Repurposing established drugs for new indications: leflunomide and hydroxychloroquine combination therapy for Sjögren's syndrome

Nederlandse vertaling

Nieuwe indicatie voor oude medicatie: leflunomide en hydroxychloroquine combinatie therapie voor het syndroom van Sjögren

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof …. Ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op 27 oktober om 16.15 uur

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Contents

Chapter 1

Introduction

Primary Sjögren's syndrome (pSS) is an auto-immune disease affecting approximately 0.1-0.5% of the population, mainly women (with a female:male ratio of 9:1). This debilitating disease has widespread implications for patients on several area's such as general health, professional and private life.

In patients with pSS, chronic inflammation of exocrine glands results in impaired function of those glands. As a consequence, patients experience invalidating dryness of eyes and mouth. In addition, approximately one third of the patients develop extra-glandular manifestations, whereby practically all organ systems can be involved. The dryness is often accompanied by severe fatigue, arthralgia and myalgia.

Chronic inflammation in pSS is characterized by pronounced B cell hyperactivity with subsequent auto-antibody production and in a part of the patients eventually formation of germinal center like structures in the exocrine gland. As a result, patients with pSS have a strongly increased risk of developing B-cell non-Hodgkin lymphoma (NHL), especially Marginal Zone (MZ) B-cell lymphoma of the MALT-type (mucosal-associated lymphoid tissue). The process underlying this cascade of B cell hyperactivity involves several cell types such as T-cells, B-cells, dendritic cells, monocytes/macrophages and NK cells and their effector molecules in a complex interplay (see **Chapter 2**). In addition, it has been demonstrated that several inflammatory pathways can contribute to immunopathology in pSS. Hence, possibly, targeting only one cell type or immunopathological pathway using a single drug might not be sufficient to inhibit the complex process underlying the chronic inflammation in pSS. This might be the reason that to this date, an effective therapeutic option for pSS does not exist. Therefore, the central hypothesis to this thesis is that in order to successfully inhibit the excessive immune-activation in pSS, a combination of drugs targeting both overlapping and distinct immune-pathological pathways that jointly results in broad immune inhibition, is required,.

For this purpose we considered to combine several anti-rheumatic drugs with complementary properties, able to inhibit multiple inflammatory pathways to culminate in improved disease. inhibition.

In this thesis a literature search is performed to identify anti-rheumatic drugs that target unique inflammatory pathways and two of these candidates were tested in vitro. Based on their immunopathological properties, targeting both overlapping and distinct pathways involved in pSS pathology, a combination of the cDMARDs leflunomide and hydroxychloroquine is tested as a novel therapeutic option. Furthermore, it was studied whether biomarkers such as interferon-associated markers and proteomic markers could be used to predict and monitor disease activity.

Thesis outline

This thesis provides an in depth literature survey on currently used conventional DMARDs (see **Chapter 2**). Numerous disease-modifying anti-rheumatic drugs (DMARDS), both conventional and biological DMARDs, have been tested in pSS patients with rather disappointing results. Biologicals did not outperform the conventional DMARDs (cDMARDs), and considering their high costs compared to cDMARDs it seems worthwhile to further explore the efficacy of a combination of cDMARDs. Combination of cDMARDs to achieve additive immune inhibition has proven to be a successful strategy in rheumatoid arthritis (RA).

In **Chapter 2** we provide a complete overview of the immunopathology underlying pSS and a review of the current knowledge on efficacy and safety of different treatment options. As can be concluded from this chapter, a combination of cDMARDs leflunomide and hydroxychloroquine has potential to result in immune inhibition and subsequent clinical efficacy in pSS. Leflunomide strongly targets T cells and to a lesser extent B cells, hydroxychloroquine targets activated B cells, pDCs, NK cells and ILCs and to a lesser extent T cells. Hence, combining these agents might result in additive and broad immune-inhibition involving several crucial players in pSS pathology.

Preceding a clinical trial investigating this theory, we tested the in vitro effects of leflunomide and hydroxychloroquine combination therapy on immune cells from pSS patients and healthy controls. We assessed the inhibitory effects of leflunomide, hydroxychloroquine and their combination by measurement of cell proliferation, cytokine-(IFNα, IFNγ, and CXCL13) and

immunoglobulin (IgM, IgG) production in peripheral blood mononuclear cells (PBMCs) of pSS patients and healthy controls (**Chapter 3**).

Following this in vitro study, we set to investigate the clinical efficacy and safety of leflunomide and hydroxychloroquine combination therapy in pSS patients in a randomized, double-blind, placebo-controlled trial. The results are described in **Chapter 4**. In this chapter, we also tried to develop a model that predicts clinical response in the leflunomide-hydroxychloroquine treated patients with clinically meaningful accuracy. To this end serum proteome analysis was performed at baseline and differentially expressed proteins were used to built a prediction model.

The marked upregulation of IFN induced genes and IFN-associated pathways has been indicated to be strongly linked to B cell hyperactivity and immunopathology in pSS (see **Chapter 2** and **Chapter 6**). Considering the inhibitory effect of leflunomide and hydroxychloroquine on B cell activation and pDC activity and IFN production in vitro (**Chapter 3**) we tested whether the IFN signature and IFN-associated biomarkers could serve to predict and monitor disease activity in **Chapter 5**. The IFN-associated biomarkers are assessed using both genomic and proteomic methods and are investigated in relationship to clinical efficacy upon leflunomide-hydroxychloroquine combination therapy. In this respect Galectin-9 was also studied as it is a promising IFN associated biomarker and its response to leflunomide-hydroxychloroquine therapy in pSS is highlighted. In **Chapter 6** the role of the IFN pathway and the IFN-associated biomarkers including Galectin-9 both in pSS as well as in systemic lupus erythematosus (SLE) are studied.

Chapter 7 provides a summary and discussion of the findings in this thesis. Implications and future steps are discussed in view of the current status in treatment options for pSS.

Chapter 2

Optimizing conventional DMARD therapy for Sjögren's syndrome

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Chapter overview

Primary Sjögren's syndrome (pSS) is an auto immune disorder characterized by exocrine dysfunction as a result of chronic inflammation of the glands. Part of the patients also develops inflammation in other organs. In a complex interplay of different cell types such as T-cells, B-cells, dendritic cells, monocytes/macrophages and NK cells and their effector molecules, all contribute to one of the ultimate hallmarks of pSS: B-cell hyperactivity, subsequent autoantibody production and eventually formation of germinal center-like structures in the salivary gland. Effective treatment options for this disease are currently lacking.

Biological DMARDs (bDMARDs) including those targeting B-cells or B-cell activation (directly or indirectly) have been studied, so far with limited efficacy. Besides that, their high costs provide a major drawback for implementation. Relatively inexpensive conventional DMARDs (cDMARDs) with well-known safety profiles have been shown efficacious in numerous clinical studies in multiple (rheumatic) diseases. cDMARDs target several pathways that are crucial in pSS immunopathology and some have proven to effectively inhibit B-cell hyperactivity and immune activation when given to patients. However, strong conclusions about potential efficacy are hampered by lack of standardization of inclusion criteria and outcome measures, dosing and validated biomarkers for patient selection. Proper implementation of these could help to optimize the use of cDMARDs in pSS treatment. In analogy with effective treatment strategies in for example rheumatoid arthritis, combination of two cDMARDs targeting different dysregulated pathwaysmight result in additive or synergistic inhibition of immune activation. In view of this and the unique and potent mechanisms of action to target immunopathology in pSS, optimizing cDMARDs for treatment of pSS is worthwhile.

1. Introduction

Primary Sjögren's syndrome (pSS) is the second most prevalent systemic rheumatic auto-immune disease, after rheumatoid arthritis (RA), with a prevalence of 0.5%[1] affecting mainly women (female/male ratio 9:1).[2] Hallmark of the disease is exocrine dysfunction leading to severe dryness of eyes and mouth. Although other mechanisms have been suggested, dryness to a considerable extent is the result of chronic inflammation, associated with B-cell hyperactivity, a key feature of the disease. As a consequence, B-cell non-Hodgkin lymphoma (NHL), especially Marginal Zone (MZ) B-cell lymphoma of the MALT-type (mucosal-associated lymphoid tissue), develop in 5-10% of the pSS patients.

Although chronic inflammation of the exocrine glands and dryness are key features of pSS, approximately one third of the patients also exhibits extraglandular manifestations, and involvement of practically every organ is possible.[3] In addition, invalidating fatigue is a common feature of pSS. The severe dryness with its accompanying complications and the debilitating fatigue make pSS a disease with a great impact on social, professional, and private life.

Despite an extensive search for a clinically effective treatment for common symptoms such as dryness, fatigue, myalgia and arthralgia, to this day symptomatic treatment consisting of saliva- and tear substitutes and supportive advice concerning lifestyle is the best that can be offered (except in cases of serious complications of internal organs that require strong immunosuppression). Various curative options that target the immune activation seen in pSS have been evaluated in the past, all with relatively disappointing results. Biologicals (bDMARDS, targeting specific molecules) were expected to have great potential and their efficacy is the focus of a large amount of research effort. In recent years numerous studies have been initiated to test the effects of biologicals targeting B-cells directly or indirectly by interfering with immune cells that drive B-cell hyperactivity or soluble factors that mediate their activation.[4-12] However, besides showing limited and inconsistent efficacy, lack of long-term safety assessments and their high costs provide major drawbacks in their clinical applicability in pSS.

Considering the high costs of biologicals (€10.000-€15.000 per year) versus the low costs of conventional DMARDs (cDMARDs) (in general some hundreds of euros per patient per year), evaluation of cDMARD therapy for pSS and comparison with bDMARDs using the same clinical

measures and inclusion criteria should be further explored. cDMARDs have been shown efficacious in numerous clinical studies in multiple (rheumatic) diseases and detailed safety profiles are available. Several of these drugs target pathways that are crucial in pSS immunopathology. However, their potential use in pSS is under debate yet not well studied.

This paper reviews our current knowledge on cDMARD function and use in rheumatic disease and pSS. It aims to describe the potential of cDMARD therapy for treatment of pSS, and discusses the current lack of knowledge and potential therapeutic strategies that might be applied.

2. (Targeting) immunopathology in pSS

2.1 pSS immunopathology

pSS is considered to be a multifactorial disease, in which environmental factors trigger inflammation in genetically prone individuals.[1] The pathological process is the resultant of the involvement of both the innate and the acquired immune system. The exact pathophysiological mechanisms of the disease have not been fully elucidated, but significant progress has been made in the understanding of the complex interplay between the different cell types involved and the way they become activated. Characteristic for pSS is focal infiltration of target organs with mononuclear cells, mainly CD4 and CD8 T-cells, B-cells and to a lesser extent dendritic cells (DC's), monocytes/macrophages and natural killer (NK) cells.[13] In a complex interplay these different cell-types all contribute to one of the hallmarks of pSS: B-cell hyperactivity, subsequent autoantibody production and ultimately formation of germinal center-like structures in the salivary gland.[14,15]

Genome Wide Association Studies (GWAS) in Sjögren's syndrome[16,17] support the involvement of multiple components of both the innate and adaptive immune systems. Most significantly associated with pSS is the HLA/MHC region. However, polymorphisms in six other, non-HLA loci proved to be associated with pSS. These loci are involved in the IFN pathway, TLR signaling, activation of B and T-cells and dysregulation of the NFKB pathway, which supports the existing knowledge on the pathogenesis of pSS.

The exact origin of the pathological process in pSS remains to be elucidated, but various mechanisms that initiate the inflammation in pSS patients have been suggested and experimentally supported. Thus, tissue cells and cells of the innate and adaptive immune system have been shown to be critical players in the initiation and perpetuation of the inflammatory processes in pSS. Their potential roles are discussed below.

2.2 Epithelial cells

Instead of being passive victims in the inflammatory process of pSS, salivary gland epithelial cells (SGECs) are indicated to play an active role in the pathophysiology. Epithelial cells in pSS patients express toll-like receptors (TLRs) and in response to TLR ligands produce a variety of pro-inflammatory mediators enhancing inflammation in the glands. Thus, it is assumed that epithelial cells in the salivary gland become activated by certain environmental factors such as viruses and chemical compounds.[1] Indeed, these cells were shown to overexpress MHC class II, adhesion and co-stimulatory molecules (e.g. CD40 to costimulate Th cells). Also, they secrete a variety of chemokines (CXCL13, CCL17, CCL19, CCL21 and CCL22) to recruit inflammatory cells and activate and orchestrate the inflammation.

SGECs are able to produce IL6, and in this way proved to contribute to differentiation of Tfh cells. Furthermore, the expression of ICOSL by SGECs is involved in IL21 production by Tfh cells.[18] Ultimately, epithelial cells produce cytokines such as IL-7[19], and BAFF[20,21] known to contribute to germinal center formation.[20] All together epithelial cells are indicated to be involved in recruitment of a variety of innate and lymphoid cells and by cytokine secretion, antigen presentation and co-stimulation contribute to activation and differentiation of innate and acquired immune cells.

2.3 Innate immunity in pSS

Recent studies have shown that the interferon pathway is involved in the pathophysiology of pSS.[22-25] More than half of the patients systemically exhibit a type 1 interferon signature, an upregulation of several interferon type I inducible genes in whole blood or isolated cells such as

monocytes. Locally, an upregulation of interferon type II inducible genes was demonstrated.[26] The presence of an interferon signature is associated with a higher disease-activity (ESSDAI scores), auto-antibody titers and serum IgG levels and with lower C3 levels, absolute lymphocyte and neutrophil counts.[27] The trigger for activation of the interferon pathway has not yet been identified, although some studies point towards viral infection. Epstein-Barr virus, hepatitis C virus, retroviruses and Coxsackie A virus have all been suspected, however no clear association between these viruses and pSS could be demonstrated.[28] Possibly this is due to the fact that it takes some years before clinically manifested disease has developed, whereby the original stimulus is no longer demonstrable at a later timepoint. Viral infection leading to epithelial activation and injury may lead to a release of pSS specific ribonucleoprotein auto-antigens (Ro/SSA and La/SSB) and chemokines resulting in migration of DCs and pDCs to the infected glands.[3] Viral RNA and DNA might activate TLRs and subsequent production of type I interferon by pDCs and mDCs and other myeloid cells.

Innate cells such as pDCs, cDCs, and macrophages are increased in LSG of pSS patients.[22,29-31] Upon activation of cDCs and pDCs both type I and type II interferon pathways are activated. In addition, activated conventional DCs produce IL-12, promoting activation of NK-cells and Th1 cells thereby further stimulating the production of IFN-γ, a type II IFN, which predominates in the salivary glands of pSS patients.[26] Both type I and II IFNs promote the production of B-cell activating factor (BAFF), an essential cytokine for maturation, proliferation and survival of B-cells and both BAFF and IFN type I and II play a critical role in (ectopic) germinal center formation.[32,33] Several pSS models support the important role of pDCs and cDCs in pSS immunopathology. ID3 is an inhibitory transcription factor suggested to be involved in inhibition of pDC development.[34,35] The ID3 knockout mouse spontaneously develop pSS-like symptoms.[36] Another model is the DC immunoreceptor (Dcir) knockout mouse model. Dcir is a type C lectin receptor with inhibitory function, mainly expressed in DCs. It negatively regulates DC expansion and therefore is important in maintenance of self-tolerance. Mice that are Dcir-deficient develop lymphocytic infiltrates in salivary glands accompanied by destruction of the tissue, anti-Ro/SSA and anti-La/SSB autoantibodies and increased numbers of T-cells with an activated phenotype and cDCs in lymph nodes when compared to normal mice.[37]

In the LSG of pSS patients increased numbers of NK cells are found as compared to non-Sjögren sicca patients. These innate NK cells reside mainly outside the aggregates of lymphocytic cells (foci) that are typically found in the glands of pSS patients. Also, the number of NK cells outside the foci correlates with the focus score. Several findings implicate that NK cells seem to be involved in the pathogenesis of pSS.[38] Release of autoantigens as a result of epithelial cells undergoing apoptosis could possibly lead to activation of NK cells by engagement of apoptosis-induced molecules to their activating receptors. Upon this activation, NK cells subsequently activate DCs residing in the lymph nodes, leading to T-cells being primed and executing their effector functions in the peripheral tissues.

2.4 Acquired immunity in pSS

Infiltrates found in target organs are dominated by activated T-cells (predominantly CD4 but also CD8) and B-cells. The exact composition of the infiltrate varies with the severity of the lesion.[30,39] Apart from the huge body of evidence that shows B-cell hyperactivity and autoantibody production, numerous studies indicate that T-cells may play a pivotal role in the induction and perpetuation of glandular inflammation. T-cell intrinsic defects were shown to trigger pSS-like disease. In mice with T-cells lacking class IA phosphoinositide-3-kinase (1A PI3K), an enzyme involved in regulation of proliferation and survival of among others T-cells, infiltration and destruction of lacrimal glands develops and the pSS specific autoantibody anti-SS-A is produced.[40] Possibly, loss of central and/or peripheral tolerance plays a role, however, the process of thymic negative and positive selection in this model remains to be studied.

Cheng et al[41] provide further evidence for an important role for aberrant T-cell regulation. In a mouse model with T-lymphocyte specific deletion of stromal interaction molecule (STIM) 1 and STIM2, key components that are critically involved in T-cell activation and function, the store-operated calcium entry (SOCE) and the cytokine production that is dependent on this process were heavily attenuated. This was accompanied by a decrease in number and function of regulatory T-cells. The mice spontaneously produced pSS-specific autoantibodies, developed inflammation and destruction of salivary glands, and loss of (stimulated) saliva production. In addition, they demonstrated that PBMCs from pSS patients show decreased levels of STIM1 and

STIM2 and SOCE in T-cells. In addition, minor salivary gland biopsies of pSS patients with relatively high focus scores (>3) showed a marked reduction in STIM1 expression in the infiltrating cells compared to healthy controls and patients with a low focus score.

