38+ rel	,514**	0,009
Th17 Ki67 CD38+ rel	,484*	0,014
CD8+ CD38+ rel	,424*	0,035
DN CD38+ rel	,495*	0,012
CD4 central memory rel	-,437*	0,029
#CD4 central memory	-,500*	0,011
Marginal zone rel	-,470*	0,018
CD3/CD28 IL 9	-,511*	0,018
CD3/CD28 TNFα	-,553**	0,009
CON A 10 t cells	-,590**	0,008
CON A 5 t cells	-,491*	0,033
ODN 0125 naive prolif	,667**	0,003

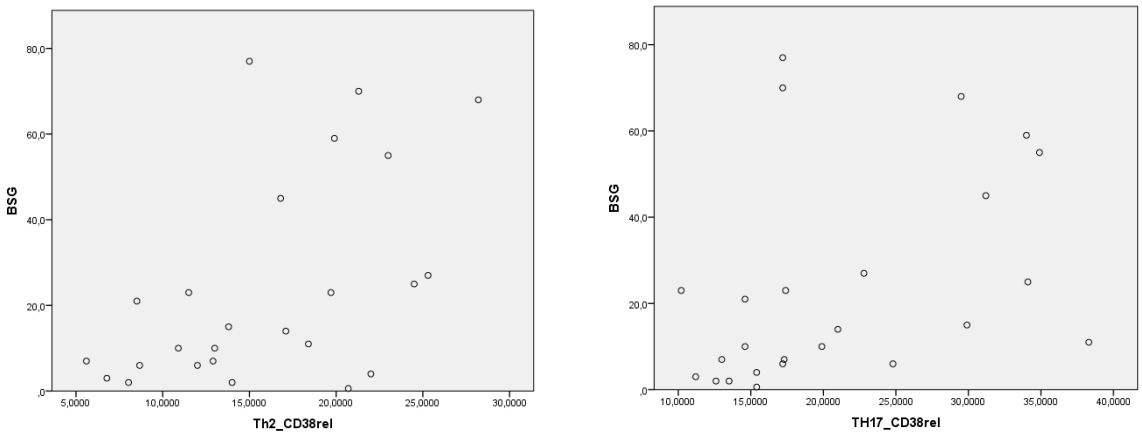


Figure 31 Correlation between BSG and proportional count of activated Th2 cells (left) and proportional count of activated Th17 cells

4.9.5 Correlation with ANA

Correlation between lymphocyte populations, zytokine levels and ANA titers were mainly invers. Only proliferation of marginal zone B cells showed a positive correlation.

Table 26 Correlation of lymphocyte populations with ANA titers in SS patients

	Correlation	P
T/UIL 9	-,463*	0,035
T/UIL13	-,468*	0,032
T/UIl 23	-,570**	0,007
CD3/CD28Il 23	-,457*	0,037
CD3/CD28 GranzymeB	-,485*	0,026
CON A 10 CD8 stim	-,544*	0,016
CON A 10 t cells	-,489*	0,033
ODN 025 Marginal Zone	,498*	0,030
B unstim switched prolif	-,489*	0,034
#CD21neg	-,509**	0,008
#Marginal zone	-,399*	0,044
#CD27-_IgD-	-,425*	0,03

5 Discussion

The aim of this study was to identify biomarkers in lymphocytes populations in the peripheral blood in order to improve the diagnosis and the prognosis of Sjögren's syndrome patients.

MSG lesions of pSS patients mainly consist of T and B lymphocytes. The majority of the T cell population is composed of CD4 + cells and a minority of CD8+ cells representing 15% of all MNC cells. Characteristic changes in the composition of infiltrating cells can be observed in MSG tissues varying with infiltration severity (mild, intermediate or severe lesions). Lesion severity and the extent of salivary gland damage is directly linked to the grade of disease activity.

In contrast to T cells, the amount of B cells correlates directly with lesion severity and B cells are the main population in severe lesions. NK cells and Treg cells represent a small, but very particular population in MSG lesions. Previous studies have shown that the percentage of NK correlates directly with lesion severity. In contrast, Treg cells were found to be most abundant in lesion of intermediate grade. (46, 125-127)

CD4 lymphocytopenia and general lymphocytopenia is a well-known and frequent finding in pSS. (125) In this study, we found a highly significant decrease in the absolute count of lymphocytes ($p = 0{,}000097$) in SS patients. This was mostly the result of an absolute decrease of the CD4 + cell ($p= 0, 0000067$) count in SS.

For the identification of lymphocyte biomarkers it's important to know how changes in lymphocyte composition in the lesion correlate with changes in the distribution of peripheral blood lymphocytes.

A recently published paper compared peripheral blood cell distribution with lymphocyte composition in affected exocrine glands of pSS patients. A strong inverse correlations between number of CD4+cells found in the blood and amount of CD4+ cells found in MSG lesions was reported. Also, a correlation between blood CD4+ cell numbers and disease activity were observed.

The main reason for the lack of lymphocytes in the blood is thought to be a migration from blood CD4+ cells into the target tissue. (126) Other theories explain the decline of CD4+ cells due to the presence of CD4+ autoantibodies, which were found in SS patients of a Swedish study. (128)

Further we identified a highly significant invers correlation between the amount of CD4+ cells and the ESSDAI. Overall, this supports the idea that lymphocytopenia is a marker for disease severity. Aside from its diagnostic character, lymphocytopenia is also considered to be one of several prognostic markers highlighting the risk of developing a NHL. (93)

The decline of the CD4+ population is a result of changes in CD4+ subpopulations. We found a decrease of the absolute numbers of CD4+ effector cells (p=0,0004), CD4 + naïve cells (p=0,00014), Th2 cells (p= 0,0001), Th1/17 cells (p= 0,01), and CD4+ CD28- cells (p= 0,007). This findings confims the findings of Michael Mingueneau et al (126) who explained the CD4+ lymphocytopenia as a result of a decline in the naïve and effector subsets .
This was mostly the result of a selective decrease in numbers of CD4+ T lymphocytes (Fig 1, C). NK cell counts were slightly but significantly reduced in patients with anti-SSA+ pSS. Naive, memory, and effector subsets of CD4+ T lymphocytes were all significantly decreased in patients with anti-SSA+ pSS, but the naive subset was the most affected
Reduction in the absolute count led to a proportional increase of numerically stable populations: Th1/17 cells and Th1 cells both are gated from the parent generation of CXCR3+ cells. The reduction of TH1/17 cells consequently led to a proportional increase of Th1 cells.