In addition, a role for auto-reactive CD4 T-cells was demonstrated by Arakaki et al.[42] In their murine pSS model, adoptive transfer of autoreactive CD4 T-cells recognizing α-fodrin (previously shown to function as an autoantigen in the pathogenesis of pSS) induced significant cytotoxicity against salivary gland cells and the mice developed autoimmune lesions characteristic for pSS.

Also, cytokines such as IL-7 that are capable of inducing T-cell activation have been demonstrated to play a role in pSS. IL-7 is a potent pro-inflammatory cytokine primarily acting on T-cells expressing high levels of the IL7 receptor α-chain (IL-7Rα), and it induces T-cell-dependent activation of B-cells, DCs and macrophages.[43] In the labial salivary gland (LSG) specimens of pSS patients, an increased number of IL-7 producing cells and IL7R-expressing cells was found as compared to patients with sicca symptoms without any sign of auto-immunity (nSS patients), and these increased numbers correlated with the lymfocytic focus score (LFS) and increased B-cell hyperactivity in the gland.[44,45] Moreover, levels of soluble IL-7 receptor (sIL-7R), considered a biomarker of IL-7 activity, are increased in pSS patients in serum and salivary gland tissue supernatant, and correlate to markers of inflammation.[46] Interestingly, IL-7 overexpression in mice plays a pivotal role in GC formation in lymphoid organs, but also in mucosa-associated tissue such as salivary glands.[43]

Also Sjögren-like disease can be simulated by TLR3 triggering of epithelial cells inducing IL-7/T-cell-driven immune activation and Sjögren-like disease features in a mouse model of pS.[19] NK-derived IFN-g, CXCL9 and CXCL10 induced an influx of T-cells into the mouse salivary gland. At a later stage, IL-7 induced by poly I:C stimulation enhanced IFN-g production by these T-cells, resulting in amplification of CXCL9 production and thereby ongoing T-cell infiltration.

Finally, CXCR5-expresssing follicular helper T (Tfh) cells are cells specialized in providing B-cell help by promoting activation, differentiation of to plasma cells and memory B-cells, and somatic hypermutation. Increased CXCL13 production in pSS patients by follicular dendritic cells (fDCs) and epithelial cells in the salivary gland is capable to attract Tfh cells into B-cell follicles via CXCR5. Tfh cells are crucial for the formation and maintenance of GCs or GC like structures and are

increased in the glands and in peripheral blood of pSS patients.[47] They are characterized by expression of ICOS, PD-1, transcription factor Bcl-6[48], and production of IL-21. IL21, produced by Tfh and a crucial cytokine for formation of GC-like structures, was demonstrated to be elevated in serum of pSS patients and IL21 expression within the salivary glands tended to increase with higher LFS.[18,49]

pSS patients with elevated IL21 levels in the serum showed significantly higher systemic activity measured by ESSDAI.[18]

Recently, a new subset of Th cells, the so-called CCR9-expressing Tfh-like effector cells as well as the chemokine that specifically attracts them, CCL25, were found increased in blood and salivary glands of pSS patients. CCR9 Th cells were found to express high levels of IL-7Rα, and produce high levels of IFNγ, IL-17, IL-21 and IL-4, typical of pathogenic effector Th cells. Like Tfh cells, CCR9 Th cells potently stimulated B-cells and increased proportions of ICOS-expressing CCR9 Th cells were found in the circulation and salivary glands of pSS patients.[50] Indicating their potential pathogenic capacity in pSS, in mice CCR9 Th cells induced inflammation and tissue destruction in mucosa-associated tissues, including salivary glands.[51]

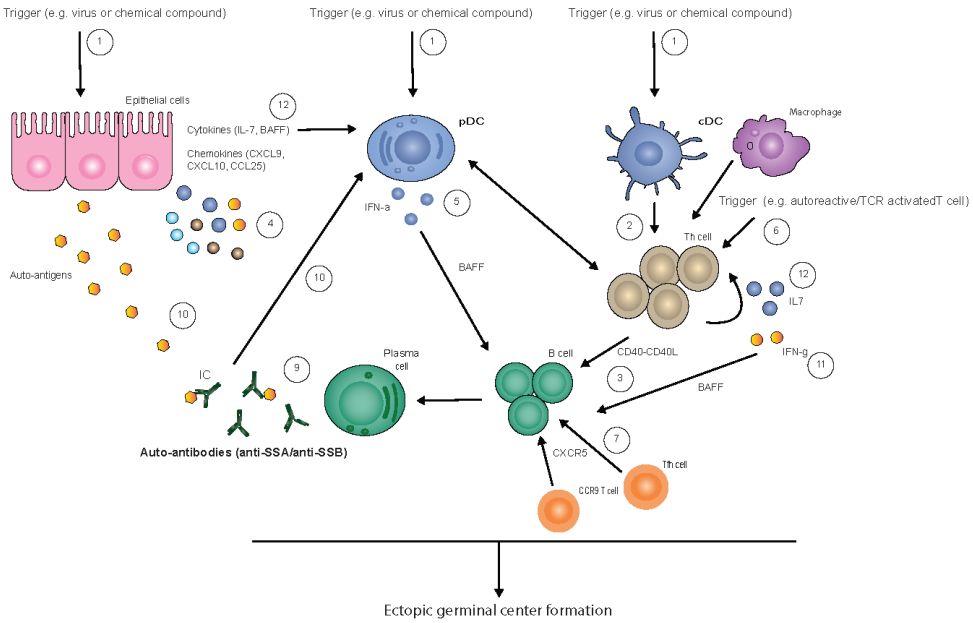

Figure 1 The initiation and perpetuation of immunopathology of primary Sjögren's syndrome involves multiple pathways that additively/synergistically trigger inflammation. Initiation of the pathological process might occur in different ways. Environmental factors such as viruses or chemical compounds can trigger activation of epithelial cells,mDCs or pDCs (1), leading to activation of Th1 cells (2) associatedwith B-cell help (via IFNg, CD40L, ICOSL upregulation) (3), production of chemokines to recruit inflammatory cells (4), contributing to the formation of lymphocytic aggregates. In addition, B-cell help comes frompDC by production of IFN-αassociatedwith BAFF induction and by activation of Th cells (5). Inflammation can also be initiated by antigen-driven (TCR) activation of resident effector T-cells or newly recruited (via chemokines CXCL9 and 10) effector Th1 cells that have been educated by antigen-primed DCs in secondary lymphoid organs (6). B-cell differentiation is specifically promoted by CXCR5 Tfh cells and CCR9 Th cells (7) that are recruited to the inflamed tissue by specific chemokines CXCL13 and CCL25, respectively (4). The formation of (auto-)antibody producing plasma cells (9) subsequently leads to further activation of antigen presenting cells (DCs and macrophages) associated with further maturation/activation of these cells e.g. via intracellular TLR stimulation (via self or non-self RNA or DNA), resulting in a self-perpetuating loop (10). Environmental triggers, e.g. a virus, damaging epithelial cells could lead to release of non-self but also self-antigens, contributing to the chronicity of the inflammation (10). IFN-γ produced by Th1 cells and cytotoxic molecules like granzymes from CD8 T-cells finally can result in significant tissue damage in the glands (11). In approximately 25% of the patients lymphocytes are ultimately organized in germinal center like structures. Increased production of IL-7 and BAFF by epithelial cells and fibroblasts play key roles in these processes and have been associated with B-cell hyperactivity, extraglandular manifestations and lymphoma development (12).

3. cDMARDs in pSS

As indicated above, cDMARDs have been shown effective in numerous clinical studies in generalized auto-immune diseases including RA, with detailed safety profiles. In addition, several of these drugs target several of the above-mentioned immunopathological pathways. In the following section, the cDMARDs that have been evaluated in relation to pSS (in vitro and animal models, case reports, clinical trials) will be discussed. Their mechanisms of action, components of the immune system that are being targeted, and their efficacy in other auto-immune diseases and in pSS are reviewed.

3.1.1 Methotrexate

Methotrexate (MTX) at a low dose has proven to be clinically effective in RA. Its broad mechanisms of actions have not yet been fully elucidated, but MTX is best known for its ability to inhibit T and B-cell proliferation. It does so by multiple mechanisms including inhibition of de novo pyrimidine and purine synthesis, increase in adenosine levels[52] causing immunosuppression by inhibition of proinflammatory cytokines such as TNF-α, downregulation of adhesion molecules and chemokines resulting in a decreased influx of leukocytes into tissues and induction of anti-inflammatory cytokines such as IL-10[53,54] , increased apoptosis of memory T-cells via fas-dependent mechanisms[55] and inhibition of the JAK-STAT signaling pathway.[56] The achieved inhibition of pro-inflammatory T-cells leads to less activation of B-cells and subsequent antibody production.

Treatment with MTX in RA patients reduced the levels of IgA-rheumatoid factor (RF) and IgM-RF.[57] It also restored the imbalance between Th17/Treg cells in PBMCs of early RA patients in an in vitro system, and it reduced the levels of Th17 effector cells from early RA patients. MTX was shown to restore the regulatory T (Treg) cell function, that is known to be defective in RA patients.[57-59]

IgG containing immune complexes, such as rheumatoid factor (RF), also present in a substantial proportion of pSS patients, potently can activate monocytes.[60] Treatment-naive RA patients, showing higher expression levels of FcγRs of peripheral blood monocytes, compared to RA patients on DMARD therapy[61], showed downregulation of expression levels of activating FcγRs

on peripheral blood monocytes after treatment with MTX. An exclusive effect of MTX on these expression levels was demonstrated in an in vitro setting.

Overall, these results indicate that MTX is a very potent immune-suppressive drug with effects on multiple pathways of both acquired and innate immune cells.

MTX applied in low dose proved to have a favorable safety profile. Most frequent side effects are nausea, mucosal ulcers, mild bone marrow depression and dose-dependent hepatotoxity reflected by mildly elevated levels of ALAT leading to cirrhosis in <1% of the cases. Frequency of side-effects can be lowered by administration of folic acid.[62-64]

3.1.2 MTX in pSS

MTX has been poorly investigated in pSS. Only empirical use, described in case-reports, and one open study have provided some information about its efficacy in pSS. Its use (often in combination with corticosteroids) has been described in case reports concerning patients with pSS-associated myelopathy[65] and inclusion body myositis associated with pSS[66] demonstrating MTX to have little to moderate clinical effects.

Efficacy and safety of MTX was studied in an open pilot study with 17 pSS patients, receiving 0.2 mg/kg body weight once a week for one year.[67] Subjective parameters, consisting of dryness of eyes and mouth, ameliorated, according to the frequency of parotid gland enlargement, dry cough and purpura. In contrast, no improvement in objective measures of dryness of eyes and mouth (Schirmer's test, saliva flow rate) was found. IgG and ESR, elevated in 82% and 65% of the patients respectively at baseline, remained unchanged.

3.2.1 Sulfasalazine

Sulfasalazine (SSZ) is a combination of sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA), linked by an azobond.[68] Alike MTX, SSZ increases extracellular adenosine, which has immunosuppressing properties as is described before, mainly targeting lymphocytes. Besides this, sulfasalazine inhibits nuclear factor kappa B (NF-KB, a central pro-inflammatory transcription factor), which is increased in pSS patients.[16,69]

SSZ was shown to inhibit in vitro TNF-α production in monocyte-derived macrophages stimulated with LPS and was associated with induction of apoptosis of the macrophages. This apoptosis was also demonstrated in vivo, using a mouse model and seemed to be a result caspase 8-induced apoptosis and TNF inhibition.[70]

Profound inhibiting effects of SSZ and of its metabolites SP and 5-ASA on IgG and IgM secretion by -cells were found in an in vitro system, in which stimulated B-cells from healthy individuals were treated with pharmacologically attainable concentrations SSZ.[71]

A meta-analysis of 15 randomized controlled trials in RA patients (including 8 trials comparing SSZ to placebo) showed that in RA patients SSZ significantly decreases ESR, duration of morning stiffness, pain and swollen joints compared to placebo.[72] In current rheumatological practice SSZ is applied mainly in combination with other cDMARDs such as MTX, hydroxychloroquine (HCQ) and leflunomide (LEF).

Common side effects of sulfasalazine are headache, gastro-intestinal complaints, dizziness and rash. Also myelodepression can occur.[73]

3.2.2. Sulfasalazine in pSS

The efficacy of SSZ in pSS-like disease was researched in New Zealand Black/New Zealand White (NZB/NZW) F1 hybrid mice, which show deviations in their salivary and lacrimal glands resembling those seen in pSS patients. SSZ treatment at 14-42 weeks or from 26-42 weeks of age did not decrease lymphocytic infiltration in the gland.[74] Other parameters such as IgG were not evaluated.

Clinical effects of SSZ have been poorly studied. Imai et al[75] describe eleven pSS patients with hypergammaglobulinemia (>30 g/L) that were treated with 1000 mg SSZ/day. Statistically significantly decreased serum levels of IgG and IgA after 8 weeks of administration were observed. Side effects were mild and restricted to skin rash in 4 out of the 11 patients.

No randomized placebo-controlled clinical studies were performed investigating the effects of SSZ in patients with primary Sjögren's syndrome.

3.3.1 Azathioprine

Azathioprine (AZA) is a pro-drug that after several conversion steps yields its active metabolites that disturb the function of enzymes essential for replication and repair and cause DNA damage. Moreover, de novo purine synthesis is inhibited. Finally, the small GTP-ase Rac1 that regulates multiple signaling pathways that amongst other things rule cell proliferation[76], is inhibited, resulting in enhanced cell apoptosis in activated T-lymphocytes. AZA was shown to modulate CD28 induced Rac1 activation in T lymphocytes in both healthy individuals and patients with Crohn's disease, resulting in enhanced apoptosis of particularly memory, but also naive T-cells.[77-81] As stated before, endothelial cells are actively involved in the chronic inflammation process. To be able to migrate through the vascular wall into the tissues, leukocytes need to adhere to endothelial cells, upon which adhesion molecules such as VCAM-1 and ICAM-1 are induced. Rac1 is crucial for the formation of these adhesion molecules, and therefore inhibition of Rac1 could reduce downstream

transcription of proinflammatory proteins.[82] Indeed, it was shown that TNF-α induced Rac1 activation in an endothelial cell line is inhibited by treatment with 6-MP and 6-T-GTP, metabolites of AZA. Also TNF-α induced NF-Kb activation was inhibited by 6-MP and 6-T-GTP. This was accompanied by a reduction of VCAM-1 expression and production of pro-inflammatory cytokines.

Taken together, azathioprine in pSS could be effective by its inhibition on T and B-cell lymphocytes. The safety profile of AZA is mainly researched in IBD patients. Most frequent side effects are myelotoxicity and hepatotoxicity and they typically occur in the first 4 months of treatment. Other side effects that can occur are gastro-intestinal complaints, alopecia. AZA treatment is associated with a potential risk of developing lymphoma in patients with IBD, however the absolute risk remains small.[83-85]

3.3.2 Azathioprine in pSS

The use of AZA in pSS is described in numerous case reports. Pragmatic use of AZA (often in combination with corticosteroids) is being applied in clinical practice for a variety of

extraglandular manifestations, including pulmonary hypertension[86,87], interstitial cystitis[88], interstitial pneumonia/interstitial lung disease[89], interstitial nephritis[90] and myelopathy.[91,92]

Yeoman et al. investigated the effect of AZA on lymphocytic focus score in submandibular and lacrimal glands, again in NZB/NZW mice. A decrease in the number of lymphocytic foci in the group of mice treated with AZA was observed. This accounted for both mice that were treated from the age of 14 weeks onwards, as for mice that started treatment at 26 weeks of age.[93]

Clinical efficacy of AZA in pSS was studied in a double blind placebo controlled trial. Twenty-five patients with pSS were enrolled in this study (12 placebo, 13 AZA). Patients received treatment with 1 mg/kg bodyweight AZA during 6 months.[94] There was no significant beneficial effect of AZA on clinical parameters, serology or histological findings. Six patients (all receiving the active drugs) withdrew from the study because of side-effects.

In conclusion, no beneficial effect on common symptoms such as dryness, fatigue, myalgia and arthralgia could be demonstrated in this study. However, as described before, AZA is used in clinical practice as a corticosteroid sparing drug to treat serious extraglandular manifestations.

3.4.1 Cyclosporine

Cyclosporine A (CyA) is a cyclic polypeptide, consisting of 11 amino acids that exerts its effect by inactivating the serine-threonine phosphatase calcineurin. This results in inhibition of activity of Nuclear Factor of Activated T-cells (NFAT) which is essential for the expression of genes encoding amongst others IL-2, IFN-γ and granulocyte-macrophage colony-stimulating-factor (GM-CSF).[95,96] This is substantially different from the upper mentioned cDMARDs that target both T and B-cells by affecting purine synthesis. CyA seems to more specifically target T-cells and T-cell-dependent responses.

In a recent study the in vitro effect of CyA on the activation of Th17 cells was assessed [97]. CyA decreased elevated levels of IL-17 and ROR-γt mRNA levels in PBMCs of pSS patients with active disease but not in PBMCs of pSS patients with inactive disease and healthy individuals. Considering the proposed role of Th1 and Th17 cells in pSS.[98,99] CyA could be an interesting treatment modality.

Peripheral T-cells co-stimulated with CD3 and CD28 and simultaneously were treated with CyA showed decreased IL-2 expression and thereby less proliferation by inhibition of nuclear translocation of NF-AT and NF-κB p65/RelA.[100]

(Reversible) nephrotoxicity is the most important side effect of CyA. Dose-related elevation of creatinine levels occurred in 48% of the patients in a study involving 154 RA patients treated with CyA. Other side effects that were frequently reported were new onset hypertension, gastro-intestinal complaints, headache and hypertrichosis.[101]

3.4.2 Cyclosporine in pSS

Efficacy and safety of CyA in pSS patients in vivo were evaluated in a double blind, placebo-controlled trial with 20 pSS patients.[102] Ten patients were treated for six months with a CyA dosage of 5 mg/kg bodyweight, versus ten that received placebo. Significant clinical improvement in the CyA-treated group was restricted to xerostomia, this was not seen in the placebo group. All other subjective and objective parameters did not significantly change after treatment in both groups (Table 1). Histological deterioration was observed in the placebo group, while the CyA treated group showed unaltered histological lesions after six months of treatment. This suggests that CyA retarded the natural course of the disease. Serum IgG levels were not evaluated.

Topical use of CyA has been investigated quite extensively. However, inclusion in most studies was not restricted to pSS patients but also patients with undefined dry eyes disease were incorporated. Sall et al[103] reported on two identical randomized, double-blind vehicle-controlled trials investigating efficacy and safety of CyA cyclosporine A 0.05% and 0.1% ophthalmic emulsion compared to vehicle emulsion. A total of 877 patients was included, 30% being diagnosed with pSS based on presence of oral symptoms, ocular symptoms and a Schirmer test ≤5 mm as well as the presence of rheumatoid factor, antinuclear antibodies, anti-Ro/SSA or anti-La/SSB autoantibodies. Significantly greater improvements in both the 0.05% as in the 0.1% CyA group compared to the vehicle group were observed for the objective measures corneal staining and categorized Schirmer values, but not for tear break-up time. Subjective parameters blurred vision, use of lubricating eye drops and the physician's evaluation of global response to treatment showed a greater improvement

for the group treated with 0.05% CyA emulsion compared to the group treated with vehicle. This study was followed by an open-label extended study[104] which enrolled 412 patients with dry eyes disease (% of patients diagnosed with pSS is unknown), all treated with CyA 0.1% ophthalmic emulsion during three extension periods of 12 months each in order to assess long-term safety. Topical CyA treatment proved to be safe. Mainly mild to moderate side effects were reported, only one was infectious of nature.

3.5.1 Leflunomide

Leflunomide (LEF) is an immune-suppressive drug, structurally not related to other immune-suppressants. It is an isoxazole derivate, which becomes active in vivo after conversion to its active form (A77 1726) by opening of the isoxazole ring. LEF is a known inhibitor of dihydro-orotate dehydrogenase (DHODH), the rate-limiting enzyme of de novo biosynthesis of pyrimidines, leading to inhibition of proliferation of B-cells and both naive and memory CD4 T-cells.[105]

In B10.A mice that were immunized with a T-cell dependent and a T-cell independent antigen, LEF inhibited both T-cell dependent and T-cell independent B-cell antibody production, indicative of direct inhibition of B-cell responses.[106] This was due to the capacity of LEF to inhibit B-cell proliferation by blocking cell cycle transition from G1 to S phase and from entering G2/M phase. Thus, B-cell antibody production is reduced by LEF by blocking expansion of antibody-secreting cells.

A non-cell specific inhibitory effect of LEF on activation of the transcription factor NF-ƙB was demonstrated in vitro.[107] Acute human T-cell leukemia cells (Jurkat cell line), pre-treated with LEF, were stimulated with different activators of NF-ƙB. Activation of NF-ƙB induced by these agents was remarkably reduced in the LEF pre-treated cells compared to the non-treated cells, indicating that LEF exerts its effect on a common step in the transduction pathway. The inhibition of NF-ƙB activation was not restricted to Jurkat cells but was also demonstrated in different cell types (myeloid (U0937), epithelial (HeLa) and glioma (H4)). By preventing translocation of NF-ƙB to the nucleus LEF influences downstream (inflammation-associated) gene expression.

3.5.2 Leflunomide in pSS

Clinical effectiveness of LEF in patients with pSS was investigated by Van Woerkom et al. in an open label pilot study.[108] Fifteen patients diagnosed with early pSS and showing active disease were treated with LEF 20 mg once a day for 24 weeks. Modest clinical improvement was observed. After 24 weeks of treatment, patients reported less 'general fatigue', a subscore from the Multidimensional Fatigue Inventory (MFI). Mean values of serum IgG, IgA and IgM levels decreased significantly from 8 weeks onwards compared to baseline measurement. Also, a significant reduction in RF levels was seen after 24 weeks of treatment. There was a tendency towards increased mean values for the Schirmer's test. Repeated labial gland specimens in 5 of the 15 patients showed a decrease of 1 focus/4mm2 in 4 of them. The remaining one patient exhibited an increase of 1 focus/4mm2. Moreover, 3 patients with leucocytoclastic vasculitis prior to start of the study showed a remarkable improvement of their vasculitic purpura after treatment.

Side-effects were restricted to mild gastro-intestinal symptoms, alopecia, transient increase of liver enzymes (ALAT), aggravation of pre-existent hypertension, mild leucopenia and skin-lesions well responding to topical corticosteroid therapy.

Cytokine analysis of circulating cells of 13 of these LEF-treated patients was performed.[109] LEF suppressed TNFα and IL1β production by PBMC from pSS patients after 24 weeks of treatment. In addition, serum concentrations of IFN-g, IL-2, TNF-α, IL-6 and IL-10 decreased after treatment. CD40L expression on CD4 T-cells also was suppressed by LEF treatment. Serum IgG, IgM, IgA levels and autoantibody production were significantly reduced. This could possibly be due to a direct effect of LEF on B-cell activity, but also due to reduced T-cell activation as can be deduced from the decreased levels of T-cell cytokines IFN-g and IL-2, the reduced expression of CD40L (costimulatory molecule enhancing B-cell maturation, important in formation of germinal centers) and B-cell activating cytokines IL-6 and IL-10.

Of the 13 LEF treated patients, 7 patients were considered clinical responders, based on 50% or more improvement of two out of three disease domains (ocular dryness, oral dryness and laboratory parameters). These responders, unlike the non-responders, showed a significant decrease of the production of T-cell associated cytokines IFN-γ and TNF-α in PBMCs upon

stimulation with CD3/CD28, indicating that LEF-induced inhibition of T-cell activation contributes to disease improvement.

3.6.1 Hydroxychoroquine

Hydroxychloroquine (HCQ) is historically used as an anti-malarial and[110] several mechanisms have been proposed that possibly lead to immune inhibition by HCQ. By increasing pH within lysosomes, lysosomal degradation is inhibited and the process of autophagy, important for the synthesis of new macromolecules and creating a source of energy, is blocked, thereby creating a less optimal environment for cell proliferation.[111] Alteration of lysosome function in T-cells affects their capacity to degrade material that has been phagocytosed. The alteration in endocytic pH also affects cytokine production, and results in reduced production of e.g. IL-1, IL-6 and TNF.[112] Wallace et al showed long-lasting suppressive effects of HCQ on IL-6 levels in patients with SLE.[113]

HCQ mainly targets antigen-presenting cells such as dendritic cells and monocytes, concentrations of the drug are higher in these cells. Antigen presentation is decreased by HCQ, since MHC II molecules after synthesis are moved to the endocytic compartments. This mainly affects binding of low affinity self-peptides instead of antigenic pathogen derived peptides.[114]

HCQ inhibits TLR mediated immune responses and subsequent induction of type I interferon and other pro-inflammatory cytokines. The mechanisms behind this inhibition are not yet fully elucidated. Two possible mechanisms have been proposed. First of all, the alteration of endosomal pH could lead to dysfunctioning of endosomal TLRs (non-competitive mechanism). Proper functioning of TLRs is pH dependent, requiring an acidic environment. By raising the endosomal pH, HCQ could possibly interfere with the functional transformation of the TLRs that is needed for their activation.[112] Also a competitive mechanism could be underlying inhibition by HCQ. Kuznik et al showed that anti-malarials directly interact with nucleic acid TLR ligands in vitro. As a result of this interaction, structural alterations of the nucleic acid occur, preventing its binding to and subsequent activation of TLRs.[115] The capacity of HCQ to inhibit TLR mediated immune responses makes it a cDMARD with unique characteristics compared to other cDMARDs.

HCQ has a favorable safety profile. Common side effects of HCQ are gastro-intestinal complaints, rash and alopecia. A rare but serious adverse effect is retinopathy. The incidence of HCQ retinopathy is very low in the first 5 years of usage (approximately 0.3%), and increases slightly after this time period.[116]

3.6.2 Hydroxychloroquine in pSS

Cholinesterase is an enzyme that degrades the parasympathic neurotransmitter acetycholine (ACh), whose binding to cell surface muscarinic type-3 receptors is a critical step in the initiation of fluid secretion by salivary acinar cells. Elevated cholinesterase levels diminish production of saliva and thus salivary gland hypofunction. The effect of HCQ on cholinesterase was studied. Salivary cholinesterase levels were determined in 9 pSS patients and 9 healthy individuals. Cholinesterase activity was significantly elevated in the pSS patients and HCQ in clinically attainable levels decreased this cholinesterase activity in vitro. Additional studies have to be performed to confirm this result In vivo.[117]

When compared to pSS patients not receiving any treatment, pSS patients treated by HCQ showed lower levels of MxA, which is a functional biomarker indicative of IFN type 1 activity.[118]

Several clinical trials investigated the effect of HCQ in patients with pSS.[119-123] In 1993, Kruize et al performed a double-blinded, placebo-controlled cross-over trial in 19 pSS patients with high disease activity parameters witnessed by high sIgG levels (mean 20.4 g/L), ESR (mean 32.5 mm/h).[119] One group was treated with 400 mg HCQ once a day during 1 year, the second year they received placebo. The other patients had the same treatment, but in reverse order. No significant effect of HCQ on tear production or salivary scintigraphy was seen. Patients did not experience any difference during treatment with hydroxychloroquine of placebo. However, HCQ significantly reduced IgG and IgM levels and there was a trend towards a decrease in ESR.

Recently, the clinical effect of HCQ in pSS patients was investigated in a double-blind, placebo-controlled trial of 120 patients with low disease activity (ESSDAI median 2.5 and 2.0 for placebo group and treated group resp, sIgG mean 14.2 g/l and 14.5 g/L for placebo group and treated group resp).[120] Patients were randomized and allocated to HCQ or placebo treatment until week 24. From week 24 to 48, all patients received HCQ. HCQ did not improve the most commonly

present symptoms, being dryness of eyes and mouth, arthralgia/general pain and fatigue. ESR, IgM and IgG levels were reduced but these reductions did not reach significance.

4. Optimizing DMARD therapy for pSS

Data on cDMARDs from both experimental and clinical studies in multiple autoimmune diseases demonstrated unique and overlapping immunosuppressive properties. Several cDMARDs in relation to primary Sjögren's syndrome were reviewed in this literature study, showing that knowledge on effectivity of cDMARDs in pSS is quite limited. It is difficult to draw strong and definite conclusions about the potential efficacy of cDMARDs in pSS, for several reasons.

First of all, standardized outcome measures were lacking in the past. All studies performed to investigate the efficacy of the different cDMARDs used different outcome measures, resulting in results that are difficult to compare. Only recently the EULAR Sjögren's syndrome disease activity index (ESSDAI), a standardized measure for systemic disease activity in pSS patients was introduced[124], providing the opportunity to standardize studies and compare results of the different treatment options that are studied. In addition, other measures to indicate effects on disease activity or immune activation are often lacking. Immunoglobulin levels for example, an important hallmark of disease activity and immune activation in pSS, often are not evaluated in these studies.

In line with the lacking knowledge on efficacy of cDMARDs in pSS, little is known about the optimal dosing of these drugs in this disease. Possibly other dosage regimes (higher or lower) should have been used in the studies that have been conducted so far. Usage of suboptimal study designs makes it hard to evaluate the efficacy of cDMARDs in pSS. Double-blind, placebo-controlled trials are lacking for most drugs and dosing-studies have not been performed. E.g. for MTX, only one open clinical study was performed, using a moderate dosage regimen (0.2 mg/kg/wk, corresponding to 14 mg/wk for a patient with an average weight of 70 kg) [67]. Better effects might be reached using higher doses, however this will have to be tested. Of interest in this respect, in RA patients optimal disease inhibition was found at a dosage of 20 mg/wk.[125] A minimal dose of 15 mg/week for oral administration and 12 mg/week for parental administration is recommended.[126] In view of its good safety profiles higher dosages of MTX or combinations

with other cDMARDs or biologicals could be worthwhile to investigate. Likewise overdosing might occur. Recently our group observed that suboptimal dosages of HCQ (10-30-fold lower than reported mean serum concentrations) extremely potently inhibit in vitro production of IgG and IgM levels by antigen/TLR stimulated PBMCs.[127] This implies that lower concentrations of HCQ than currently used in clinical practice might be sufficient to control B-cell hyperactivity and subsequently B-cell associated immunopathology such as lymphoma and extra glandular manifestations, thereby minimalizing the risk on side effects.

In addition to the lack of knowledge on optimal dosing of cDMARDs in pSS, also safety profiles of the various drugs in patients with pSS are not investigated thoroughly. This is related to the fact that only a few (small) double-blind placebo-controlled trials are performed investigating efficacy of cDMARDs. AZA seems to have a relatively unfavorable safety profile in patients with pSS. Relevant myelotoxicity, hepatotoxicity and increased risk of lymphoma may hamper its suitability as a treatment modality for pSS. In a clinical study investigating AZA therapy in pSS, 6 out of 13 patients using the active drug withdrew because of side effects. LEF and HCQ, also investigated in a double-blind placebo controlled trial, proved to have favorable safety profiles. Common side effects of LEF are typically mild and include gastro-intestinal symptoms, alopecia, transient increase of liver enzymes (ALAT), aggravation of pre-existent hypertension, mild leucopenia and skin-lesions. For HCQ, common side effects are restricted to gastro-intestinal complaints, rash and alopecia. The risk on retinopathy is small especially in the first five years of usage.

Another drawback in comparing results of cDMARD treatments is lack of standardized inclusion criteria. Inclusion criteria between studies investigating cDMARDs were different; making comparison with recently conducted clinical trials difficult. For example this has resulted in selection of patient groups that were comprised of patients with rather stable disease and low to moderate activity at moment of enrollment. Logically, this can hugely influence the effect of the investigated treatment. For example, in a recent RCT[120] investigating HCQ in pSS, HCQ did not improve the most commonly present symptoms. ESR, IgM and IgG levels were reduced although not significant. It is important to realize that it was the goal of this study to investigate the clinical efficacy of hydroxychloroquine on symptoms that are widespread under pSS patients but are not indicative of current disease activity. As a result, mainly patients with low or mild disease activity

were enrolled (ESSDAI median 2.5 and 2.0 for placebo group and treated group resp). In this cohort, mean IgG, IgM and IgA levels at baseline were within the normal range, and thus not likely to decrease upon treatment with HCQ. Sub analyses using several clinical characteristics were performed, including a subdivision of patient groups with low or elevated IgG levels (≥12.6 g/L), observing no differences between HCQ and placebo groups. However, in our opinion, this cut-off point for defining hypergammaglobulinemia was set relatively low, since IgG levels below 16 g/L are considered normal. One could speculate that patients with higher levels of immunoglobulins might have shown significant reductions. In line with this assumption HCQ did significantly reduce serum Ig levels in

patients that were included based on higher baseline serum Ig levels[119] (figure 2). As such, stratifying patients on the basis of these immunoglobulin values might have led to overlooking important effects of HCQ immune activation, which can have important implications for effects on long-term risks.

The before mentioned example indicates that no clear consensus exists on the clinical and immunological parameters that should lead to proper evaluation of efficacy of the different treatment options. In this respect it might be wise to target and evaluate different clinical outcomes separately. In particular since hallmarks of the disease such as dryness, fatigue, immune activation and clinical symptoms/disease activity as captured eg by Clinical ESSDAI (ClinESSDAI) are poorly correlating. Considering that dryness, often chosen as main outcome, has not been significantly influenced by any of the current systemic treatments, such an approach might open up ways for selective treatment modalities. In this respect, besides immunoglobulin levels, immunological outcomes of the different cDMARDs in pSS have been poorly studied. Nonetheless, if dryness is disregarded as main outcome, but for example immunoglobulin levels as a reflection of disease activity are evaluated, cDMARDs strikingly show equal results within patients with hypergammaglobulinemia compared to bDMARDS (figure 2). Given the association of B-cell hyperactivity with severe disease in particular on the long-term this is an important observation.

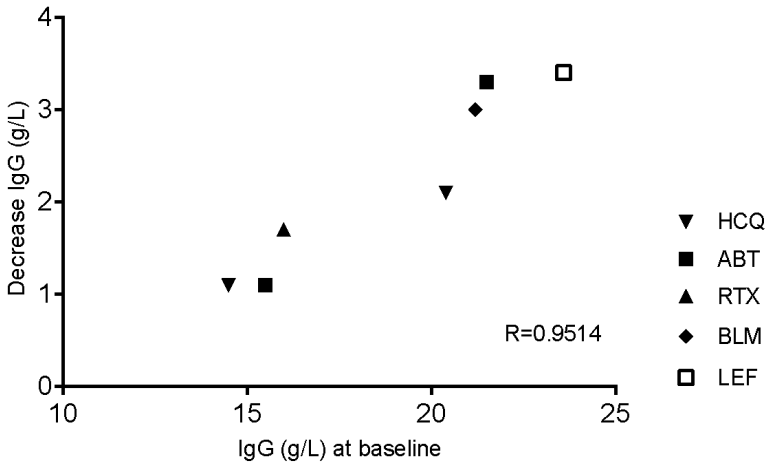

Figure 2. Clinical trials of bDMARDs and cDMARDs demonstrate inhibition of B-cell hyperactivity. Inhibition of serum IgG levels after treatment with hydroxychloroquine (HCQ), abatacept (ABT), rituximab (RTX), belumimab (BLM) and leflunomide (LEF) are shown. Correlation (r) between IgG level at baseline and decrease of IgG level is indicated (p = 0.001). cDMARDs HCQ and LEF perform equal to biologicals ABT, RTX and BLM. References: HCQ [120] [119]; ABT [7] [5]; RTX [8]; BLM [10]; LEF [108].