In contrary to another study (46), we didn't find any significant decrease in the absolute count of CD8 + cells. The frequency of CD8+ cells was significantly increased in SS patients (p= 0,00042) compared to the healthy control groups. This increase is a result of the absolute decrease of the CD4+ cells. SS patients have more activated CD8+ cells both, in absolute (p= 0,045) as well as in relative numbers (p= 0,00054). Although more activated, CD8+ cells from SS patients exhibited a profound defect in proliferation after stimulation with CD3/28 and both concentrations of Con A.
The role of CD8+ cells in the pathogenesis of immune tolerance breakdown has been studied among different autoimmune disorders (33, 38) and auto reactive CD8+ have also been observed for Sjögren's syndrome (37). Our findings are concordant with the results of a French study, where the number of activated CD8+

cells in the blood rose according to disease activity. Furthermore the percentage of activated CD8+ cells in the target tissue correlated with the severity of the focus score (126).

CD8+ cell depletion resulting in reduction of disease activity in other autoimmune diseases (40, 41) further emphasizes a possible pathogenic role of CD8+ cells.

Analyzing the CD8+ subpopulations showed interesting changes. SS patients have less CD8+ central memory cells (p= 0,019), less CD8+ naïve cells (p= 0,002) and more CD8+CD28- cells (p= 0,022). The CD8+CD28- cells are thought to have regulatory functions inhibiting the reactivity of CD4+ cells either directly or with the help of secreted cytokines (129) An increased frequency of CD8+ cells with the lack of CD28- is decribied in ageing, immunodeficiency or chronic inflammation(130). Several studies evaluating the frequency of CD8+CD28 in the peripheral blood of SLE patients or patients with other autoimmune patients resulted in conflicting outcomes (129, 131). Our results are in line with a study reporting an increase of CD8+CD28-in SLE patients compared to HCs. In addition, they found a strong correlation with the disease activity.(132)

Tregs have become the focus of interest in the last several years. Their potential to ameliorate auto-reactivity and their pivotal role in peripheral tolerance was highlighted in the last decades. In our study, we surprisingly found a highly significant increase of the frequency of Tregs in the peripheral blood of SS patients (p= 0,00001). The reduction of overall CD4+ cells in the presence of a constant number of Tregs may explain this relative increase of Tregs. Considering their inhibiting properties, we would have expected to find a decrease number of Tregs in our SS patients.

The amount of Tregs in autoimmune disorders have been controversially discussed. Some studies reported an increase in Tregs, some could not find any significant difference while others have reported a decrease in the amount of Tregs. (43, 133) One of the main problems of comparing these studies is that different authors define different pools of CD4 + cells as Tregs. In this study we used the CD4+, CD25+CD127- as markers, whereas others only used the Fox P3 as a marker and some authors used both as markers. (46) Interestingly, in the present study Treg numbers decreased with increasing SS activity as measured by the ESSDAI.

A remarkable increase in relative (p=0,000000009) and in absolute count (p=0,032) of activated DN in SS patients was found.

DN cells are defined by the lack of CD4 and CD8 and consequently may include T cells with different functions. (26) Besides CD4+, Th17 and CD8+ cells, the DN cell is descript to be a source of IL 17 production, a zytokine with a pivotal role in autoimmunity . (44) A growing body of evidence suggests that DN cells play a major role in the pathogenesis of autoimmune disorders:

Animal models with different autoimmune mices (MRL-lpr/lpr, C3H7HeJ-gld/gld BXSB, NOD, MRL-+/- and NZB/W F1) showed an intensive accumulation of DN in the liver when they became affected. Before disease onset no infiltrating DN were observed. This study also suggest the liver to be the main source of DN production, after other lymphoid organ. (134)

Elevated numbers of DN in the peripheral blood are associated with anti-DNA production and lupus nephritis in SLE. (135). Recent studies in SS showed that the amount of DN cells found in the blood correlates inversely with the number of infiltrating DN in minor salivary gland (MSG), whereas the amount of infiltrating DN cells correlates directly with the Sicca symptoms. DN also seem to be resistant to corticosteroid therapy. (26, 31, 136) Similar to our results higher percentages of activated DN (markers HLA-DR, CD69, CD28 and CTLa) are found in SLE patients compared to HCs. (137) The direct correlation with the amount of IgG, further supports the important role of DN in pSS.

In SS patients the absolute B cell count was decreased compared to HCs (p=0,009). The reduction was mainly a consequence of a decline of switched cells (p= 0,00016) marginal zone cells (p= 0,001) and CD 19+CD 21- cells (p= 0,004).

5.1 Limitations

Limitations of this work arise from the fact that pure phenotyping of lymphocytes by FACS analysis is not able to analyze altered function of these cells. It would be interesting to know if counted cells are modified or display pathogenic functions, which may play a role in pathogenesis of SS. Further investigations of these cells in mouse models could be helpful answering these questions.

Besides this, gating of the populations is sometimes subjective. Setting the gates manually allows differences among users to occur. Even, if in most of the cases populations can be easily gated, some subsets were more difficult to gate and may display different result depending on the person doing the gating.

Furthermore it was not possible to reach the expected numbers of participants for our SS group. Instead of 80 persons just 25 patients have been included in our group limiting the value of the study.

5.2 Conclusion

We found a reduction of T helper cells in SS as described by Mandl T Mandl T, Bredberg A, Jacobsson LT, Manthorpe R, Henriksson G (125) and Sudzius G, Mieliauskaite D, Siaurys A, Viliene R, Butrimiene I, Characiejus D, et al. (46), which we identified to be the result of a loss of peripheral naïve and effector cells, Th2 and Th1/17 cells. CD8+ cells in SS showed impaired proliferative capacity despite signs of activation. DN cells are a source of IL-17, which is thought to play a major role in the pathogenesis of SS. Activated DN cells seem to be a promising lymphocyte subset as they are increased in SS and correlate with serum IgG. Consequently they may be used as a biomarker in the future. These results warrant further functional investigations.

6 Literature

1. Haugen AJ, Peen E, Hulten B, Johannessen AC, Brun JG, Halse AK, et al. Estimation of the prevalence of primary Sjogren's syndrome in two age-different community-based populations using two sets of classification criteria: the Hordaland Health Study. Scandinavian journal of rheumatology. 2008;37(1):30-4.
2. Rischmueller M, Tieu J, Lester S. Primary Sjogren's syndrome. Best practice & research Clinical rheumatology. 2016;30(1):189-220.
3. Goules AV, Tzioufas AG, Moutsopoulos HM. Classification criteria of Sjogren's syndrome. Journal of autoimmunity. 2014;48-49:42-5.
4. Alamanos Y, Tsifetaki N, Voulgari PV, Venetsanopoulou AI, Siozos C, Drosos AA. Epidemiology of primary Sjogren's syndrome in north-west Greece, 1982-2003. Rheumatology (Oxford, England). 2006;45(2):187-91.
5. Patel R, Shahane A. The epidemiology of Sjogren's syndrome. Clinical epidemiology. 2014;6:247-55.
6. Garcia-Carrasco M, Ramos-Casals M, Rosas J, Pallares L, Calvo-Alen J, Cervera R, et al. Primary Sjogren syndrome: clinical and immunologic disease patterns in a cohort of 400 patients. Medicine. 2002;81(4):270-80.
7. Mathews PM, Hahn S, Hessen M, Kim J, Grader-Beck T, Birnbaum J, et al. Ocular complications of primary Sjogren syndrome in men. American journal of ophthalmology. 2015;160(3):447-52.e1.
8. Low HZ, Witte T. Aspects of innate immunity in Sjogren's syndrome. Arthritis research & therapy. 2011;13(3):218.
9. Konsta OD, Thabet Y, Le Dantec C, Brooks WH, Tzioufas AG, Pers JO, et al. The contribution of epigenetics in Sjogren's Syndrome. Frontiers in genetics. 2014;5:71.
10. Selmi C, Lu Q, Humble MC. Heritability versus the role of the environment in autoimmunity. Journal of autoimmunity. 2012;39(4):249-52.
11. Kuo CF, Grainge MJ, Valdes AM, See LC, Luo SF, Yu KH, et al. Familial Risk of Sjogren's Syndrome and Co-aggregation of Autoimmune Diseases in Affected Families: A Nationwide Population Study. Arthritis & rheumatology (Hoboken, NJ). 2015;67(7):1904-12.
12. Foster H, Walker D, Charles P, Kelly C, Cavanagh G, Griffiths I. Association of DR3 with susceptibility to and severity of primary Sjogren's syndrome in a family study. British journal of rheumatology. 1992;31(5):309-14.