In addition to the above-mentioned drawbacks, biomarkers to predict response to therapy but also to monitor therapy efficacy are lacking. In this respect promising results were shown in a double-blind, placebo-controlled trial in SLE patients investigating efficacy of IFN-α receptor blocking monoclonal antibody Anifrolumab. This study showed a significantly greater response rate in patients with a high IFN-signature at baseline compared to IFN-signature negative patients. Considering the important role of IFNs in pSS pathology, in particular on B-cell hyperactivity and severe disease[128], the presence of an IFN-signature might hold promise in pSS patients as a biomarker predicting response to IFN-inhibiting therapy, possibly also cDMARDs like HCQ which potently targets the type I IFN response. In the light of the recent association between IFN-α signatures and immunoglobulin levels it is tempting to speculate that HCQ - as inhibitor of TLR signaling - is effective in diminishing long-term disease risk in those individuals with increased immunoglobulin levels. Biomarkers could also be used to monitor efficacy of therapy. An open-label study investigating efficacy of LEF in pSS patients[108] showed that LEF significantly inhibited T-cell produced IFN-γ only in the group of pSS patients that was classified as clinical responders,

thereby validating the supposed inhibitory effect of LEF on T-cells secreting type II IFNs[109]. Possibly biomarkers like type I and II IFNs could serve as a biomarker to monitor therapy efficacy in pSS and provide tools to treat patients with tailored immunotherapy.

For improved and patient-tailored therapies it is important that novel tools are implemented and developed to improve classification of patients on the basis of their molecular profile, and to monitor cellular and molecular changes systemically and at the site of inflammation. To this end novel state of the art techniques such as CYTOF and epigenetic cell counting have proven useful to monitor a multitude of cell subsets at the site inflammation.[129,130] At the same time molecular profiling of key cell types has shown molecular aberrances in pDCs, mDC, monocytes, B-cells and T-cells of pSS patients that will be crucial to understand changes at the cellular level.[131,132] These techniques have the potential to reveal previously undiscovered aberrances in both immune and tissue cells, dissecting molecular pathways that are related to different immunopathological features. Likewise novel tools to assess the proteome at the site of inflammation and in the circulation, such as the salivary gland secretome analysis[46,133] and multi-analyte technologies assessing thousands of proteins and autoantibodies will provide the necessary information to understand the complex disease processes underlying pSS. Increased comprehension through computational approaches that integrate the multiomic datalayers constitutes a systems medicine approach to perform a desired in depth molecular patient taxonomy. As our knowledge about the molecular pathways underlying diseases progresses, repurposing of existing drugs seems a necessary and promising step. New insights into mechanisms of disease force medical researchers and physicians to look beyond conventional drugs that are indicated for a certain disease and to investigate other drugs prescribed for other diseases based on the dysregulated molecular pathways. Investigating cDMARD therapy for pSS is a perfect example of exploring the possibility to repurpose old drugs for a new indication, based on progressive insights on molecular pathways underlying the disease.

Numerous studies have shown synergism between multiple biological processes and different cell types. In pSS patients we recently showed synergism between IL-7- induced CD4 T-cell activation and TLR-7-induced B-cell activation, leading to enhanced Th cell cytokine and B-cell immunoglobulin production. The presence of antigen presenting cells (monocytes/macrophages

in this case) was proven to be critical in this.[134] Considering the fact that potential additive pathways are upregulated in pSS patients (eg. increased IL-7/IL7R[44] and TLR7 pathways[135]) it is anticipated that molecular and cellular synergism between the different key cell subsets in pSS will occur and might be of great importance. Hence it seems reasonable to conclude that targeting pathways affecting T-cell and B-cell activation, and at the same time also DC activation could be essential for successful treatment of pSS. Considering the different mechanisms of action of the different cDMARDs, combination therapy with two complementary cDMARDs might be worthwhile. MTX, SSZ, LEF and CyA, based on their shared immunomodulatory effects and favorable safety profiles, all are good candidates to be combined with a DMARD with a distinct mechanism of action, in order to target distinct immunopathological pathways in pSS pathology. The ability of HCQ to inhibit this and

TLR-mediated immune responses that are suggested to induce IFNs and B-cell hyperactivity, makes HCQ an attractive DMARD with unique properties as compared to other cDMARDs. HCQ mainly targets antigen-presenting cells such as DCs, monocytes/macrophages and also B-cells, pivotal cells that play crucial roles in driving and sustaining T and B-cell responses.

The combination of LEF, targeting mainly activated T-cells and to a lesser extent B-cells, and HCQ, targeting mainly B-cells and TLR-mediated processes, is a promising option that we recently tested in vitro. In a culture system mimicking pSS pathology by TCR/TLR9 stimulation of PBMCs of pSS patients, a combination of LEF and HCQ complementarily inhibited T and B-cell proliferation, production of IFN-α, CXCL13, IgG and IgM in clinically relevant concentrations. IFN-γ production persisted however additive inhibition was seen using a substantial concentration of HCQ in combination with a clinically relevant concentration of LEF.[127] These results indicate that a combination of LEF and HCQ inhibits the activity of the important players in pSS pathology, namely DCs, T and B-cells. Moreover, the positive clinical results of these separate cDMARDs in pSS are encouraging. It is striking how the clinical results of LEF and HCQ resemble those of the biologicals Rituximab, Abatacept and Belimumab (table 1). When considering safety and clinical experience of these drugs in pSS treatment, a combination of LEF and HCQ seems a good therapeutic option and is currently being explored in a double blind placebo-controlled trial (EudraCT 2014-003140-12).

5. Conclusion

pSS is a multifactorial disease with an immunopathology that is the resultant of an interplay between tissue cells and both the innate and the acquired immune system. Several cDMARDs target many of the immunopathological pathways involved in pSS with unique mechanisms of action and therefore are potentially effective in inhibiting pSS immunopathology. Efficacy of cDMARDs in pSS is poorly studied and deserves proper comparison with bDMARDs by applying the same inclusion criteria and clinical outcome measures and by designing well-controlled clinical (dose finding) studies and employing biomarkers to stratify patients to optimize treatment efficacy. In this respect targeting multiple immunopathological pathways by combining complementary cDMARDs could be a promising strategy. The results of a clinical study in pSS investigating such a combination (leflunomide and hydroxychloroquine), with comparable inclusion and response criteria as used for bDMARDs, are underway. Clinical results of these cDMARDs separately show promising results on clinical parameters, in particular inhibition of B-cell hyperactivity, comparable to those of recently tested biologicals. Considering their harmonizing mechanisms of action, it is hypothesized that a combination will result in improved inhibition of disease activity and immune activation. If positive

this might set the stage for combination trials of cDMARDs for treatment of pSS and might renew the interest in cDMARDs as therapeutic options for pSS, optimizing their efficacy.

	Ref	ESSDAI	ESSPRI	LFS	Oral dryness (subjective)	Ocular dryness (subjective)	Saliva prodution	Schirmer	ESR	CRP	IgG
MTX	Skopouli et al [63]	NI	NI	NI	Improved	Improved	No change	No change	No change	NI	No change
AZA	Price et al [90] RCT	NI	NI	NI	No change	No change	NI	No change	No change	NI	No change
CYA	Drosos et al [98] RCT	NI	NI	Stable in CyA group, worse in placebo group	Significant improvement compared to placebo	No change	No change	No change	NI	NI	NI
LEF	Van Woerkom et al [104] Open study	NI	NI	Improved in 4 out of 5 patients	No change	No change	No change	Trend towards increase	No change	No change	Significant decrease
HCQ	Kruize et al [115] RCT	NI	NI	NI	No change	No change	NI	No change	Trend towards decrease	NI	Significant decrease
	Gottenberg et al [116]	No change	No change	NI	No change	No change	No change	No change	Significant decrease	NI	No change
ABT	Meiners et al [5] Open study	Significant decrease	Significant decrease	NI	NI	NI	No change	No change	NI	NI	Significant decrease
	Adler et al [7] Open study	NI	NI	Significant decrease	No change	No change	Significant increase(*)	NI	NI	NI	No change
BLM	Mariette et al [10] Open study	Significant decrease	Significant decrease	NI	Significant improvement (dryness in general)	Significant improvement (dryness in general)	No change	No change	NI	NI	Significant decrease

RTX+ pred	Devauchelle-Pensec [8] RCT	No change	NI	No change	No change	No change	No change	No change	No change	No change	Significant decrease
	Carubbi et al [6] Prospective follow-up study (**)	Significant decrease	NI	Significant decrease	Significant improvement (dryness in general)	Significant improvement (dryness in general)	Significant increase	Significant increase	NI	NI	No change
	Dass et al [130] RCT	NI	NI	NI	NI	NI	No change	No change	NI	NI	No change
	Meijer et al [4] RCT	NI	NI	NI	Significant improvement	Significant improvement	Significant increase	No change	NI	NI	Significant decrease

Table 1. Clinical and immunological effects of different treatment options in pSS.

ABT, abatacept; AZA, azathioprine; BLM, belumimab; CRP, C-reactive protein; CYA, cyclosporine A; ESR, erythrocyte sedimentation rate; HCQ, hydroxychloroquine; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; LEF, leflunomide; MTX, methotrexate; NI, no information; pred, prednisone; RTX, rituximab; SSZ, sulfasalazine.

* Only reached significance when corrected for disease duration.

** Results were compared to cDMARDs (HCQ, MTX or CYA): DMARD therapy also caused significant improvement in dryness and ESSDAI score.

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Chapter 3

Additive immunosuppressive effect of leflunomide and hydroxychloroquine supports rationale for combination therapy for Sjögren's syndrome

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Abstract

Objective Effective treatment for primary Sjögren's syndrome (pSS) is not available. pSS immunopathology involves a variety of immune-cells and dysregulated pathways, targeting several pathways instead of only one could therefore be effective. Treatment with leflunomide (LEF) and hydroxychloroquine (HCQ) might be successful given their unique immunosuppressive properties. In this chapter, we aimed to study the *in vitro* effects of LEF, HCQ and their combination on T and B-cell proliferation, cytokine and immunoglobulin production by activated PBMCs.

Methods PBMCs of six healthy individuals and nine pSS patients were stimulated with superantigen and TLR9 agonist to mimic the hallmark features. LEF, HCQ and their combinations were tested at clinically observed concentrations and proliferation, cytokine and immunoglobulin production were measured.

Results TCR/TLR9 activation of PBMCs induced strong proliferation of T and B-cells and production of CXCL13, IFN-α, IFN-γ, IgG and IgM. LEF dose-dependently inhibited all measured parameters, where HCQ potently and dose-dependently decreased B cell proliferation, CXCL13, IFN-α, IgG and IgM production. At different concentration combinations HCQ and LEF inhibited several immune hallmark features more potently than each single compound.

Conclusion A combination of LEF and HCQ at clinically applicable concentrations additively inhibits immune activation, supporting a potential implementation of this drug combination in pSS treatment.

1. Introduction

Despite an extensive search for a clinically effective treatment for primary Sjögren's syndrome (pSS), to this day standard care comprises only symptomatic treatment with saliva- and tear substitutes to relieve the hallmark symptoms of pSS, severe dryness of eyes and mouth. Even though the precise immunopathological processes of pSS remain to be elucidated, evolving knowledge in recent years has prompted many efforts to target one or more of these processes specifically, either by biological DMARDs (bDMARDs), small drug molecules or repurposing existing drugs like classical synthetic DMARDs (csDMARDs).[1]

The salivary glands of patients with pSS are infiltrated by a range of immune cells, mainly CD4 and CD8 T-cells, B-cells, and to a lesser extent dendritic cells (DCs), monocytes/macrophages and NK-cells.[2] A complex interplay between these cells and their effector molecules results in chronic inflammation with B cell hyperactivity, auto-antibody production and ultimately formation of ectopic germinal centers.[3,4] Reflecting B cell hyperactivity, ~30% of pSS patients develops extraglandular manifestations and 5-10% of patients with pSS develop B-cell lymphoma, in particular those patients with high numbers of lymphocytic foci or germinal centers.[2,5-7]

A clear role for the interferon pathway has been shown in pSS.[8-11] The presence of an interferon signature is associated with B cell hyperactivity and clinical parameters.[12] Systemically, an interferon type I is present in approximately 55% of pSS patients. This type I IFN signature has been attributed to production of type I IFNs (eg IFN-α) by plasmacytoid DCs (pDCs). In target organs, a predominant upregulation of interferon type II inducible genes was demonstrated[13], which is associated with the abundant presence of IFN-γ-producing T cells.[14] IFN-α induces Tfh cells expressing Bcl6 and triggers B cell activation.[15] Interestingly, recent studies demonstrate that also IFN-γ is a critical inducer of germinal center formation and autoimmunity. Furthermore, pathogenic Tfh cells co-express IL-21 and IFN-γ.[16-18]

Ectopic germinal centers, that develop in 25-30% of pSS patients[3-5], exhibit functional characteristics of lymphoid germinal centres, including B cell affinity maturation and clonal selection[3]. Presence of GCs in pSS is associated with high disease activity and strongly increased risk of lymphoma.[5,19] CXCL13 and its unique receptor CXCR5 are crucial in formation, function

and maintenance of GCs by regulating homing and tissue localization of CXCR5-expressing Tfh cells and B cells. In pSS patients, CXCR5 Tfh cells in peripheral blood and labial salivary gland (LSG) are increased and in LSG CXCR5 Tfh cells correlate with percentages of memory B cells and plasma cells. In line with this observation, the degree of cellular organization of the lymphocytic aggregate is positively associated with the CXLC13 expression. These data implicate a critical role for the CXCL13/CXCR5 axis in B cell hyperactivity and GC formation in pSS patients.[4]

The involvement of different immune cells and effector mechanisms in pSS pathology indicates that targeting multiple immunopathological pathways could lead to an improved inhibition of disease activity. Combining csDMARDs with overlapping but also distinct anti-inflammatory activities has proven to be a successful strategy in for instance rheumatoid arthritis. In pSS, the csDMARDs leflunomide (LEF) and hydroxychloroquine (HCQ), when given as monotherapy, inhibited immunological parameters in clinical trials[20,21], although suboptimal clinical effects were seen for these monotherapies[20-22]. Considering their complementary mechanisms of action, a combination of LEF and HCQ could have beneficial effects on immunological and clinical parameters in patients with pSS.

Leflunomide (LEF) is an isoxazole derivate that, after in vivo conversion to its active form (A77 1726), becomes biologically active. It inhibits dihydro-orotate dehydrogenase (DHODH), which is the rate limiting enzyme of de novo synthesis of pyrimidines causing inhibition of (CD4) T cell proliferation.[23] In mice, LEF inhibits both T cell-dependent and T cell-independent B cell antibody production by blocking cell cycle transition, thereby limiting the expansion of antibody producing cells.[24]

Hydroxychloroquine (HCQ), initially used as an anti-malarial drug, also exhibits immunomodulatory effects. HCQ mainly targets antigen presenting cells such as dendritic cells and monocytes, illustrated by the fact that after exposure these cells have higher intracellular concentrations of the drug.[25] Antigen presentation is decreased by HCQ, as MHC class II molecules are moved to the endocytic compartments after synthesis. This mainly affects binding of low affinity self-peptides instead of antigenic pathogen-derived peptides.[25] HCQ inhibits activity of pDCs, which are the strongest producers of type I IFN, and of B cells, by inhibiting Toll

Like receptor (TLR)-mediated responses.[25,26] This is due to the HCQ-mediated raise of endosomal pH leading to dysfunction of endosomal TLRs. By raising endosomal pH, HCQ interferes with the functional transformation of the TLRs needed for their activation.[27] Also, HCQ directly interferes with nucleic acid TLR ligands, resulting in structural alterations of the nucleic acid and prevention of its binding to TLRs.[28] In addition, HCQ inhibits autophagy, resulting in less cell proliferation.[29]

Considering the distinct immune cells and effector mechanisms targeted by LEF and HCQ and the potential for their combined use, we here assessed the in vitro effects of a combination of these drugs on TCR/TLR-activated PBMCs. We show that the combined use of LEF and HCQ leads to strong additive inhibition of T and B cell proliferation, resulting in decreased production of T follicular helper (Tfh)-related cytokine CXCL13, type I and type II IFNs and immunoglobulins.

2. Materials and methods

2.1 Patients

Nine pSS patients who fulfilled the American-European Consensus Group classification criteria for primary Sjögren's syndrome[30] as well as six healthy individuals were included. Characteristics of the patients and healthy controls are given in Table 1. The study was performed according to the regulations of the medical ethical committee of the University Medical Centre Utrecht. All patients and healthy individuals gave their written informed consent.

Table I. Characteristics of the controls (n=6) and patients (n=9)

Age (mean, SD)	59.3 (16.5)	40.0 (12.3)
Female (n, %)	9 (100)	5 (83.3)
Disease duration (mean, SD)	18.2 (11.0)	N.a.
ESSDAI (mean, SD)	6.2 (4.7)	N.a.
SSA and/or SSB positivity (n, %)	8 (88.9)	N.i.
Serum IgG g/L (mean, SD)	21.6 (11.6)	N.i.

Table 1. Characteristics of the six healthy controls and nine pSS patients. ESSDAI, EULAR Sjögren Syndrome Disease Activiy Index. SSA, anti-Sjögren's-syndrome-related antigen A; SSB, anti-Sjögren's-syndrome-related antigen B

2.2 Cell culture

Mononuclear cells (MCs) from patients and controls were isolated from heparinized peripheral blood (PB) by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MC isolation, PB was diluted 1:1 in PBS. Peripheral blood mononuclear cells (PBMCs) were stimulated with superantigen Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, Zwijndrecht, the Netherlands), CPG-C ODN-M362 (Invivogen, San Diego, USA) and their combination, in the presence or absence of clinically relevant concentrations of HCQ (Sigma-Aldrich) and A77 1726 (MedChemExpress, Monmouth Juntion, USA), the active metabolite of LEF. For analyses of cytokine production and cell proliferation, supernatant and cells were harvested after four days of culture. For analyses of immunoglobulin production, supernatants were harvested after 10 days of culture. Culture supernatants were rendered cell free and stored at -80C prior to analysis.