13. Lessard CJ, Li H, Adrianto I, Ice JA, Rasmussen A, Grundahl KM, et al. Variants at multiple loci implicated in both innate and adaptive immune responses are associated with Sjogren's syndrome. Nature genetics. 2013;45(11):1284-92.
14. Li Y, Zhang K, Chen H, Sun F, Xu J, Wu Z, et al. A genome-wide association study in Han Chinese identifies a susceptibility locus for primary Sjogren's syndrome at 7q11.23. Nature genetics. 2013;45(11):1361-5.
15. Quddus J, Johnson KJ, Gavalchin J, Amento EP, Chrisp CE, Yung RL, et al. Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice. The Journal of clinical investigation. 1993;92(1):38-53.
16. Moss WN, Steitz JA. Genome-wide analyses of Epstein-Barr virus reveal conserved RNA structures and a novel stable intronic sequence RNA. BMC genomics. 2013;14:543.
17. Nakamura H, Takahashi Y, Yamamoto-Fukuda T, Horai Y, Nakashima Y, Arima K, et al. Direct infection of primary salivary gland epithelial cells by human T lymphotropic virus type I in patients with Sjogren's syndrome. Arthritis & rheumatology (Hoboken, NJ). 2015;67(4):1096-106.
18. Nakamura H, Shimizu T, Takagi Y, Takahashi Y, Horai Y, Nakashima Y, et al. Reevaluation for clinical manifestations of HTLV-I-seropositive patients with Sjogren's syndrome. BMC musculoskeletal disorders. 2015;16:335.
19. Triantafyllopoulou A, Tapinos N, Moutsopoulos HM. Evidence for coxsackievirus infection in primary Sjogren's syndrome. Arthritis and rheumatism. 2004;50(9):2897-902.
20. Triantafyllopoulou A, Moutsopoulos HM. Autoimmunity and coxsackievirus infection in primary Sjogren's syndrome. Annals of the New York Academy of Sciences. 2005;1050:389-96.
21. Gottenberg JE, Pallier C, Ittah M, Lavie F, Miceli-Richard C, Sellam J, et al. Failure to confirm coxsackievirus infection in primary Sjogren's syndrome. Arthritis and rheumatism. 2006;54(6):2026-8.
22. Chaigne B, Lasfargues G, Marie I, Huttenberger B, Lavigne C, Marchand-Adam S, et al. Primary Sjogren's syndrome and occupational risk factors: A case-control study. Journal of autoimmunity. 2015;60:80-5.

23. Barrera MJ, Bahamondes V, Sepulveda D, Quest AF, Castro I, Cortes J, et al. Sjogren's syndrome and the epithelial target: a comprehensive review. Journal of autoimmunity. 2013;42:7-18.
24. Humphreys-Beher MG, Peck AB. New concepts for the development of autoimmune exocrinopathy derived from studies with the NOD mouse model. Archives of oral biology. 1999;44 Suppl 1:S21-5.
25. Hernandez JB, Newton RH, Walsh CM. Life and death in the thymus--cell death signaling during T cell development. Current opinion in cell biology. 2010;22(6):865-71.
26. Alunno A, Bistoni O, Bartoloni Bocci E, Caterbi S, Bigerna B, Pucciarini A, et al. IL-17-producing double-negative T cells are expanded in the peripheral blood, infiltrate the salivary gland and are partially resistant to corticosteroid therapy in patients with Sjogren's syndrome. Reumatismo. 2013;65(4):192-8.
27. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4(+)T cells: differentiation and functions. Clinical & developmental immunology. 2012;2012:925135.
28. Kuriya G, Uchida T, Akazawa S, Kobayashi M, Nakamura K, Satoh T, et al. Double deficiency in IL-17 and IFN-gamma signalling significantly suppresses the development of diabetes in the NOD mouse. Diabetologia. 2013;56(8):1773-80.
29. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, et al. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. Nature immunology. 2008;9(2):166-75.
30. Fei Y, Zhang W, Lin D, Wu C, Li M, Zhao Y, et al. Clinical parameter and Th17 related to lymphocytes infiltrating degree of labial salivary gland in primary Sjogren's syndrome. Clinical rheumatology. 2014;33(4):523-9.
31. Katsifis GE, Rekka S, Moutsopoulos NM, Pillemer S, Wahl SM. Systemic and local interleukin-17 and linked cytokines associated with Sjogren's syndrome immunopathogenesis. The American journal of pathology. 2009;175(3):1167-77.
32. Russ B, Prier J, Rao S, Turner S. T cell immunity as a tool for studying epigenetic regulation of cellular differentiation. Frontiers in genetics. 2013;4(218).
33. Gravano DM, Hoyer KK. Promotion and prevention of autoimmune disease by CD8+ T cells. Journal of autoimmunity. 2013;45:68-79.

34. Lucchinetti CF, Popescu BF, Bunyan RF, Moll NM, Roemer SF, Lassmann H, et al. Inflammatory cortical demyelination in early multiple sclerosis. The New England journal of medicine. 2011;365(23):2188-97.
35. Nakao H, Eguchi K, Kawakami A, Migita K, Otsubo T, Ueki Y, et al. Phenotypic characterization of lymphocytes infiltrating synovial tissue from patients with rheumatoid arthritis: analysis of lymphocytes isolated from minced synovial tissue by dual immunofluorescent staining. The Journal of rheumatology. 1990;17(2):142-8.
36. Chabot S, Fakhfakh A, Beland K, Lamarre A, Oldstone MB, Alvarez F, et al. Mouse liver-specific CD8(+) T-cells encounter their cognate antigen and acquire capacity to destroy target hepatocytes. Journal of autoimmunity. 2013;42:19-28.
37. Fujihara T, Fujita H, Tsubota K, Saito K, Tsuzaka K, Abe T, et al. Preferential localization of CD8+ alpha E beta 7+ T cells around acinar epithelial cells with apoptosis in patients with Sjogren's syndrome. Journal of immunology (Baltimore, Md : 1950). 1999;163(4):2226-35.
38. Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. Arthritis and rheumatism. 2005;52(1):201-11.
39. Malmestrom C, Lycke J, Haghighi S, Andersen O, Carlsson L, Wadenvik H, et al. Relapses in multiple sclerosis are associated with increased CD8+ T-cell mediated cytotoxicity in CSF. Journal of neuroimmunology. 2008;196(1-2):159-65.
40. Coles AJ, Compston DA, Selmaj KW, Lake SL, Moran S, Margolin DH, et al. Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. The New England journal of medicine. 2008;359(17):1786-801.
41. Reynolds J, Norgan VA, Bhambra U, Smith J, Cook HT, Pusey CD. Anti-CD8 monoclonal antibody therapy is effective in the prevention and treatment of experimental autoimmune glomerulonephritis. Journal of the American Society of Nephrology : JASN. 2002;13(2):359-69.
42. Zehn D, Bevan MJ. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. Immunity. 2006;25(2):261-70.
43. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmunity reviews. 2014;13(6):668-77.

44. Alunno A, Carubbi F, Bistoni O, Caterbi S, Bartoloni E, Mirabelli G, et al. T Regulatory and T Helper 17 Cells in Primary Sjogren's Syndrome: Facts and Perspectives. Mediators of inflammation. 2015;2015:243723.
45. Passerini L, Santoni de Sio FR, Roncarolo MG, Bacchetta R. Forkhead box P3: the peacekeeper of the immune system. International reviews of immunology. 2014;33(2):129-45.
46. Sudzius G, Mieliauskaite D, Siaurys A, Viliene R, Butrimiene I, Characiejus D, et al. Distribution of Peripheral Lymphocyte Populations in Primary Sjogren's Syndrome Patients. Journal of immunology research. 2015;2015:854706.
47. van der Vliet HJ, von Blomberg BM, Nishi N, Reijm M, Voskuyl AE, van Bodegraven AA, et al. Circulating V(alpha24+) Vbeta11+ NKT cell numbers are decreased in a wide variety of diseases that are characterized by autoreactive tissue damage. Clinical immunology (Orlando, Fla). 2001;100(2):144-8.
48. Illes Z, Kondo T, Newcombe J, Oka N, Tabira T, Yamamura T. Differential expression of NK T cell V alpha 24J alpha Q invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. Journal of immunology (Baltimore, Md : 1950). 2000;164(8):4375-81.
49. Maeda T, Keino H, Asahara H, Taniguchi M, Nishioka K, Sumida T. Decreased TCR AV24AJ18+ double-negative T cells in rheumatoid synovium. Rheumatology (Oxford, England). 1999;38(2):186-8.
50. Demoulins T, Gachelin G, Bequet D, Dormont D. A biased Valpha24+ T-cell repertoire leads to circulating NKT-cell defects in a multiple sclerosis patient at the onset of his disease. Immunology letters. 2003;90(2-3):223-8.
51. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, et al. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. Nature. 1998;391(6663):177-81.
52. Verge CF, Gianani R, Yu L, Pietropaolo M, Smith T, Jackson RA, et al. Late progression to diabetes and evidence for chronic beta-cell autoimmunity in identical twins of patients with type I diabetes. Diabetes. 1995;44(10):1176-9.
53. Abdulahad WH, Kroese FG, Vissink A, Bootsma H. Immune regulation and B-cell depletion therapy in patients with primary Sjogren's syndrome. Journal of autoimmunity. 2012;39(1-2):103-11.

54. Cornec D, Devauchelle-Pensec V, Tobon GJ, Pers JO, Jousse-Joulin S, Saraux A. B cells in Sjogren's syndrome: from pathophysiology to diagnosis and treatment. Journal of autoimmunity. 2012;39(3):161-7.
55. Varin MM, Le Pottier L, Youinou P, Saulep D, Mackay F, Pers JO. B-cell tolerance breakdown in Sjogren's syndrome: focus on BAFF. Autoimmunity reviews. 2010;9(9):604-8.
56. Yao Y, Liu Z, Jallal B, Shen N, Ronnblom L. Type I interferons in Sjogren's syndrome. Autoimmunity reviews. 2013;12(5):558-66.
57. Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, et al. The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjogren's syndrome. Annals of the rheumatic diseases. 2003;62(2):168-71.
58. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. The Journal of clinical investigation. 2002;109(1):59-68.
59. Szodoray P, Alex P, Brun JG, Centola M, Jonsson R. Circulating cytokines in primary Sjogren's syndrome determined by a multiplex cytokine array system. Scandinavian journal of immunology. 2004;59(6):592-9.
60. Testar J. Cytokines: Introduction London: British Soviety for Rheumatology; 2016. Available from: http://bitesized.immunology.org/receptors-and-molecules/cytokines/.Accessed July 15, 2016
61. Holdgate N, St Clair EW. Recent advances in primary Sjogren's syndrome. F1000Research. 2016;5.
62. Dudakov JA, Hanash AM, van den Brink MR. Interleukin-22: immunobiology and pathology. Annual review of immunology. 2015;33:747-85.
63. Ciccia F, Guggino G, Rizzo A, Ferrante A, Raimondo S, Giardina A, et al. Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjogren's syndrome. Annals of the rheumatic diseases. 2012;71(2):295-301.
64. Brkic Z, Maria NI, van Helden-Meeuwsen CG, van de Merwe JP, van Daele PL, Dalm VA, et al. Prevalence of interferon type I signature in CD14 monocytes of patients with Sjogren's syndrome and association with disease activity and BAFF gene expression. Annals of the rheumatic diseases. 2013;72(5):728-35.