2.3 Flow cytometry

Proliferation of T cells and B cells was assessed, using the Cell Trace Violet technique (Life technologies, Carlsbad, California, USA). This intracellular dye dilutes out following every cell division and is measured by Fluorescence-activated cell sorting (FACS). For analysis of cytokine production and proliferation, a viability dye staining (Fixable Viability Dye eFluor©780, Thermo Fisher, Waltham, USA) was added to exclude cell death as an explanation for inhibition of read-outs. To distinguish cell subsets in flow-cytometry analyses, the following antibody panel was used: CD3 V500 (BD Biosciences), CD4 PerCP-Cy5.5 (Sony Biotechnology), CD45ROPE-Cy7 (BD Bioschiences), CD19 AF700 (eBioscience), CD14 FITC (Miltenyi) and CD25 BV711 (BD Biosciences). Data were analyzed using FlowJo (version 10).

2.4 Measurement of cytokines and immunoglobulins

Cytokine production was measured using an in-house developed and validated multiplex immunoassay as described previously.[31] IgG and IgM levels in the supernatants taken after ten

days of culture were determined by ELISA according to manufacturer's instructions (Bethyl Laboratories, Montgomery, USA).

2.5 Statistics

To compare groups and conditions, the paired T-test was used. To test the additive effects of LEF and HCQ, paired T-tests were applied. Differences were considered to be statistically significant at $p<0.05$.

3. Results

Stimulation of PBMCs with SEB and TLR-9L induces strong proliferation of T and B cells, as well as production of cytokines and immunoglobulins

To study the combined effects of LEF and HCQ in vitro, we sought to set up a culture system that reflects activation of multiple immune cell types and effector molecules as observed in patients with pSS. As SEB strongly induces T cell receptor-mediated T cell activation in a large proportion of T cells and TLR9 ligand CPG-C is a robust activator of B cells and pDCs, we investigated whether the combination of these stimuli resulted in activation of these three cell subsets. After four days of culturing PBMCs in the presence of both SEB and TLR9 ligand, we observed strong proliferation of both T cells and B cells (figure 1A). This was true for cells from both healthy controls and patients with pSS and there were no apparent differences between patients and controls (figure 1B). In line with the robust T and B cell proliferation after four days, high levels of Tfh-related cytokine CXCL13 and type I and II interferons IFN-α and IFN-γ, were measured upon activation. After ten days of culture, we observed strongly increased levels of IgG and IgM production upon stimulation (figure 1C). Judging from the strongly enhanced proliferation and production of IFNs and immunoglobulins, this culture system was used as a model to study the inhibitory effect of LEF and HCQ on activation of T cells, B cells and pDCs.

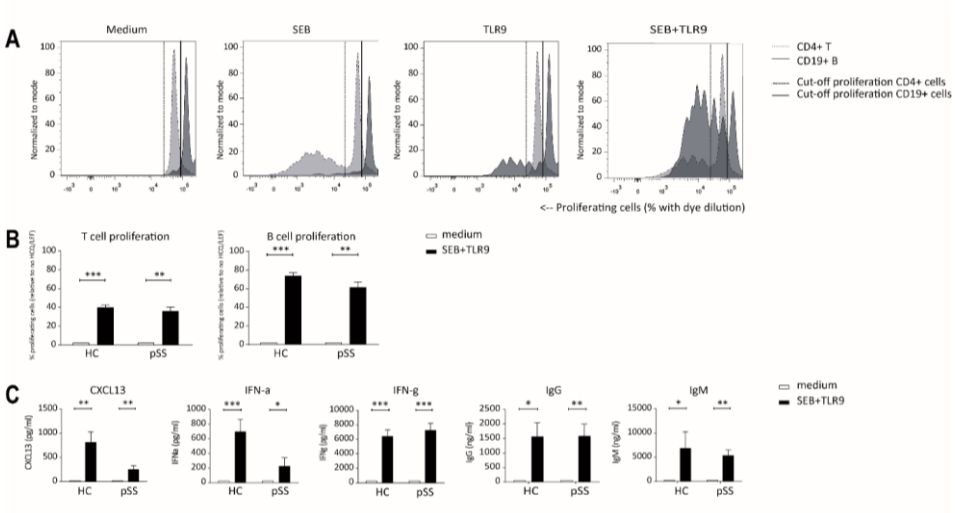

Figure 1. In vitro model to assess efficacy of csDMARDs to inhibit lymphocyte proliferation, cytokine- and immunoglobulin production. (a) Induction of both T- and B-cell proliferation upon TCR stimulation with SEB en TLR9 stimulation with CpG-C. % proliferating T cells and B cells are indicated in light grey and dark grey, respectively. (b) Induction of T and B cell proliferation and (c) cytokine and immunoglobulin production upon stimulation with SEB and TLR9in healthy controls (HC) and pSS patients. *, **, *** indicate statistical significant differences of p<0.05, 0.01 and 0.001.

3.1. HCQ and LEF distinctly inhibit lymphocyte proliferation, cytokine and Ig production

We next investigated the dose dependency of LEF or HCQ to inhibit activation of PBMCs cultured with SEB and TLR-9L. Several concentrations of either cDMARD were tested on cells from both patients and controls. For both drugs, the highest condition was chosen based on mean serum levels that are seen in patients treated with clinically usual dosages. For LEF, mean serum levels are approximately 30-40 mg/l[21,32] (this corresponds to 111-148 µM) and a minimum concentration of 13-16 mg/l (48-59 µM) is considered minimally required for clinical response.[32] Therefore, 100 µM was chosen as the highest concentration. Studies in SLE patients suggest a minimum effective blood concentration of 500 ng/ml (1.15 µM) of HCQ, however others claim that a minimum of 1000 ng/ml (2.3 µM) is required for therapeutic efficacy.[33,34] Therefore, the optimal therapeutic range of HCQ remains to be determined.[33] However, a great variability is

seen in serum HCQ concentration among individuals, more than a 10-fold range of drug concentrations was found in patients all receiving similar doses.[34] Therefore, a concentration of 4300 ng/ml (this corresponds to 10 µM) was chosen as the highest condition.

HCQ by itself potently inhibited B cell proliferation (figure 2A), IFN-α, CXCL13 (figure 2B), and immunoglobulin production (figure 2C) in a dose dependent manner. T cell proliferation was only inhibited at the highest concentration of HCQ. LEF alone dose-dependently inhibited proliferation of T and B cells, production of cytokines CXCL13, IFN-α and IFN-γ and production of IgG and IgM.

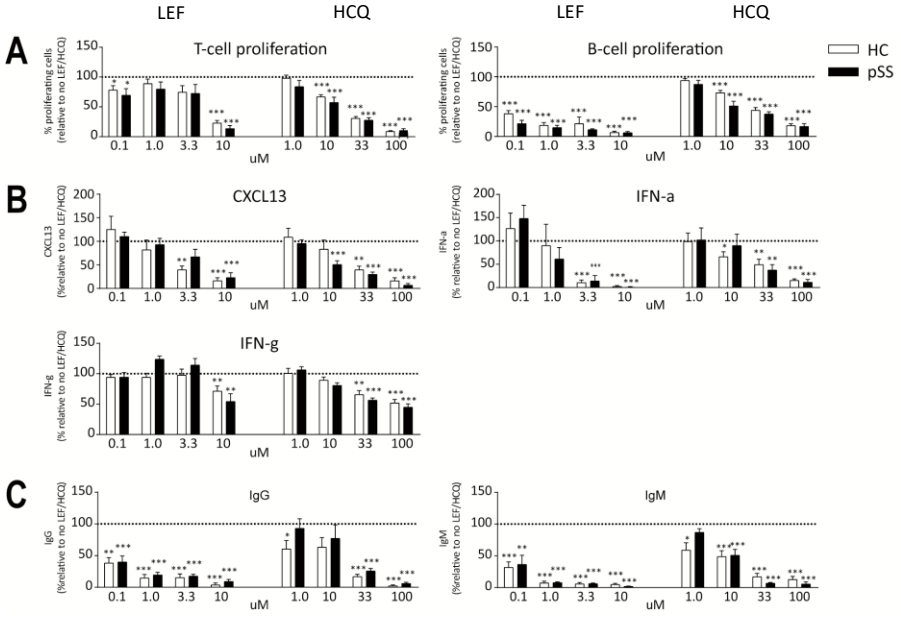

Figure 2. Inhibition of proliferation, cytokine and immunoglobulin production by HCQ and LEF in HC and pSS-patients. HCQ alone potently inhibited B cell proliferation (A), IFN-α, CXCL13 (B), and immunoglobulin production (C) in a dose-dependent manner. T cell proliferation was only inhibited at the highest concentration of HCQ. LEF alone dose-dependently inhibited proliferation of T and B cells (a), production of cytokines CXCL13, IFN-α and IFN-γ (b) and production of IgG and IgM (c). *, **, *** indicate statistical significant differences of $p < 0.05$, 0.01 and 0.001.

3.2. Suboptimal concentrations of the HCQ/LEF combination additively inhibit T cell proliferation, B cell proliferation and CXCL13 production (in pSS patients) and inhibit IFN-α and immunoglobulins

Next, we investigated the effects of the combination of csDMARDs in this culture system. We tested different combinations of LEF and HCQ in a checkerboard setup and determined the concentration at which optimal additive inhibition of T and B cell proliferation was observed. At a concentration of 3.3 µM HCQ and 10 µM LEF, which are submaximal dosages of each drug, T cell and B cell proliferation were additively inhibited in HCs and pSS patients, compared to the addition of either HCQ or LEF alone (figure 3A).

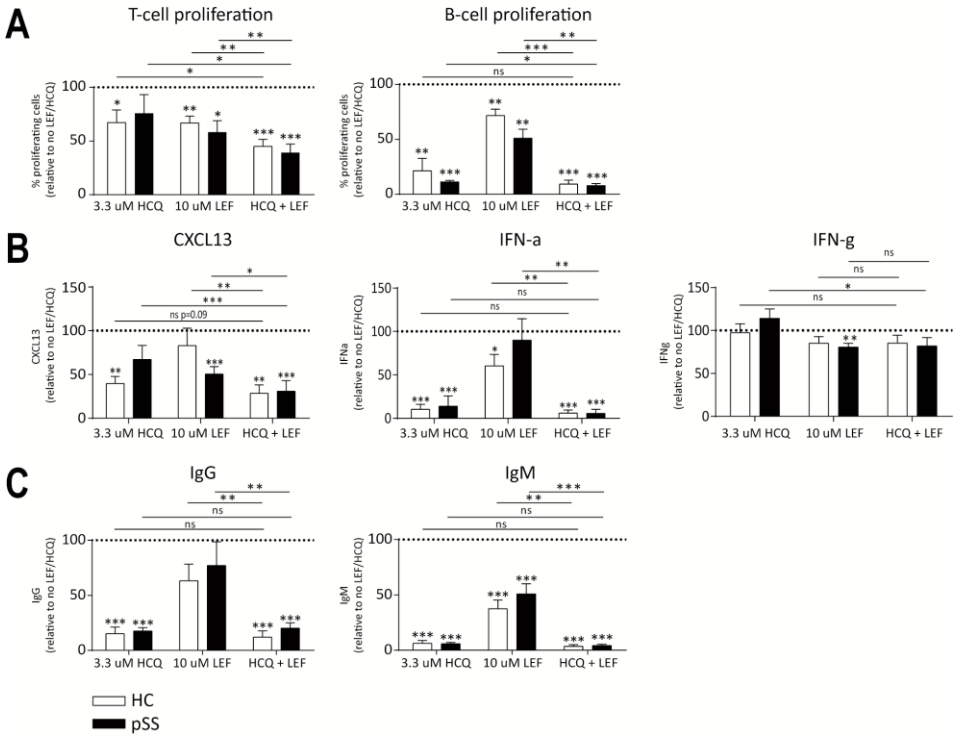

Figure 3. Additive inhibition of proliferation and CXCL13 production and robust inhibition of Igs and IFN-a. Additive inhibition of T- and B-cell proliferation and production of CXCL13 and robust inhibition of IgG, IgM, and IFN-a is achieved by suboptimal concentrations of HCQ and LEF in HC and pSS patients. (a): T cell proliferation is additively inhibited by HCQ and LEF both in HC and in pSS patients. Combination of HCQ and LEF leads to profound inhibition of B cell proliferation (b): Robust inhibition of CXCL13 and IFN-a production in HC and pSS patients. IFN-g production remains unchainged (c): IgG and IgM are strongly inhibited by HCQ and LEF in HC and pSS patients.
*, **, *** indicate statistical significant differences of $p < 0.05$, 0.01 and 0.001.

In pSS patients, CXCL13 production was also strongly and additively inhibited by the combination of 3.3 µM HCQ and 10 µM LEF. A trend towards additive inhibition was seen in HCs, but statistical significance was not reached. The combination of 3.3 µM HCQ and 10 µM LEF induced robust inhibition of IFN-α, but no additive effect was observed since HCQ alone already induced strong almost complete inhibition.

Inhibition of IFN-γ was seen at a concentration of 10 µM HCQ (clinically relevant concentration) and 33 and 100 µM LEF (suboptimal and clinically relevant concentration respectively) (figure 2A, B). At 3.3 µM HCQ and 10 µM LEF inhibition of IFN-γ production was absent or only minor. For both IgG and IgM, the combination of 3.3 µM HCQ and 10 µM LEF strongly reduced production, but this was not significantly different from HCQ alone since this by itself strongly diminished Ig production (figure 2B, C).

Concentrations of LEF and HCQ that optimally inhibited lymphocyte proliferation did not show significant additive effects on cytokine and immunoglobulin production, because HCQ had a very strong effect by itself. As such, we investigated whether suboptimal concentrations of HCQ would show more pronounced additive effects on Ig production. Using a concentration of 0.1 µM HCQ and 33 µM LEF significant additive inhibition of IgG for both healthy individuals and pSS patients was observed. This was also seen in pSS patients for IgM production, although in healthy individuals only a trend towards additive inhibition was seen. Similar results were seen for combinations of LEF with 1.0 µM HCQ (data not shown).

3.3. Optimal concentrations of LEF and HCQ additively inhibit production of IFN-γ and immunoglobulins

The combination of the highest concentrations of the two drugs, 10 µM HCQ and 100 µM LEF, resulted in significant additive and profound inhibition of IFN-γ (figure 4).

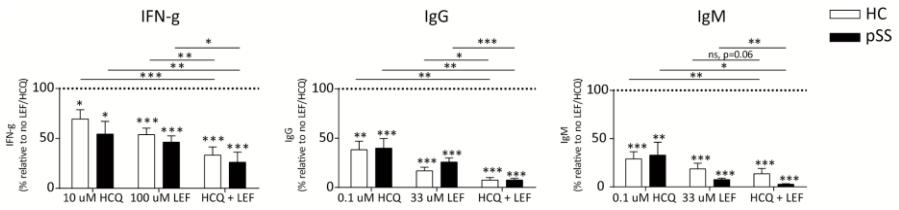

Figure 4. Additive inhibition of IFN-γ and B cell activity (IgG and IgM). Additive inhibition of IFN-γ and immunoglobulins is achieved using either higher (IFN-γ) or suboptimal (IgG and IgM) concentrations of HCQ and LEF.
*, **, *** indicate statistical significant differences of $p < 0.05$, 0.01 and 0.001.

Cell death was measured in all conditions in patients and healthy donors and did not significantly differ between donors and patients. In none of the conditions cell death exceeded 8% on average (figure 5) not correlating with immune inhibitory effects, thus excluding cell death as a cause for inhibition of proliferation, cytokine and immunoglobulin production.

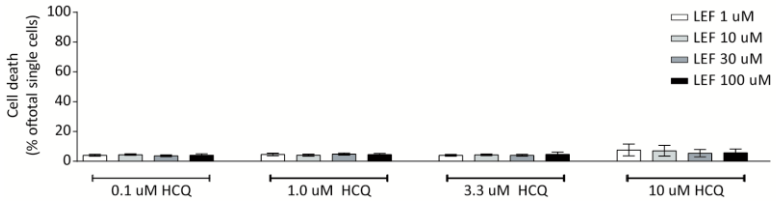

Figure 5. Cell death induced by LEF and HCQ is minimal and does not correlate with immunosuppressive effects . Mean cell death did not differ between HC and pSS patients (mean plus SEM, pooled data of n=3 HC and n=3 pSS patients are shown) and was only minimal even at the highest concentrations of LEF and HCQ, not correlating with immune inhibitory effects.

4. Discussion

Both HCQ and LEF separately inhibit T and B cell activation, with HCQ extremely potently inhibiting B cell activation and LEF more potently inhibiting T cell activation. At different combinations of HCQ and LEF, additive inhibition of in vitro proliferation of T and B cells, production of Tfh-associated cytokine CXCL-13, IFN-γ, and B cell activity was observed.

Dosages of HCQ and LEF were chosen based on serum levels seen in patients treated with the usual dosage of these drugs, with the highest concentration *in vitro* set as the highest concentration observed *in vivo* and subsequent testing of lower concentrations. With respect to HCQ, studies in SLE patients suggest a minimum effective blood concentration of 500 ng/ml (1.15 μM)[33], others claim that a minimum of 1000 ng/ml (2.3 μM) is required for therapeutic efficacy.[33,34] Our data show that at 3.3 μM HCQ *in vitro* robustly inhibits B cell activity, IFN-α and to a lesser extent CXCL13 production. Also at the lower clinically suggested concentration (1 μM) HCQ in vitro extremely well inhibits B cell activation. This is line with the observed effect of HCQ[20] on sIgG levels in pSS patients that resembles those of the biologicals Rituximab[35], Abatacept[36,37] and Belimumab[38]. Interestingly, our study shows that HCQ in much lower concentrations, even up to 0.1 μM, is capable of inhibiting B cell activation. Since at this concentration no inhibition of T cell proliferation, CXCL13, IFNγ or IFNα production was observed HCQ seems to target B cells independent of these processes.