65. Hall JC, Baer AN, Shah AA, Criswell LA, Shiboski CH, Rosen A, et al. Molecular Subsetting of Interferon Pathways in Sjogren's Syndrome. Arthritis & rheumatology (Hoboken, NJ). 2015;67(9):2437-46.
66. Mitarbeiter GHu. Innere Medizin 2016. Köln2016.
67. Brito-Zeron P, Theander E, Baldini C, Seror R, Retamozo S, Quartuccio L, et al. Early diagnosis of primary Sjogren's syndrome: EULAR-SS task force clinical recommendations. Expert review of clinical immunology. 2016;12(2):137-56.
68. Thanou-Stavraki A, James JA. Primary Sjogren's syndrome: current and prospective therapies. Seminars in arthritis and rheumatism. 2008;37(5):273-92.
69. van Nimwegen JF, Arends S, van Zuiden GS, Vissink A, Kroese FG, Bootsma H. The impact of primary Sjogren's syndrome on female sexual function. Rheumatology (Oxford, England). 2015;54(7):1286-93.
70. Abrol E, Gonzalez-Pulido C, Praena-Fernandez JM, Isenberg DA. A retrospective study of long-term outcomes in 152 patients with primary Sjogren's syndrome: 25-year experience. Clinical medicine (London, England). 2014;14(2):157-64.
71. Nannini C, Jebakumar AJ, Crowson CS, Ryu JH, Matteson EL. Primary Sjogren's syndrome 1976-2005 and associated interstitial lung disease: a population-based study of incidence and mortality. BMJ open. 2013;3(11):e003569.
72. Goroshi M, Khare S, Jamale T, Shah NS. Primary Sjogren's syndrome presenting as hypokalemic paralysis: A case series. Journal of postgraduate medicine. 2016.
73. Skopouli FN, Barbatis C, Moutsopoulos HM. Liver involvement in primary Sjogren's syndrome. British journal of rheumatology. 1994;33(8):745-8.
74. Tsianos EV, Hoofnagle JH, Fox PC, Alspaugh M, Jones EA, Schafer DF, et al. Sjogren's syndrome in patients with primary biliary cirrhosis. Hepatology (Baltimore, Md). 1990;11(5):730-4.
75. Chai J, Logigian EL. Neurological manifestations of primary Sjogren's syndrome. Current opinion in neurology. 2010;23(5):509-13.
76. Locht H, Pelck R, Manthorpe R. Clinical manifestations correlated to the prevalence of autoantibodies in a large (n=321) cohort of patients with primary Sjogren's syndrome: a comparison of patients initially diagnosed according to the

Copenhagen classification criteria with the American-European consensus criteria. Autoimmunity reviews. 2005;4(5):276-81.
77. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Annals of the rheumatic diseases. 2002;61(6):554-8.
78. Brun JG, Madland TM, Gjesdal CB, Bertelsen LT. Sjogren's syndrome in an out-patient clinic: classification of patients according to the preliminary European criteria and the proposed modified European criteria. Rheumatology (Oxford, England). 2002;41(3):301-4.
79. Ramos-Casals M, Brito-Zeron P, Perez-De-Lis M, Jimenez I, Blanco MJ, Bove A, et al. Sjogren syndrome or sjogren disease? The histological and immunological bias caused by the 2002 criteria. Clinical reviews in allergy & immunology. 2010;38(2-3):178-85.
80. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjogren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & rheumatology (Hoboken, NJ). 2016.
81. Hung T, Pratt GA, Sundararaman B, Townsend MJ, Chaivorapol C, Bhangale T, et al. The Ro60 autoantigen binds endogenous retroelements and regulates inflammatory gene expression. Science (New York, NY). 2015;350(6259):455-9.
82. Bournia VK, Vlachoyiannopoulos PG. Subgroups of Sjogren syndrome patients according to serological profiles. Journal of autoimmunity. 2012;39(1-2):15-26.
83. Ramos-Casals M, Solans R, Rosas J, Camps MT, Gil A, Del Pino-Montes J, et al. Primary Sjogren syndrome in Spain: clinical and immunologic expression in 1010 patients. Medicine. 2008;87(4):210-9.
84. Vermeersch P, Bossuyt X. Prevalence and clinical significance of rare antinuclear antibody patterns. Autoimmunity reviews. 2013;12(10):998-1003.
85. Theander E, Jonsson R, Sjostrom B, Brokstad K, Olsson P, Henriksson G. Prediction of Sjogren's Syndrome Years Before Diagnosis and Identification of

Patients With Early Onset and Severe Disease Course by Autoantibody Profiling. Arthritis & rheumatology (Hoboken, NJ). 2015;67(9):2427-36.
86. Trier NH, Nielsen IO, Friis T, Houen G, Theander E. Comparison of antibody assays for detection of autoantibodies to Ro 52, Ro 60 and La associated with primary Sjogren's syndrome. Journal of immunological methods. 2016;433:44-50.
87. Kyriakidis NC, Kapsogeorgou EK, Tzioufas AG. A comprehensive review of autoantibodies in primary Sjogren's syndrome: clinical phenotypes and regulatory mechanisms. Journal of autoimmunity. 2014;51:67-74.
88. Seror R, Bootsma H, Bowman SJ, Dorner T, Gottenberg JE, Mariette X, et al. Outcome measures for primary Sjogren's syndrome. Journal of autoimmunity. 2012;39(1-2):97-102.
89. Lendrem D, Mitchell S, McMeekin P, Gompels L, Hackett K, Bowman S, et al. Do the EULAR Sjogren's syndrome outcome measures correlate with health status in primary Sjogren's syndrome? Rheumatology (Oxford, England). 2015;54(4):655-9.
90. Seror R, Bowman SJ, Brito-Zeron P, Theander E, Bootsma H, Tzioufas A, et al. EULAR Sjogren's syndrome disease activity index (ESSDAI): a user guide. RMD open. 2015;1(1):e000022.
91. Seror R, Theander E, Bootsma H, Bowman SJ, Tzioufas A, Gottenberg JE, et al. Outcome measures for primary Sjogren's syndrome: a comprehensive review. Journal of autoimmunity. 2014;51:51-6.
92. Fragkioudaki S, Mavragani CP, Moutsopoulos HM. Predicting the risk for lymphoma development in Sjogren syndrome: An easy tool for clinical use. Medicine. 2016;95(25):e3766.
93. Nishishinya MB, Pereda CA, Munoz-Fernandez S, Pego-Reigosa JM, Rua-Figueroa I, Andreu JL, et al. Identification of lymphoma predictors in patients with primary Sjogren's syndrome: a systematic literature review and meta-analysis. Rheumatology international. 2015;35(1):17-26.
94. Zintzaras E, Voulgarelis M, Moutsopoulos HM. The risk of lymphoma development in autoimmune diseases: a meta-analysis. Archives of internal medicine. 2005;165(20):2337-44.

95. Hochberg Md Mph Macp MCSM, Alan J ; Smolen Md Frcp, Josef S ; Weinblatt Md, Michael E ; Weisman Md, Michael H. Rheumatology 3rd, editor: Mosby; 2003.
96. Jung HH, Ji YS, Sung MS, Kim KK, Yoon KC. Long-Term Outcome of Treatment with Topical Corticosteroids for Severe Dry Eye Associated with Sjogren's Syndrome. Chonnam medical journal. 2015;51(1):26-32.
97. Lin T, Gong L. Topical fluorometholone treatment for ocular dryness in patients with Sjogren syndrome: a randomized clinical trial in China. Medicine. 2015;94(7):e551.
98. Li J, Zhang X, Zheng Q, Zhu Y, Wang H, Ma H, et al. Comparative Evaluation of Silicone Hydrogel Contact Lenses and Autologous Serum for Management of Sjogren Syndrome-Associated Dry Eye. Cornea. 2015;34(9):1072-8.
99. Foulks GN, Forstot SL, Donshik PC, Forstot JZ, Goldstein MH, Lemp MA, et al. Clinical guidelines for management of dry eye associated with Sjogren disease. The ocular surface. 2015;13(2):118-32.
100. Wu AJ. Optimizing dry mouth treatment for individuals with Sjogren's syndrome. Rheumatic diseases clinics of North America. 2008;34(4):1001-10, x.
101. Ramos-Casals M, Tzioufas AG, Stone JH, Siso A, Bosch X. Treatment of primary Sjogren syndrome: a systematic review. Jama. 2010;304(4):452-60.
102. Fox RI, Konttinen Y, Fisher A. Use of muscarinic agonists in the treatment of Sjogren's syndrome. Clinical immunology (Orlando, Fla). 2001;101(3):249-63.
103. Papas AS, Sherrer YS, Charney M, Golden HE, Medsger TA, Jr., Walsh BT, et al. Successful Treatment of Dry Mouth and Dry Eye Symptoms in Sjogren's Syndrome Patients With Oral Pilocarpine: A Randomized, Placebo-Controlled, Dose-Adjustment Study. Journal of clinical rheumatology : practical reports on rheumatic & musculoskeletal diseases. 2004;10(4):169-77.
104. Fife RS, Chase WF, Dore RK, Wiesenhutter CW, Lockhart PB, Tindall E, et al. Cevimeline for the treatment of xerostomia in patients with Sjogren syndrome: a randomized trial. Archives of internal medicine. 2002;162(11):1293-300.
105. Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P, Khamashta MA. Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. Annals of the rheumatic diseases. 2010;69(1):20-8.

106. Kruize AA, Hene RJ, Kallenberg CG, van Bijsterveld OP, van der Heide A, Kater L, et al. Hydroxychloroquine treatment for primary Sjogren's syndrome: a two year double blind crossover trial. Annals of the rheumatic diseases. 1993;52(5):360-4.
107. Mavragani CP, Moutsopoulos HM. Conventional therapy of Sjogren's syndrome. Clinical reviews in allergy & immunology. 2007;32(3):284-91.
108. Skopouli FN, Jagiello P, Tsifetaki N, Moutsopoulos HM. Methotrexate in primary Sjogren's syndrome. Clinical and experimental rheumatology. 1996;14(5):555-8.
109. Carubbi F, Alunno A, Cipriani P, Bartoloni E, Ciccia F, Triolo G, et al. Rituximab in primary Sjogren's syndrome: a ten-year journey. Lupus. 2014;23(13):1337-49.
110. Carubbi F, Cipriani P, Marrelli A, Benedetto P, Ruscitti P, Berardicurti O, et al. Efficacy and safety of rituximab treatment in early primary Sjogren's syndrome: a prospective, multi-center, follow-up study. Arthritis research & therapy. 2013;15(5):R172.
111. Souza FB, Porfirio GJ, Andriolo BN, Albuquerque JV, Trevisani VF. Rituximab Effectiveness and Safety for Treating Primary Sjogren's Syndrome (pSS): Systematic Review and Meta-Analysis. PloS one. 2016;11(3):e0150749.
112. Seror R, Nocturne G, Lazure T, Hendel-Chavez H, Desmoulins F, Belkhir R, et al. Low numbers of blood and salivary natural killer cells are associated with a better response to belimumab in primary Sjogren's syndrome: results of the BELISS study. Arthritis research & therapy. 2015;17:241.
113. Sandhu A, Harford A, Singh P, Alas E. Is thymoglobulin or rituximab the cause of this serum sickness? A case report of serum sickness dilemma and literature review. Case reports in medicine. 2012;2012:234515.
114. Nguyen CV, Miller DD. Serum sickness-like drug reaction: two cases with a neutrophilic urticarial pattern. Journal of cutaneous pathology. 2016.
115. Murphy K. Janeway's Immunobiology. © 2012 by Garland Science TFG, LLC, editor. New York Garland Science, Taylor & Francis Group; July 24, 2011.
116. Low WS, Wan Abas WA. Benchtop technologies for circulating tumor cells separation based on biophysical properties. BioMed research international. 2015;2015:239362.

117. Inc. TFS. Dynabeads® Human T-Expander CD3/CD2 Waltham, Massachusetts,: Thermo Fisher Scientific Inc.; 2012 [cited 2016]. Available from: https://tools.thermofisher.com/content/sfs/manuals/Dynabeads_hu_Expander_CD3CD28_man.pdf. Accessed October 21, 2016
118. Palacios R. Concanavalin A triggers T lymphocytes by directly interacting with their receptors for activation. Journal of immunology (Baltimore, Md : 1950). 1982;128(1):337-42.
119. Invivogen. 2395C lass C CpG oligonucleotide; a human/murine TLR9 ligand San Diego: Invivogen; 2016. Available from: http://www.invivogen.com/PDF/ODN2395_TDS.pdf. Accessed November 28, 2016
120. Inc. TFS. CellTrace Reagents for Cell Proliferation Waltham, Massachusetts,: Thermo Fisher Scientific Inc.; 2016. Available from: https://www.thermofisher.com/at/en/home/life-science/cell-analysis/flow-cytometry/cell-health-and-viability-assays-for-flow-cytometry/cell-proliferation-assays-for-flow-cytometry/celltrace-reagents-for-cell-proliferation.html. Accessed November 29, 2016
121. Inc TFS. The New MAGPIX Multiplexing System Waltham, Massachusetts,: Thermo Fisher Scientific Inc; 2017. Available from: https://www.thermofisher.com/at/en/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-issues-2011/bioprobes-66-october-2011/magpix-multiplexing-system-luminex.html. Accessed December 5, 2016
122. Inc TFS. How Luminex® Technology Works Waltham, Massachusetts,: Thermo Fisher Scientific Inc; 2016. Available from: https://www.thermofisher.com/at/en/home/references/protein-analysis-guide/multiplex-assays-luminex-assays/how-luminex-technology-works.html. Accessed December 6, 2016
123. Yau N. The box plot Los Angeles2017 [2007]. Available from: https://flowingdata.com/2008/02/15/how-to-read-and-use-a-box-and-whisker-plot/. Accessed December 7, 2017
124. School NP. The box plot Monterey: Naval Postgraduated School; 2017. Available from: http://faculty.nps.edu/mjdixon/styled-11/styled-13/styled-18/files/pasted-graphic.jpg. Accessed December 12, 2016