We demonstrate that both LEF and HCQ inhibit T and B cel proliferation and production of CXCL13, IFN-α, IFN-γ and immunoglobulins IgG and IgM, all of which are important contributors to pSS pathology, albeit with different potencies. LEF dose-dependently inhibits proliferation of T- and B cells, CXCL13, IFN-α, IFN-γ and immunoglobulins IgG and IgM. HCQ in a dose-dependent manner inhibits proliferation of B cells, production of CXCL13, IFN-α and immunoglobulins, however T cell proliferation and IFNγ were inhibited much less potent, only at the highest HCQ concentration.

For the first time we showed that a combination of LEF and HCQ induced complementary and additive inhibition of TCR/TLR9 activated immune activation. We observed the most robust additive effects on B and T cell proliferation and CXCL13 using suboptimal dosages of LEF and

HCQ. In addition, optimal inhibition of IFN-α and B cell activity reflected by IgG and IgM production was achieved at these concentrations. Hence, the key pathways involved in pSS pathology are successfully targeted by this combination.

Recently it was shown that IFN-γ plays a critical role in GC formation and induction of autoimmunity. IFN-γ was one of the more persistent cytokines in our in vitro model. Significant inhibition of IFN-γ production required higher concentrations of HCQ (10 μM) and LEF (100 μM), concentrations that in vivo can be easily reached for LEF, but less easy for HCQ when patients are treated with the usual dosage. A combination of 10 μM HCQ and 100 μM LEF did result in significant and additive inhibition of IFN-γ production. Trends for additive inhibition were observed when using a lower concentration of HCQ, but did not reach statistical significance. This suggests that the local IFN-γ production in pSS could persist despite broad immune inhibition with HCQ and LEF in clinically relevant concentrations, requiring higher concentrations of HCQ and LEF.

Despite huge efforts to successfully inhibit disease activity in pSS, results of numerous clinical studies, including those testing biologicals such as rituximab, have been rather disappointing[1,39], and new promising drugs require confirmation in larger studies. Notwithstanding, treatment with for example Rituximab has been demonstrated to lead to biological effects, including mitigation of T and B cell activation, glandular inflammation, formation of ectopic lymphoid structures and B cell hyperactivity (reflected by reduction of serum IgG and RF) .[40-43] Still, inhibition of B cell hyperactivity may only be partially, insufficient to fully tackle immunopathology and disease activity.[35,44,45] Interestingly, upon investigation of the biological effects of LEF/HCQ combination therapy in a double-blind randomized controlled clinical trial we recently performed (EudraCT 2014-003140-12) we found an unprecedented strong inhibition of B cell hyperactivity as reflected by reduction in serum IgG.[46] This was associated by significant inhibition of several clinical measures, including the primary outcome, the ESSDAI score[47] (ms submitted for publication). Whether LEF/HCQ combination therapy truly culminates into higher clinical efficacy remains to be confirmed in larger clinical trials.

5. Conclusion

Our data show that the combination of LEF and HCQ potently inhibits several hallmark immune responses *in vitro* that are indicated to play key roles in immunopathology of pSS. To confirm these favourable *in vitro* results, we are currently investigating the clinical and immunological effects of combination therapy with HCQ and LEF in pSS patients. Publication of the results of this double-blind, randomized controlled clinical trial is eagerly awaited.

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Chapter 4

Leflunomide-hydroxychloroquine combination therapy in patients with primary Sjögren's syndrome (RepurpSS-I): a placebo-controlled, double-blinded, randomised clinical trial

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Abstract

Background Primary Sjögren's syndrome is a systemic autoimmune disease characterised by secretory gland dysfunction, for which no effective therapy is available. Based on the complementary properties of leflunomide and hydroxychloroquine in inhibiting activation of key immune cells in primary Sjögren's syndrome, we aimed to evaluate the clinical efficacy and safety of leflunomide–hydroxychloroquine combination therapy in patients with primary Sjögren's syndrome.

Methods We did a placebo-controlled, double-blinded, phase 2A randomised clinical trial in patients with primary Sjögren's syndrome at the University Medical Center Utrecht (Utrecht, Netherlands). Eligible patients were aged 18–75 years, had a European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index (ESSDAI) score of 5 or higher, and a lymphocytic focus score of 1 or higher in labial salivary gland biopsy specimens. Patients were randomly assigned (2:1) with block randomisation (block size of six) to receive leflunomide 20 mg and hydroxychloroquine 400 mg daily or placebo for 24 weeks. The primary endpoint was the between-group difference in change in ESSDAI scores from 0 to 24 weeks, adjusted for baseline ESSDAI score. Patients were analysed according to the intention-to-treat principle. This study is registered with EudraCT, 2014–003140–12.

Findings Between March 7, 2016, and Nov 30, 2017, 37 patients were screened, of whom 29 patients (28 women and one man) were enrolled. 21 patients were assigned to receive leflunomide–hydroxychloroquine and eight patients were assigned to receive placebo. One patient in the placebo group required high-dose prednisone to treat polymyalgia rheumatica at week 13 and was excluded from the primary analysis. From 0 to 24 weeks, the mean difference in ESSDAI score, adjusted for baseline values, in the leflunomide–hydroxychloroquine group compared with the placebo group was –4.35 points (95% CI –7.45 to –1.25, p=0.0078). No serious adverse events occurred in the leflunomide–hydroxychloroquine group and two serious adverse events occurred in the placebo group (hospital admission for pancreatitis and hospital admission for nephrolithiasis). The most common adverse events in the leflunomide– hydroxychloroquine group were gastrointestinal discomfort (11 patients [52%] *vs* two [25%] in the placebo group),

modest transient increases in alanine aminotransferase (ten [48%] *vs* one [13%]), and short episodes of general malaise and shivering (nine [43%] *vs* one [13%]).

Interpretation Leflunomide–hydroxychloroquine was safe and resulted in a clinical response in patients with primary Sjögren's syndrome. These results warrant further evaluation of leflunomide–hydroxychloroquine combination therapy in larger clinical trials.

Introduction

Primary Sjögren's syndrome is a chronic autoimmune disease characterised by lymphocytic infiltration and functional impairment of exocrine glands, leading to dryness of eyes and mouth. Extraglandular manifestations occur in approximately one-third of patients with primary Sjögren's syndrome, involving a wide range of organs, and 5–10% of patients develop B-cell lymphomas.[1] The immunopathological mechanisms in primary Sjögren's syndrome have only partially been elucidated, but an understanding of the complex interplay between the different cell types involved is progressing rapidly. Target organs are infiltrated with mononuclear cells, mainly CD4+ and CD8+ T cells, B cells, dendritic cells, monocytes and macrophages, and natural killer cells.[2] The coordinated actions of these cells result in immune activation that drives the prominent B-cell hyperactivity seen in primary Sjögren's syndrome patients, leading to increased serum IgG levels, auto-antibody production, immune-complex formation, and complement activation.[3,4] To date, no effective treatment for primary Sjögren's syndrome is available, despite the many clinical trials that have tested the efficacy of both conventional and biological disease-modifying antirheumatic drugs (DMARDs).[5] The absence of clinical efficacy in trials to date is probably explained in part by the vast redundancy of the immune system.[6] Therefore, we hypothesised that simultaneous targeting of several cell types and inflammatory pathways involved in primary Sjögren's syndrome immunopathology is a prerequisite for potent immune inhibition and effective treatment. To test this hypothesis, we proposed treatment of patients with primary Sjögren's syndrome with a combination of two classical synthetic DMARDs: leflunomide and hydroxychloroquine.

Leflunomide and hydroxychloroquine target both overlapping and distinct immunopathological pathways. The primary mechanism of leflunomide is the inhibition of pyrimidine biosynthesis, which affects the proliferation of naive and memory CD4+ T cells and B cells[7]. Leflunomide also inhibits activation of the transcription factor NF-κB, which drives the expression of pro-inflammatory cytokines and mediators.[8] Hydroxychloroquine increases endosomal pH, interfering with signaling via endosomal Toll-like receptors (TLRs), including TLRs 3, 7, 8, and 9. These endosomal TLRs recognise endogenous nucleic acids, such as those contained within immune complexes and apoptotic cell particles, resulting in activation of primarily plasmacytoid

dendritic cells and B cells and perpetuation of the immune response in primary Sjögren's syndrome.[9–11] Inhibition of this pathway profoundly reduces the production of type I interferons and other inflammatory cytokines.[12] In previous clinical trials in patients with primary Sjögren's syndrome,[13,14] both leflunomide and hydroxychloroquine monotherapies were shown to be safe and to lead to inhibition of immune activation, including B-cell activation. Despite these immunosuppressive effects, hydroxychloroquine was not clinically effective in a double-blind, randomised, placebo-controlled trial.[14] In an open-label clinical trial, a clinical response to leflunomide was seen only in patients with significant inhibition of type 1 T helper cell activation.[13] To our knowledge, the combination of leflunomide and hydroxychloroquine has not been tested in a clinical study. However, in an in vitro study by our group,[15] we showed a complementary and additive immune-inhibitory effect of leflunomide and hydroxychloroquine. Given these data, as well as the acceptable safety profiles of the two drugs and their previous approval for use in multiple rheumatic musculoskeletal

diseases, including rheumatoid arthritis and systemic lupus erythematosus, we aimed to investigate the efficacy and safety of leflunomide and hydroxychloroquine as a combination therapy for primary Sjögren's syndrome.

Methods

Study design

We did a placebo-controlled, double-blinded, phase 2A randomised clinical trial (RepurpSS-I) to evaluate the efficacy and safety of leflunomide and hydroxychloroquine as a combination therapy for primary Sjögren's syndrome. The study was done at the University Medical Center Utrecht (Utrecht, Netherlands). The study protocol was reviewed and approved by the medical ethical committee of the University Medical Center Utrecht.

Patients

Patients were selected from the outpatient clinic of the University Medical Center Utrecht or referred to our outpatient clinic from other hospitals in the Netherlands. The inclusion criteria were age 18–75 years; primary Sjögren's syndrome diagnosed according to the American-

European Consensus Criteria (2002 revision)[16]; moderate to active disease reflected by a European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index (ESSDAI) score of 5 or higher; a lymphocytic focus score of 1 or higher in labial salivary gland biopsy specimens that were obtained at any time before inclusion; and use of a reliable method of contraception for female participants. Exclusion criteria included the use of hydroxychloroquine within 6 months before inclusion; and the use of other immunosuppressive drugs, with the exception of a stable, low dose of non-steroidal anti-inflammatory drugs or a stable, low dose (≤7.5 mg) of oral corticosteroids. A full list of exclusion criteria is provided in the supplementary material. All patients provided written informed consent.

Randomisation and masking

Patients were randomly allocated in a ratio of 2:1 to leflunomide–hydroxychloroquine or placebo–placebo. Block randomisation (block size of six) was done by the manufacturer of the study medication (Basic Pharma, Geleen, Netherlands). All investigators, patients, and laboratory staff were masked to the treatment allocation. Capsules were manufactured specifically for the trial, had identical appearance, and were packaged in identical glass containers by Basic Pharma.

Procedures

Patients in the leflunomide–hydroxychloroquine group received 20 mg leflunomide and 400 mg hydroxychloroquine (200 mg twice or 200 mg once if bodyweight was <60 kg) daily. Patients in the placebo–placebo group received placebo in place of both hydroxychloroquine and leflunomide. Patients were treated for 24 weeks. Clinical assessments were done at baseline and after 8, 16, and 24 weeks of treatment.

Outcomes

The primary endpoint was the between-group difference in change in ESSDAI scores from baseline to 24 weeks, corrected for baseline values.

Secondary endpoints were between-group differences in the EULAR Sjögren's syndrome patient

reported index (ESSPRI), including subscores of pain, fatigue, and dryness; measurement of stimulated and unstimulated whole saliva production; visual analogue scales of oral and ocular dryness; lachrymal flow as measured by Schirmer's test; serum IgG; patient and physician global assessment; quality of life assessed by the Short Form-36 survey; and fatigue, as measured by the Multidimensional Fatigue Inventory, measured at baseline, and at 8, 16, and 24 weeks. Concentrations of complement factors 3 and 4 (C3 and C4) and serum IgM rheumatoid factor were determined at baseline and at 24 weeks.

A post-hoc analysis was done comparing baseline ESSDAI scores to ESSDAI scores after 24 weeks within the two treatment arms. This analysis was done using a random intercept model, taking into account repeated measures. Clinical responders were defined as having a decrease in ESSDAI of 3 or more points at 24 weeks according to EULAR recommendations.[17] To understand whether the difference in ESSDAI scores reflected symptomatic improvement or was largely dependent on changes in the biological domain, ClinESSDAI scores (ESSDAI scores without the biological domain[18]) were calculated and a post-hoc analysis was performed using the same statistical methods as for the analysis of ESSDAI scores. In addition, percentages of patients that showed no, low, or moderate activity in the different domains were calculated. To monitor local treatment response as an exploratory outcome, inflammation was quantified in parotid gland biopsy specimens taken at baseline and after 24 weeks of treatment.

The occurrence of adverse events was evaluated at 8, 16, and 24 weeks by assessment of patient-reported physical symptoms and laboratory testing. Serious adverse events were immediately reported to the investigators.

Proximity extension immunoassay and prediction model

Serum was collected from all patients at baseline. Concentrations of 368 inflammation-related proteins encompassed in four panels (cardiometabolic, immune response, inflammation, and metabolism, each consisting of 92 proteins) were measured for patients in the leflunomide–hydroxychloroquine group by proximity extension immunoassay (OLink Bioscience, Uppsala, Sweden), as previously described.[19] Normalised protein expression data were filtered to include only proteins expressed in more than 60% of samples, leaving 338 proteins for further analysis.

Quantile-normalisation was performed on filtered protein expression values. Proteins that were differentially expressed in responders versus non-responders were identified using likelihood ratio test (p<0,05). Unsupervised hierarchical clustering on Z-transformed values based on Euclidian distance was performed using Ward's method. Principal component analysis was used to visualise data.

A prespecified random forest machine-learning model was established to identify the proteins that best predict response to treatment using the baseline serum proteome and cross-validated using 500 leave-seven-out iterations. A model based on the ten most significantly differentially expressed proteins (based on p value) reached the highest mean accuracy.

Statistical analysis

Based on an allocation ratio of leflunomide–hydroxychloroquine to placebo of 2:1, two-tailed testing, α=0.05, and 80% power, we calculated that 20 patients in the leflunomide–hydroxychloroquine group and ten patients in the placebo group would be sufficient to detect an effect size of 2.3 ESSDAI points, assuming a standard deviation of 2.0, based on values from previous trials.[20,21] Expiration of the last batch of the study medication caused a stop of inclusion at 29 patients. Expiration of the first batch of study medication caused unanticipated allocation to both groups.

All patients were analysed according to the intention-to-treat principle. To evaluate the difference in ESSDAI scores between the leflunomide–hydroxychloroquine group and the placebo group from baseline to 24 weeks, analysis of covariance was done with ESSDAI scores as the independent variable and baseline ESSDAI scores as the covariate (primary analysis). As a secondary analysis, mixed model analysis was done, with the post-baseline ESSDAI measurements (8, 16, and 24 weeks) as the dependent variable and baseline ESSDAI score and visit week as covariates. This model also included the interaction between visit week and treatment. A difference between groups was considered statistically significant at p<0,05. This latter analysis was applied to all secondary outcomes, except for the concentrations of C3 and C4, because data were available for only two timepoints. For C3 and C4 concentrations, linear

regression adjusted for baseline values was done. Data were normalised using square-root or log transformation when applicable.

No correction for multiple comparisons was applied when analysing secondary endpoints. Therefore, results are reported as point estimates with 95% CIs. To assess whether the number of responders at 24 weeks was different between the treatment groups, a Fisher's exact test was performed. For histological markers, differences over time within the groups were assessed using Wilcoxon signed-rank test. Statistical analysis was done using SPSS (version 25) and SAS (version 9.4).

Patients that prematurely stopped the medication had a close-out visit if possible. The results of this close-out visit were carried forward for the 24-week measurement, and they were analysed as such in the primary and secondary analysis. Patients who did not have a close-out visit were excluded from the primary endpoint analysis, but they were included in the secondary analyses, which also encompass data from the 8-week and 16-week measurements.

This study is registered with EudraCT, 2014–003140–12.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Patients were enrolled between March 7, 2016, and Nov 30, 2017, and the study was completed on May 18, 2018. 37 patients were assessed for eligibility, of whom 29 patients (28 women and one man) who fulfilled the 2002 classification criteria for primary Sjögren's syndrome[16] were enrolled (figure 1). All patients also fulfilled the 2016 classification criteria,[22] which became available after the initiation of this study. 21 patients were randomly allocated to leflunomide–hydroxychloroquine and eight patients were randomly allocated to placebo treatment.

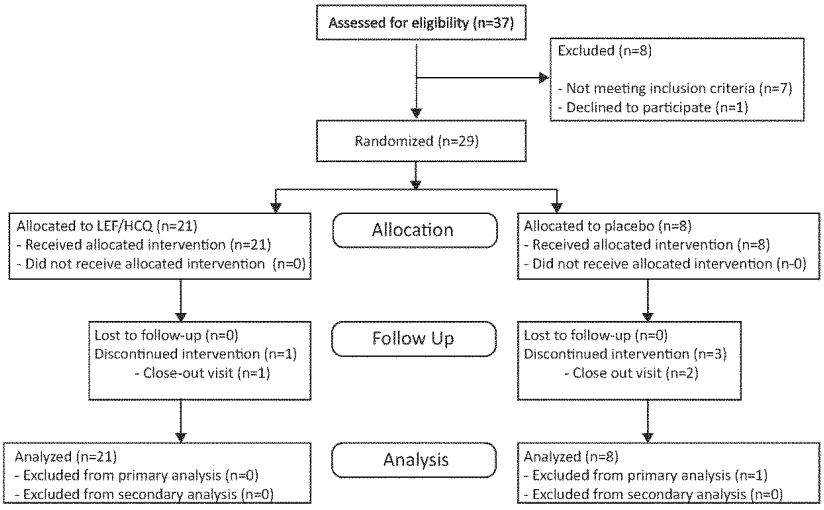

Figure 1. Trial profile

One patient in the placebo group was excluded from analysis after 8 weeks of treatment because of the need for high-dose prednisone at week 13 to treat polymyalgia rheumatica. The patient left the study at week 13 and no close-out visit was done. Therefore, 21 patients in the leflunomide–hydroxychloroquine group and seven patients in the placebo group were assessed for the primary endpoint. Three other patients terminated medication early; two of these patients, one from the leflunomide–hydroxychloroquine group and one from the placebo group, had blinded 16-week visits and close-out visits. One patient from the placebo group completed the 16-week visit but had an unblinded close-out visit because of pancreatitis requiring de-blinding. This patient did not receive additional treatment until after the close-out visit.