125. Mandl T, Bredberg A, Jacobsson LT, Manthorpe R, Henriksson G. CD4+ T-lymphocytopenia--a frequent finding in anti-SSA antibody seropositive patients with primary Sjogren's syndrome. The Journal of rheumatology. 2004;31(4):726-8.
126. Mingueneau M, Boudaoud S, Haskett S, Reynolds TL, Nocturne G, Norton E, et al. Cytometry by time-of-flight immunophenotyping identifies a blood Sjogren's signature correlating with disease activity and glandular inflammation. The Journal of allergy and clinical immunology. 2016;137(6):1809-21.e12.
127. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjogren's syndrome. Journal of autoimmunity. 2010;34(4):400-7.
128. Henriksson G, Manthorpe R, Bredberg A. Antibodies to CD4 in primary Sjogren's syndrome. Rheumatology (Oxford, England). 2000;39(2):142-7.
129. Tulunay A, Yavuz S, Direskeneli H, Eksioglu-Demiralp E. CD8+CD28-, suppressive T cells in systemic lupus erythematosus. Lupus. 2008;17(7):630-7.
130. Arosa FA. CD8+CD28- T cells: certainties and uncertainties of a prevalent human T-cell subset. Immunology and cell biology. 2002;80(1):1-13.
131. Lee GH, Lee WW. Unusual CD4+CD28- T Cells and Their Pathogenic Role in Chronic Inflammatory Disorders. Immune network. 2016;16(6):322-9.
132. Zabinska M, Krajewska M, Koscielska-Kasprzak K, Klinger M. CD3(+)CD8(+)CD28(-) T Lymphocytes in Patients with Lupus Nephritis. Journal of immunology research. 2016;2016:1058165.
133. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos NM, Moutsopoulos HM. Foxp3+ T-regulatory cells in Sjogren's syndrome: correlation with the grade of the autoimmune lesion and certain adverse prognostic factors. The American journal of pathology. 2008;173(5):1389-96.
134. Masuda T, Ohteki T, Abo T, Seki S, Nose S, Nagura H, et al. Expansion of the population of double negative CD4-8- T alpha beta-cells in the liver is a common feature of autoimmune mice. Journal of immunology (Baltimore, Md : 1950). 1991;147(9):2907-12.
135. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. Journal of immunology (Baltimore, Md : 1950). 2008;181(12):8761-6.

136. Alunno A, Carubbi F, Bistoni O, Caterbi S, Bartoloni E, Bigerna B, et al. CD4(-)CD8(-) T-cells in primary Sjogren's syndrome: association with the extent of glandular involvement. Journal of autoimmunity. 2014;51:38-43.
137. Anand A, Dean GS, Quereshi K, Isenberg DA, Lydyard PM. Characterization of CD3+ CD4- CD8- (double negative) T cells in patients with systemic lupus erythematosus: activation markers. Lupus. 2002;11(8):493-500.

7 Appendix

The ESSDAI score (adopted from EULAR Sjögren's task force – September 2009)

Constitutional domain [3] *Please be careful of not rating constitutional symptoms not related to the disease (such as fever of infectious origin, voluntary weight loss)*		
No activity	Absence of the following symptoms	☐
Low activity	Mild or intermittent fever (37.5°-38.5°C) / night sweats Involuntary weight loss of 5 to 10% of body weight	☐
Moderate activity	Severe Fever (>38.5°C) / night sweats Involuntary weight loss of >10% of body weight	☐

Lymphadenopathy domain [4]		
No activity	Absence of the following features	☐
Low activity	- Lymphadenopathy ≥ 1cm in any nodal region or ≥ 2cm in inguinal region	☐
Moderate activity	- Lymphadenopathy ≥ 2cm in any nodal region or ≥ 3cm in inguinal region, - and/or splenomegaly (clinically palpable or assessed by imaging)	☐
High activity	- Current malignant B-cell proliferative disorder	☐

Glandular domain [2] *Please be careful of not rating glandular swelling not related to the disease (such as stone or infection)*		
No activity	Absence of glandular swelling	☐
Low activity	Small glandular swelling with: - enlarged parotid (≤ 3cm), - or limited submandibular or lachrymal swelling[1]	☐
Moderate activity	Major glandular swelling with: - enlarged parotid (>3cm) - or important submandibular or lachrymal swelling[1]	☐

[1]Distinction between limited and important submandibular or lachrymal swelling is left to the physician judgment

Articular domain [2] *Please be careful of not rating articular involvement not related to the disease, such as osteoarthritis*		
No activity	Absence of currently active articular involvement	☐
Low activity	Arthralgias in hands, wrists, ankles and feet accompanied by morning stiffness (>30 min)	☐
Moderate activity	1 to 5 synovitis among a 28 count	☐
High activity	≥ 6 synovitis among a 28 count	☐

Cutaneous domain [3] *Please be careful of rating as "No activity" stable long lasting features that are related to damage rather than disease activity, or cutaneous involvement not related to the disease*		
No activity	Absence of currently active cutaneous involvement	☐
Low activity	Erythema multiforme	☐
Moderate activity	Limited cutaneous vasculitis, including urticarial vasculitis[2], or purpura limited to feet and ankle, or subacute cutaneous lupus	☐
High activity	Diffuse cutaneous vasculitis, including urticarial vasculitis[2], or diffuse purpura or ulcers related to vasculitis	☐

[2] Limited cutaneous vasculitis involve <18% body surface area; Diffuse Cutaneous vasculitis involve >18% body surface area

Body surface area (BSA) is defined using the rules of nines (used to assess extent of burns) as follows: Palm (excluding fingers) = 1% BSA; each lower limb = 18% BSA; each upper limb = 9% BSA; torso (front) = 18% BSA; torso (back) = 18% BSA

Pulmonary domain [5] *Please be careful of rating as "No activity" stable long lasting features that are related to damage rather than disease activity, or respiratory involvement not related to the disease, (tobacco…)*		
No activity	Absence of currently active pulmonary involvement	☐
Low activity	Persistent cough or bronchial involvement with no radiographic abnormalities on X-ray Or radiological or HRCT evidence of interstitial lung disease[3] with: • No breathlessness, • And normal lung function test.	☐
Moderate activity	Moderately active pulmonary involvement, such as interstitial lung disease proven by HRCT[3] with • shortness of breath on exercise (NHYA II) • or abnormal lung function tests restricted to: – 70% >DL_{CO} ≥ 40% and/or 80% > FVC ≥ 60%	☐
High activity	Highly active pulmonary involvement, such as interstitial lung disease proven by HRCT[3] with : • shortness of breath at rest (NHYA III, IV) • or with abnormal lung function tests: - DL_{CO} < 40% and/or FVC < 60%	☐

[3]For diagnosis of interstitial lung disease HRCT or radiography is required and must have been performed in the last 2 years

DLCO= diffusing CO capacity; FVC= forced vital capacity; HRCT= high-resolution computed tomography; NHYA= New York heart association classification;

Renal domain [5] *Please be careful of rating as "No activity" stable long lasting features that are related to damage rather than disease activity, and renal involvement not related to the disease* If biopsy has been performed, please rate activity based on **histological** features first		
No activity	Absence of currently active renal involvement: - Proteinuria< 0.5g/d, no hematuria, no leucocyturia, no acidosis. - Or long lasting stable proteinuria due to damage	☐

Low activity	Evidence of specific active renal involvement, limited to: • Tubular acidosis without renal failure (GFR[4] ⩾60ml/min) • Glomerular involvement -with proteinuria (between 0.5 and 1 g/d) -without hematuria or renal failure (GFR4 ≥60ml/min)	☐
Moderate activity	Moderately active renal involvement, such as: • Tubular acidosis with renal failure (GFR[4] < 60 ml/min) • Glomerular involvement - with proteinuria between 1 and 1.5g/d - without hematuria or renal failure (GFR4 ≥ 60ml/min) • or histological evidence of - Extra-membranous glomerulonephritis - Important interstitial lymphoid infiltrate	☐
High activity	Highly active renal involvement, such as: • Glomerular involvement -with proteinuria > 1.5 g/d -or hematuria -or renal failure (GFR[4] < 60 ml/min) • or histological evidence of -proliferative glomerulonephritis -cryoglobulinemia related renal involvement	☐

[4] Glomerular filatration rate (GFR) estimated with MDRD formula

Muscular domain [6] *Please be careful of not rating muscular involvement not related to the disease, such as weakness due to corticosteroids…*		
No activity	Absence of currently active muscular involvement	☐
Low activity	Active myositis proven by abnormal EMG or biopsy with: - no weakness - and creatine kinase (N < CK ≤ 2N)	☐
Moderate activity	Moderately active myositis proven by abnormal EMG or biopsy with: - weakness (maximal deficit of 4/5), - or elevated creatine kinase (2N < CK ≤ 4N),	☐
High activity	Highly active myositis proven by abnormal EMG or biopsy with: - weakness (deficit ≤ 3/5) - or elevated creatine kinase (> 4N)	☐

EMG= electromyogram;

Peripheral nervous system domain [5] *Please be careful of rating as "No activity" stable long lasting features that are related to damage rather than activity, or PNS involvement not related to the disease*		
No activity	Absence of currently active PNS involvement	☐
Low activity	Evidence of active peripheral nervous system involvement, such as: - - Pure sensory axonal polyneuropathy proven by NCS - - Trigeminal (V) neuralgia	☐
Moderate activity	Evidence of moderately active peripheral nervous system involvement shown by NCS, such as: - Axonal sensory-motor neuropathy with maximal motor deficit of 4/5, - Pure sensory neuropathy with presence of cryoglobulinemic vasculitis, - Ganglionopathy[5] with symptoms restricted to mild/moderate ataxia, - Inflammatory demyelinating polyneuropathy[6] (CIDP) with mild Functional impairment (maximal motor deficit of 4/5or mild ataxia), Or cranial nerve involvement of peripheral origin (except trigeminal (V) neuralgia)	☐
High activity	Evidence of highly active peripheral nervous system involvement shown by NCS, such as: - Axonal sensory-motor neuropathy with motor deficit ≤ 3/5 - Peripheral nerve involvement due to vasculitis (mononeuritis multiplex, etc…) - Severe ataxia due to ganglionopathy[5] - Inflammatory demyelinating polyneuropathy[6] (CIDP) with severe functional impairment: motor deficit ≤ 3/5 or severe ataxia	☐

NCS: Nerve conduction studies.

[5] Pure sensory impairment with ataxia and diffuse impairment or abolition of sensitive potential on NCS

[6] Polyradiculoneuropathy with suggestive clinical symptoms (4 limbs sensorimotor deficit, proximal motor deficit, generalised areflexia, initial sensory symptoms affecting the upper limbs, and/or associated involvement of cranial nerves), increased protein level and/or supportive abnormal NCS (prolonged motor distal latency, reduced nerve conduction velocity, prolonged F wave latency, conduction block and/or temporal dispersion)

Central nervous system domain [5] *Please be careful of rating as "No activity" stable long lasting features that are related to damage rather than disease activity, or CNS involvement not related to the disease*		
No activity	Absence of currently active CNS involvement	☐
Moderate activity	Moderately active CNS features, such as: - Cranial nerve involvement of central origin - Optic neuritis - Multiple sclerosis-like syndrome with symptoms restricted to pure sensory impairment or proven cognitive impairment	☐

High activity	Highly active CNS features, such as: - Cerebral vasculitis with cerebrovascular accident or transient ischemic attack - Seizures - Transverse myelitis. - Lymphocytic meningitis - Multiple sclerosis-like syndrome with motor deficit	☐

Hematological domain [2] *Please be careful :* *- considering anemia[7], thrombopenia[8] and neutropenia[9], only auto-immune cytopenia must be considered* *- not rating cytopenia not related to the disease (such as vitamin or iron deficiency, drug-induced cytopenia, as for example lymphocytopenia associated with cyclophosphamide)*		
No activity	Absence of auto-immune cytopenia	☐
Low activity	Cytopenia of auto-immune origin with: - neutropenia (1000 < neutrophils < 1500/mm3) - or anemia (10 < Hb < 12g/dl) - or thrombocytopenia (100,000 < Plt < 150,000/mm3) Or lymphopenia (500<lymphocytes<1000/mm3)	☐
Moderate activity	Cytopenia of auto-immune origin with: - neutropenia (500 ≤ neutrophils ≤ 1000/mm3), - or anemia (8 ≤ Hb ≤ 10g/dl) - or thrombocytopenia (50,000 ≤ Plt ≤ 100,000/mm3) Or lymphopenia (≤500/mm3)	☐
High activity	Cytopenia of auto-immune origin with: - neutropenia (neutrophils < 500/mm3), - or anemia (Hb < 8 g/dl) - or thrombocytopenia (Plt < 50,000/mm3),	☐

[7] Anemia with positive Coombs testing and increase reticulocyte count
[8] Thrombopenia of peripheral origin with no other etiology found, or in case of difficulty with anti-platelet auto-antibodies and/or presence of megacaryocyte on bone marrow aspirate and/or associated auto-immune anenia.
[9] Neutropenia with no other etiology found

Biological domain [1]		
No activity	Absence of any of the following biological features	☐
Low activity	- Clonal component - or hypocomplementemia (low C4 or C3 or CH50) - or hypergammaglobulinemia or IgG level between 16 and 20g/L	☐
Moderate activity	- presence of cryoglobulinemia - or hypergammaglobulinemia or high IgG level > 20g/L - or recent onset[10] hypogammaglobulinemia or recent decrease of IgG level (<5g/L)	☐

[10] In the last 6 months

SCORING METHODOLOGY:

- The score of each domain is the product of the weight of the domain by the level of activity. The domain weights are indicated in bracket for each domain.
- Activity levels should be scored as follows:
  - No activity = 0
  - Low activity = 1
  - Moderate activity = 2

    - High activity = 3
- The total score is the sum of the score of all domains.

For more information:

1: Seror R, Ravaud P, Bowman S, Baron G, Tzioufas A, Theander E, Gottenberg JE, Bootsma H, Mariette X, Vitali C. EULAR Sjogren's Syndrome Disease Activity Index (ESSDAI): Development of a consensus systemic disease activity index in primary Sjogren's syndrome. Ann Rheum Dis. 2009 Jun 28. [Epub ahead of print] PMID: 19561361.