Baseline characteristics of the patients are described in table 1**.** With the exception of serum IgG levels, baseline values were similar between the groups. Although allowed by the inclusion criteria, none of the participants used oral corticosteroids (<7.5 mg) during the trial.

	Leflunomide-hydroxychloroquine (n=21)	Placebo (n=8)
Age, years	54.7 (12.4)	53.5 (15.2)
Sex		
Men	1 (5%)	0 (0%)
Women	20 (95%)	8 (100%)
Disease duration, years	7.7 (9.8)	8.9 (6.8)
ESSDAI score	10.4 (3.9)	9.1 (3.4)
ESSPRI score	6.7 (5.3 – 7.7)	6.7 (5.9 – 7.9)
Serum IgG, g/L	19.4 (6.9)	13.8 (5.0)
Schirmer score, mm/5min	3.0 (0.0 – 11.5)	3.0 (0 – 7.5)
Unstimulated saliva flow, ul/5 min	300 (10.0 – 500)	300 (75.0 – 1275)
Stimulated saliva flow, ul/5 min	400 (200 – 1075)	700 (150 – 2250)
Anti-SSA antibodies, n (%)	18 (86%)	7 (88%)
Anti-SSB antibodies, n (%)	13 (62%)	4 (50%)
VAS ocular dryness	58.2 (31,5)	56.9 (25.9)
VAS oral dryness	56.0 (29.0)	62.5 (25.5)
Global assessment patient	71.0 (55.5 – 84.5)	75.5 (67.5 – 82.5)
Global assessment physician	69.0 (13.4)	69.6 (12.5)

Table 1. Baseline characteristics (n=29). Data are mean (SD), median (IQR), or n (%). Mean (SD) is provided for normally distributed parameters and median (IQR) for non-normally distributed parameters. ESSDAI=EULAR Sjögren's syndrome disease activity index. ESSPRI=EULAR Sjögren's syndrome patient reported index. EULAR=European League Against Rheumatism. SSA= Sjögren's syndrome-related antigen A. SSB=Sjögren's syndrome-related antigen B. VAS=visual analogue scale.

The mean difference in ESSDAI score from week 0 to 24, adjusted for baseline values, in the leflunomide–hydroxychloroquine group compared with the placebo group was –4.35 points (95% CI –7.45 to –1.25, p=0.0078). Based on mixed model analysis, the estimated mean difference in ESSDAI score in the leflunomide– hydroxychloroquine group compared with the placebo group was –1.29 (–3.98 to 1.41, p=0.34) after 8 weeks, –3.50 (–6.27 to –0.72, p=0.015) after 16 weeks and –4.29 (–7.06 to –1.53, p=0.003) after 24 weeks (figure 2A).

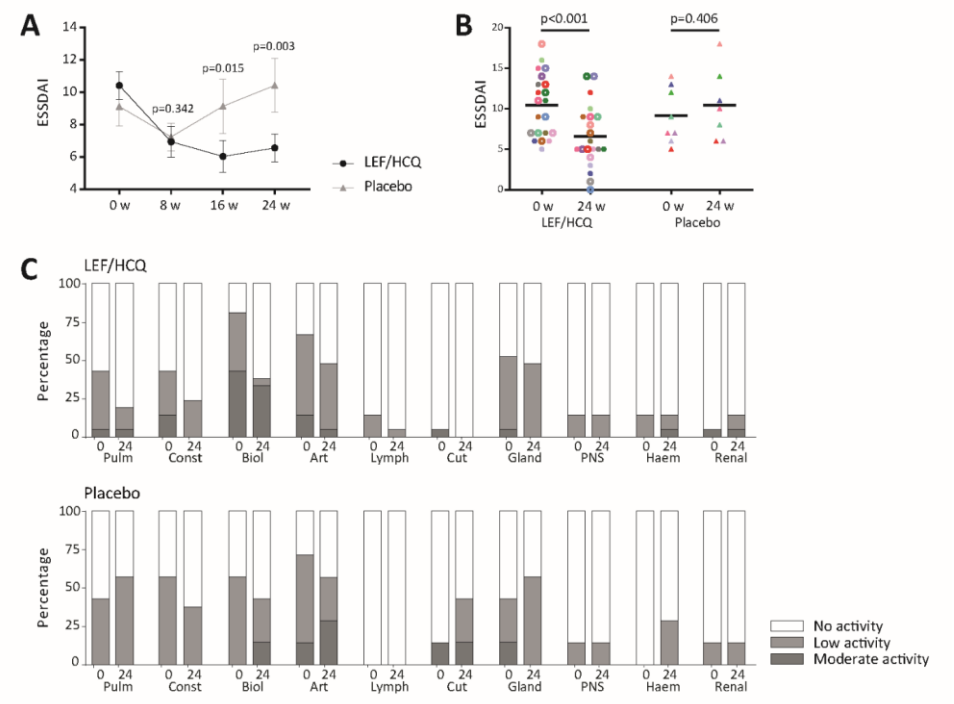

Figure 2. ESSDAI scores over 24 weeks of treatment with leflunomide–hydroxychloroquine or placebo

(A) Mean (SEM) ESSDAI scores over time. p values indicate the effect of treatment compared with placebo at 8, 16, and 24 weeks, corrected for baseline values. (B) ESSDAI scores at baseline and 24 weeks. Patients are represented by the same coloured symbol at both timepoints. Mean values are indicated. p values indicate statistical differences compared with baseline within groups. (C) Percentage of patients showing no, low, or moderate activity in the different ESSDAI domains at baseline and 24 weeks. ESSDAI=European League Against Rheumatism Sjögren's syndrome disease activity index. Lymph=lymphadenopathy and lymphoma. PNS=peripheral nervous system.

Secondary endpoints were assessed at 8, 16, and 24 weeks and corrected for baseline values. Total ESSPRI scores were lower in the leflunomide–hydroxychloroquine group compared with the placebo group at 16 weeks (table 2; table S1; figure S2). ESSPRI fatigue subscore was lower in the leflunomide–hydroxychloroquine group compared with the placebo group at 16 weeks and 24 weeks. ESSPRI pain subscore was lower in the leflunomide–hydroxychloroquine group at 8 weeks and 16 weeks. ESSPRI dryness subscore was not significantly different in the two groups at any timepoint. Unstimulated whole saliva production was increased in the leflunomide–

hydroxychloroquine group compared with the placebo group at 24 weeks and stimulated whole saliva production was increased in the leflunomide– hydroxychloroquine group at 16 weeks (table 2). Global assessment scored by the physician was increased in the leflunomide–hydroxychloroquine group at weeks 8 and 24, and global assessment scored by the patient was increased at 8 weeks. No differences between groups were seen for Schirmer score or visual analogue scores for ocular or oral dryness (table 2; table S1; figure S2), or in the Multidimensional Fatigue Inventory questionnaire (table S1; table S2). At 24 weeks, social function and mental health in the Short Form-36 questionnaire were significantly improved compared with placebo, with no significant differences in other categories (table S1; table S2).

Serum IgG was decreased at 16 and 24 weeks and rheumatoid factor was decreased at 24 weeks in the leflunomide–hydroxychloroquine group compared with the placebo group. No differences in C3 and C4 were seen between groups at 24 weeks. Mean (SD) and median (IQR) values of all secondary endpoints are provided in the supplementary material (table S1; table S2; figure S2).

Table 2. Secondary endpoints.

	8 weeks			16 weeks			24 weeks		
	Effect size	95% CI	p-value	Effect size	95% CI	p-value	Effect size	95% CI	p-value
ESSPRI	-0.97	-2.18 to 0.23	0.11	-1.66	-2.90 to -0.41	0.010	-1.11	-2.35 to 0.13	0.079
ESSPRI fatigue	-1.10	-2.81 to 0.60	0.20	-2.44	-4.17 to -0.70	0.0068	-1.78	-3.51 to -0.05	0.044
ESSPRI pain	-1.73	-3.28 to -0.17	0.030	-1.76	-3.38 to -0.13	0.035	-1.23	-2.84 to 0.39	0.13
ESSPRI dryness	-0.42	-2.15 to 1.32	0.63	-1.06	-2.85 to 0.72	0.24	-0.64	-2.42 to 1.14	0.48
UWS *	4.78	-3.27 to 12.83	0.24	4.24	-4.15 to 12.64	0.32	10.57	2.21 to 18.93	0.014
SWS *	5.13	-6.31 to 16.57	0.37	13.1	1.35 to 24.80	0.030	9.81	-1.89 to 21.51	0.098
Schirmer's test	1.38	-5.22 to 7.97	0.68	4.79	-1.95 to 11.5	0.16	3.02	-3.71 to 9.74	0.37
VAS oral	-9.86	-28.46 to 8.74	0.29	-12.08	-31.07 to 6.92	0.21	-7.17	-26.12 to 11.78	0.45
VAS ocular	-3.72	-24.22 to 16.78	0.72	-5.62	-26.66 to 15.42	0.59	-8.48	-29.45 to 12.50	0.42
GA patient	-19.25	-38.36 to -0.14	0.048	-14.01	-33.66 to 5.64	0.16	-18.80	-38.39 to 0.78	0.059
GA physician	-15.62	-29.64 to -1.60	0.030	-12.81	-27.31 to 1.69	0.082	-15.52	-29.96 to -1.08	0.036
sIgG	-0.36	-2.29 to 1.57	0.71	-2.39	-4.36 to -0.43	0.018	-3.32	-5.28 to -1.37	0.0013
IgM RF *	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-0.59	-1.06 to -0.12	0.017
C3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.08	-0.07 to 0.23	0.29
C4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.02	-0.03 to 0.06	0.43

Effect sizes show the difference in the leflunomide–hydroxychloroquine group (n=21) compared with the placebo group (n=8). ESSPRI=European League Against Rheumatism Sjogren's syndrome patient reported index. UWS=unstimulated whole saliva flow.

SWS=stimulated whole saliva flow. VAS=visual analogue scale. C3=complement factor C3. C4=complement factor C4. *Data have been transformed to normalise residuals.

Three patients in the placebo group stopped treatment prematurely due to adverse events: one patient had polymyalgia rheumatica requiring high doses of prednisone at 13 weeks; one patient had pancreatitis at 16 weeks; and one patient had pyrosis, fatigue, and dyspnoea at 16 weeks, for which no cause was found after extensive analysis. One patient in the leflunomide–hydroxychloroquine group stopped treatment prematurely due to pruritus and pre-existing gastrointestinal discomfort at 10 weeks. Two serious adverse events occurred in the placebo group, one of which required de-blinding (pancreatitis at week 16). The other serious adverse event, hospital admission for nephrolithiasis, did not result in discontinuation or de-blinding (table 3). The most common adverse events in the leflunomide–hydroxychloroquine group were gastrointestinal discomfort (11 patients [52%] versus two [25%] in the placebo group), modest transient increases in alanine aminotransferase (ten [48%] versus one [13%]), and short episodes of general malaise and shivering (nine [43%] versus one [13%]).

Table 3. Adverse events and serious adverse events (n=29)

	Leflunomide-hydroxychloroquine (n=21)	Placebo (n=8)
Adverse events		
Gastrointestinal discomfort	11 (52%)	2 (25%)
Diarrhoea	8 (38%)	0
Weight loss of >2 kg	3 (14%)	0
Anorexia	2 (10%)	0
Alopecia	1 (5%)	0
Mood changes	1 (5%)	1 (13%)
Dizziness	2 (10%)	0
Dyspnoea	3 (14%)	1 (13%)
Viral infection*	9 (43%)	1 (13%)
Sinusitis	1 (5%)	0
Cystitis	3 (14%)	0
Hyperpigmentation skin	1 (5%)	0
Pruritus	4 (19%)	0
Herpes Zoster	1 (5%)	0
Increased fatigue	0	1 (13%)
Increased arthralgia	1 (5%)	0
Pyrosis	0	1 (13%)
Persistent pain after parotid biopsy for <1 month	0	3 (38%)
Migraine	0	1 (13%)
Mouth ulcers	2 (10%)	0
Balance disorder	0	1 (13%)
Folliculitis	0	1 (13%)
Inguinal dermatitis	1 (5%)	0
Coughing	1 (5%)	0
Polymyalgia rheumatica	0	1 (13%)
Diverticulitis	1 (5%)	0
Fractured or bruised limbs after a fall	1 (5%)	1 (13%)
Triggerfinger requiring surgery	1 (5%)	0
Alanine aminotransferase 1-2 ULN	10 (48%)	1 (13%)
Alanine aminotransferase >2 ULN	1 (5%)	0
Creatinine kinase 1-2 ULN	4 (19%)	0
Creatinine kinase >2 ULN	2 (10%)	0
Elevated RR requiring (higher doses of) medication	2 (10%)	0
Allergic skin rash	2 (10%)	0
Leucopenia 3-4x10^9 per L	5 (24%)	2 (25%)
Leucopenia <3x10^9 per L	4 (19%)	0
Lymphopenia 0.5-0.99x10^9 per L	8 (38%)	1 (13%)
Neutropenia 1.0-1.5x10^9 per L	3 (14%)	0
Neutropenia 0.5-0.99x10^9 per L	1 (5%)	0
Serious Adverse Events		
Pancreatitis requiring admission to the hospital	0	1 (13%)
Nefrolithiasis requiring admission to the hospital	0	1 (13%)

ULN=upper limit of normal. * Short episodes of general malaise and shivering. This was a clinical diagnosis, viral infection was not proven.

Within the leflunomide–hydroxychloroquine group, ESSDAI scores were significantly decreased at 24 weeks compared with baseline (p<0,0001); no significant change from baseline was seen in the placebo group (p=0.41; figure 2B). Mean (SD) and median (IQR) values of ESSDAI at all timepoints are provided in the supplementary material (table S1). Of the 21 patients that received leflunomide–hydroxychloroquine, 11 (52%) were clinical responders according to EULAR criteria, defined as a decrease in ESSDAI of 3 or more points at 24 weeks. None of the patients treated with placebo were clinical responders (p=0.020).

In the leflunomide–hydroxychloroquine group, the percentage of patients with symptoms in seven ESSDAI domains decreased from 0 to 24 weeks (figure 2C), whereas in the control group, decreases were seen in three domains: constitutional, biological, and articular (although an increase in moderate activity was seen in the articular domain). None of the patients reported symptoms in the muscular or CNS domains at baseline or during the trial, so these domains are not shown in figure 2C.

To understand whether the difference in ESSDAI scores reflected symptomatic improvement or was largely dependent on changes in the biological domain, ClinESSDAI scores were calculated. At 24 weeks, the mean difference in ClinESSDAI score, adjusted for baseline values, in the leflunomide–hydroxychloroquine group compared with the placebo group was –3.84 points (95% CI –6.98 to –0.70, p=0.019). Based on mixed model analysis, the estimated mean difference in ClinESSDAI score in the leflunomide– hydroxychloroquine group compared with the placebo group was –1.12 (–3.76 to 1.51, p=0.40) after 8 weeks, –2.97 (–5.70 to –0.25, p=0.033) after 16 weeks and –3.79 (–6.50 to –1.08, p=0.0073) after 24 weeks (figure S1).

In an exploratory analysis, treatment efficacy was evaluated via inflammatory scores in parotid biopsies taken at baseline and 24 weeks. In the leflunomide– hydroxychloroquine group, the tissue area occupied by inflammatory cells decreased between 0 and 24 weeks, which was not seen in the placebo group (representative histology and mean infiltration percentages are provided in figure S3).

Clinical response was observed in 11 of 21 patients who received leflunomide–hydroxychloroquine treatment (ESSDAI scores of non-responders and responders over time are shown in figure S4). The difference in changes in ESSDAI between responders and non-responders provided an opportunity to examine biomarkers to predict treatment response. With the exception of C3, baseline laboratory and clinical markers were not significantly different between responders and non-responders (data not shown), which highlights the challenge in the prediction of therapy response. To this end, we measured the expression of 368 proteins in baseline serum samples of patients in the leflunomide– hydroxychloroquine group. 42 significantly differentially expressed proteins between responders and non-responders were identified. Visualisation of data using unsupervised hierarchical clustering (figure 3 A) and principal-component analysis (figure 3 B) showed clear differences between responders and non-responders. To assess whether clinical response could be predicted using these dissimilarities in the baseline serum proteome, we trained a machine-learning model using a random forest algorithm. A model using the ten most significantly differentially expressed proteins showed the best performance in cross-validation, with a mean accuracy of 87%, a mean true-positive rate of 95%, and a mean true-negative rate of 82% (figure 3C, D).

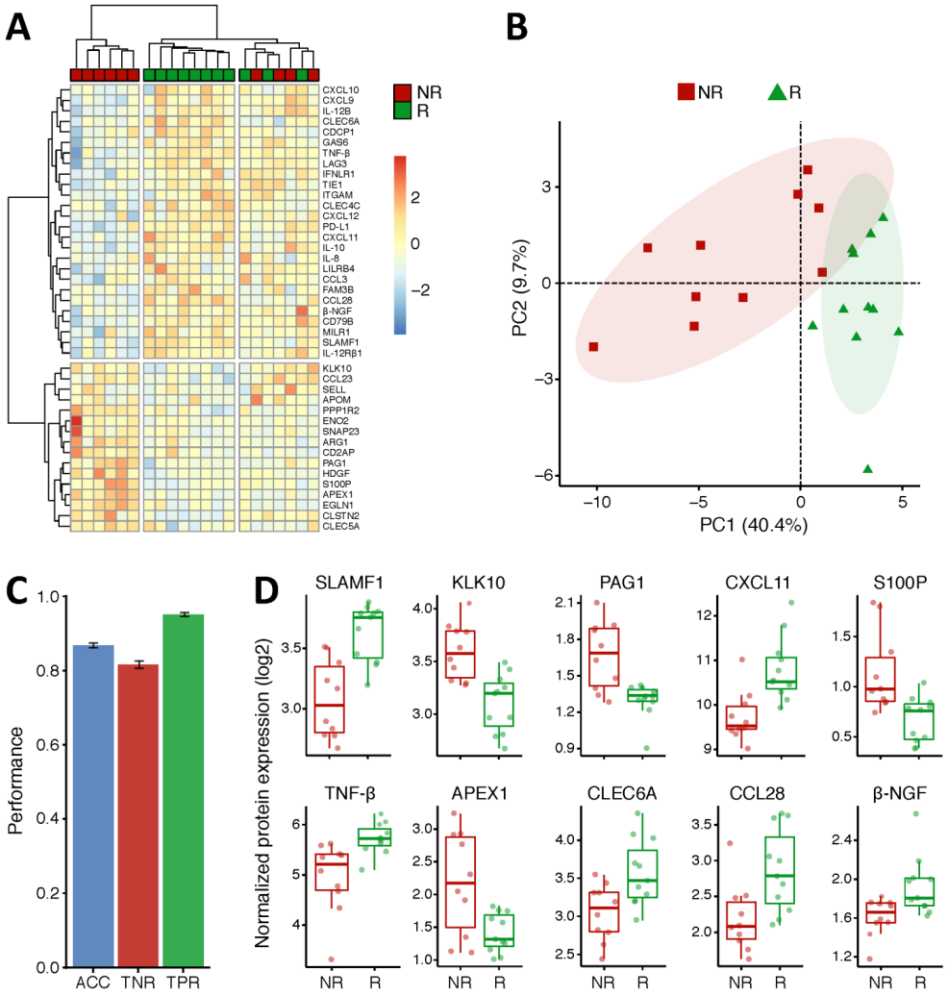

Figure 3 Prediction of clinical response to leflunomide–hydroxychloroquine with use of the baseline serum proteome

(A) Unsupervised hierarchical clustering based on the Z-transformed values of the 42 significantly differentially expressed proteins between responders and non-responders. (B) Principal component analysis. Ellipses indicate SD of 1・5 from the mean. (C) Performance of a random forest machine-learning model based on the expression of the top 10 differentially expressed proteins. Bars show the mean (SD) obtained from cross-validation analysis. (D) Expression of the 10 differentially expressed proteins on which the model is based. Proteins are ordered by significance of the difference between responders and non-responders from left to right and from top to bottom (ie, lowest p value top left, highest p value bottom right). Data are depicted using boxplots that follow the standard Tukey representation.

Discussion

Leflunomide and hydroxychloroquine combination therapy was found to be effective and safe in patients with primary Sjögren's syndrome with moderate to high disease activity. Disease activity, as measured by ESSDAI scores, significantly decreased in patients treated with leflunomide–hydroxychloroquine treatment compared with those treated with placebo (–4.35 points; 95% CI –7.45 to –1.25, p=0.0078). For ethical reasons, comparing leflunomide and hydroxychloroquine monotherapies with leflunomide–hydroxychloroquine combination therapy would not have been justified, because these monotherapies did not reach clinical endpoints in previous studies.[13,14] Therefore, although we found a significant clinical response to leflunomide–hydroxychloroquine treatment, which was not previously observed using either drug independently, no conclusions can be drawn with respect to additive clinical effects of the combination. However, reduced B-cell hyperactivity at 24 weeks in the leflunomide–hydroxychloroquine group (mean serum IgG reduction of 5.7 g/L [–29.4%] *vs* an increase of 0.7 g/L [5.1%] in the placebo group compared with baseline) suggests that inhibition could have been greater than in previous studies with leflunomide and hydroxychloroquine monotherapies (mean serum IgG reduction of 3.4 g/L [–14.4%] for leflunomide and 1.6 g/L [–7.8%] for hydroxychloroquine compared with baseline; figure S5).[13,14]

To our knowledge, this is the first randomised, placebo-controlled clinical trial in patients with primary Sjögren's syndrome that shows significant clinical efficacy, as measured by ESSDAI, and is associated with significant improvements in other clinical parameters, including dryness and fatigue. Consistent with the primary endpoint, baseline-corrected ESSPRI (including individual constituents of pain and fatigue) decreased, indicating an improvement in the disease from the patient's perspective. In addition, an unprecedented improvement in saliva output was seen with leflunomide– hydroxychloroquine treatment, and social function and mental health in the Short Form-36 questionnaire showed improvement. These positive outcomes in multiple disease parameters suggest a potent effect of leflunomide–hydroxychloroquine in patients with primary Sjögren's syndrome, which was also confirmed in the histological analysis. The results of this study warrant replication in larger, independent follow-up clinical trials.

After 8 weeks of treatment, the leflunomide–hydroxychloroquine and placebo groups showed a similar trend of decreasing ESSDAI score. A possible explanation for this is that several ESSDAI domains contain scores that are sensitive to subjective scoring (eg, the articular domain, in which patients are asked for arthralgia and morning stiffness), potentially contributing to a placebo effect. Of note, the early response reverted at 16 weeks in the placebo group, whereas the leflunomide–hydroxychloroquine group showed a further decrease in ESSDAI at 16 weeks.

Leflunomide–hydroxychloroquine combination therapy was well tolerated. Side-effects were mild and manageable and no unexpected side-effects related to study medication were observed. In addition, lupus-like skin rashes that occurred in 33% of patients in the leflunomide monotherapy trial[13] were not seen with leflunomide– hydroxychloroquine treatment.

We were able to predict response to therapy with clinically meaningful accuracy on the basis of a set of ten circulating proteins (SLAM1, KLK10, PAG1, CXCL11, S100P, TNF-β, APEX1, CLEC6A, CCL28, β-NGF), including several pro-inflammatory mediators. Overall, the significantly increased circulating concentrations of multiple inflammatory mediators in responders versus non-responders suggests an inflammatory endotype that is associated with clinical response. These mediators include chemokines CXCL9, CXCL10, CXCL11, IL-8, CCL3, and CCL28, and cytokines associated with regulation of B-cell activation and lymphoid neogenesis (CXCL12, TNF-β**,** and CD79B) and T-cell activation (IL-12B, soluble IL-12Rβ1, SLAMF1, soluble PD-L1, and soluble LAG3). In support of an inflammatory endotype, we have shown that efficacy is associated with downregulation of such inflammatory mediators (eg, CXCL10, unpublished data). Successful prediction of response at baseline could improve the cost-effectiveness of treatment and prevent unnecessary exposure of patients to drugs that will not have benefit. In addition, proteomic analysis and ongoing systems biology efforts could provide insights into the molecular mechanisms that are associated with response to therapy, possibly opening up novel therapeutic strategies.

Our study has several limitations, including the small sample size and the short duration. In addition, although significant effect estimates of several of the secondary endpoints were observed, confirmation is required in larger clinical studies. Several other secondary endpoints

were only modestly affected, supporting the need for better understanding of the underlying mechanisms.

In summary, we showed a clinical and histological response in patients with primary Sjögren's syndrome treated with the combination of leflunomide and hydroxychloroquine, and identified a set of circulating proteins that could be used to predict response to therapy. The treatment was well tolerated by patients. Leflunomide–hydroxychloroquine combination therapy warrants further evaluation in larger randomised controlled trials before its implementation in daily clinical practice.

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Supplementary material

Supplementary methods

Inclusion criteria

Inclusion criteria were slightly modified during recruitment, amendments were approved by the ethical committee. Full inclusion criteria are described below, with amendments made during recruitment in *italics*.

1. Women, 18-75 years of age

   Changed to women and men, 18-75 years of age
2. Primary Sjögren's syndrome (pSS) according to the American-European Consensus Criteria, revised in 2002
3. Lymphocytic focus score (local lymphocytic infiltrates) ≥1 in sublabial salivary gland specimen
4. ESSDAI≥5
5. Presence of autoantibodies directed to pSS-related SSA/Ro and/or SSB/La nuclear antigens

   This criterion was cancelled
6. Use of a reliable method of contraception
7. Signed written informed consent

Exclusion criteria

Exclusion criteria for enrollment to this study were:

1. Pregnancy or wishing to conceive during or within two years after the study
2. Breastfeeding
3. Therapy resistant hypertension
4. Maculopathy or retinitis pigmentosa
5. Secondary Sjögren's Syndrome (Sjögren's syndrome associated with other connective tissue disease)

6. Hepatic or renal impairment
7. Severe infection (including hepatitis B, C or HIV)
8. Presence of a malignancy other than mucosa-associated lymphoid tissue lymphoma (MALT lymphoma)
9. Significant cytopenia
10. Concomitant heart- and inflammatory bowel disease
11. Sarcoidosis
12. Usage of HCQ or LEF <6 months prior to inclusion
13. Usage of immunosuppressive drugs, with the exception of a stable dose of non-steroidal inflammatory drugs and a stable, low dose (≤7.5 mg) of oral corticosteroids
14. Inadequate mastery of the Dutch language

Patients

Patients were selected on fulfilling the 2002 classification criteria for pSS[1], however all also fulfilled the 2016 criteria, which became available after the initiation of this study.

Although allowed by the inclusion criteria, none of the patients in both arms used low dose (<7.5 mg) of oral corticosteroids during the trial.

Because the expiration date of the study medication was reached before 30 patients could be included, patient inclusion was stopped after 29 patients.

Clinical scoring

Cytopenia is a known side-effect of leflunomide[2]. Hence, according to the ESSDAI guidelines[3], decreased levels of lymphocytes and neutrophils were not taken into account when scoring the ESSDAI. As anticipated in the present study significantly reduced absolute numbers of lymphocytes were observed (mean baseline LEF/HCQ arm $1.444x10^9$/l to $1.095x10^9$/l at 24 weeks, $p<0.0001$, Wilcoxon signed rank test). Only transient increases in absolute numbers of granulocytes and monocytes were observed, not significantly different from the placebo group at 24 weeks.

Assessment of the pulmonary domain was done via extensive anamneses, and pulmonary function test, chest x-ray or HR-CT were performed when indicated.
For parotid biopsies we noticed that local anesthetics in high dosage triggered one-sided transient facial nerve paralysis and tear formation in some cases. To avoid any interference of local anesthesia on tear production, Schirmer's test at baseline and after 24 weeks (time points when biopsy was taken) was measured only in the contralateral eye, opposite to the side that was used for parotid biopsy. At 8 and 16 weeks the mean score of both eyes was used.

Biopsy procedure

Parotid biopsies were taken in local anesthesia by one of two maxillofacial surgeons (AR or NJ). After injection with 1,8 cc of articaïn 2%/adrenalin 1:80.000 (Utracain DS Forte, Sanofi Aventis, Gouda, The Netherlands) parotid samples were harvested through an infralobular incision. The parotid capsula, subcutis and cutis were closed in layers using resorbable sutures (Vicryl 4-0 and Monocryl 4-0, Ethicon, Brussels, Belgium).

Biopsy samples were washed in sterile saline, dried using filter paper circles (GE Healthcare, Eindhoven, the Netherlands), embedded in Tissue tek (Sakura Finetek Holland BV, Alphen aan de Rijn, the Netherlands), snap frozen in liquid nitrogen and stored at -80°C.

Histological analysis was performed using Zen software (Zeiss) on manually drawn images (glandular areas and lymphocytic infiltrates, with exclusion of fat). Values from Zen analysis were used to calculate the inflamed area fraction (percentage of lymphocytic infiltration /mm2 of the total glandular area) and focus score (number of lymphocytic foci[4] (>50 lymphocytes) /4mm2 glandular tissue). All analyses were performed in a blinded fashion, VI and FB were not aware of the treatment arms.
All glandular tissue available was analyzed. For biopsies presenting with area < 4mm2 a second cutting level was analyzed at a distance of more than 90 microns from the first H&E[5]. For those donors where multiple biopsies/time-point or multiple cutting levels were available, both area fraction and focus score were calculated over the total glandular area.

Statistical analysis

In case of early discontinuation of medication, a close-out visit was performed as soon as possible. The result of this last observation was carried forward for the 24 week measurement. This was done for 1 patient in the LEF/HCQ arm, who discontinued medication after 10 weeks because of (pre-existent) gasto-intestinal complaints, and 1 patient in the placebo arm who discontinued medication after 16 weeks because of dyspnea, pyrosis and increased fatigue. Also in the placebo arm, in one patient pancreatitis led to early discontinuation and de-blinding just after the 16 weeks measurement, thus the close out visit was performed de-blinded. Finally, 1 patient in the placebo arm did not have a close-out visit because of the need for high doses of prednisone, thereby violating the inclusion criteria. Baseline and 8 weeks measurements of this patient were taken along in the analysis of endpoints. Actual ESSDAI scores of the patients that had last observed values carried forward have been plotted in Figure S6 (Patient LH07, LH16, and LH29). For patient LH07 and LH29 ESSDAI scores at the close-out visit were measured blinded, for patient LH16 this was done de-blinded. Taking these actual scores into account didn't majorly change the outcomes in the primary endpoint and a significant difference between the two treatment arms remained if excluded.

Proximity extension immunoassay and prediction model

Fresh blood samples were collected at baseline and 24 weeks in Vacutainer SSTII Advance tubes (BD Biosciences, Franklin Lakes, NJ, USA). Serum was collected as per manufacturer's instructions, snap frozen in liquid nitrogen, and stored at -80°C. Serum levels of 368 inflammation-related proteins encompassed in four panels (Cardiometabolic, Immune response, Inflammation, and Metabolism; each consisting of 92 proteins) were measured by proximity extension immunoassay (OLink Bioscience, Sweden) according to manufacturer's instructions. This technique, which has been previously described in detail[6], allows simultaneous assessment of a panel of proteins using oligonucleotide-labeled antibody-probe pairs that bind to each protein within the sample. The pre-processed data are provided in normalized protein expression (NPX), an arbitrary unit which is given on a log2 scale. Additional information on methods, assay

performance, detection limits, quality control, and validation are available at the manufacturer's webpage (http://www.olink.com). All samples passed OLink quality control for all panels, with the exception of one sample from a non-responder that showed evidence of assay interference with the used spike-ins for two of the four panels measured (encompassing 184/368 measured analytes). As this sample behaved like an average sample in all descriptive statistics and multi-parametric analyses, we included it for subsequent analysis.

Measurements below limit of detection were set at the limit of detection. Duplicated proteins between panels were unified by only using the entry with highest average expression data. Only proteins quantified above the limit of detection in more than 60% of samples (338 proteins) were taken into account for further analysis. NPX values were quantile normalized and differentially expressed proteins were identified using likelihood ratio test ($p<0.05$). Unsupervised hierarchical clustering based on Euclidian distance was performed using Ward's method. Principal component analysis using the differentially-expressed proteins was used to compare the dissimilarity between the responders and non-responders. A random Forest machine-learning model was established using R package random Forest with parameters mtry=7, ntree=1000. Overall accuracy, true-positive rate, and true-negative rate of the model were evaluated using 500 iterations of leave-7-out cross-validation.

Supplementary results

ClinESSDAI

In order to exclude the possibility that the difference in ESSDAI score was solely caused by changes in the biological domain, ClinESSDAI scores[7] were calculated and analysed using the same statistical methods (figure S1**)**.

Secondary endpoints

No differences were detected between LEF/HCQ and placebo arms for Schirmer's test and VAS scores for ocular and oral dryness. Mean differences over time with 95% CI can be found in Table 2.

LEF/HCQ treatment did not alter scores on either of the subscores of health questionnaire MFI. Subscale physical function of the SF-36 questionnaire ameliorated upon LEF/HCQ treatment, all other subscales remained unchanged (Table S2).

Results of secondary endpoints that showed baseline-corrected estimated differences between placebo and LEF/HCQ arms with a confidence interval that did not include zero (as indicated in table 2), are depicted over time in figure S2A. In addition, figure S2B shows objective parameters UWS, SWS, Schirmers's test and complement factors C3 and C4.

Figure S3 shows the effect of LEF/HCQ and placebo treatment on local inflammation assessed in the parotid biopsy tissue sections. For 8 out of 29 patients, biopsy material at either baseline or 24 weeks did not allow quantification of infiltration due to tissue quality (only fat tissue), these patients were not included in the histological analyses. As patients were selected based on a previously analyzed labial salivary gland biopsies with a positive focus score (LFS ≥1), they were expected to have considerable inflammation in their parotid gland and at least 1 focus per 4 mm2. However, 8 of the 21 patients analyzed were found to have an LFS of ≤1 at baseline (3 patients in the placebo arm, 3 patients in the LEF/HCQ arm) and in some cases no inflammatory areas at all (3 patients from the LEF/HCQ arm). The mean relative area of infiltration in LEF/HCQ-

treated patients was significantly reduced at 24 weeks as compared to baseline. In the placebo arm, no changes were observed in area of infiltration at 24 weeks as compared to baseline. No changes in the number of foci per 4 mm^2 (lymphocytic focus score/LFS) were seen in either placebo or LEF/HCQ arms.

Safety of LEF/HCQ combination treatment

Two patients in the LEF/HCQ group developed an allergic skin rash. Medication was temporarily withdrawn in both patients (for 4 and 5 weeks) and re-introduced together with anti-histaminic medication. In both patients the skin lesions did not recur.

Because of viral infection, two patients from the LEF/HCQ group temporarily stopped medication (1 week and 2 weeks). An overview of all reported and observed adverse and serious adverse events can be found in Table 3 of the manuscript.

Table S1. Mean and median values of all endpoints at all individual endpoints in the LEF/HCQ arm.

	LEF/HCQ		Placebo	
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**
ESSDAI				
0	10.4 (3.9)	11.0 (7.0 – 13.5)	9.1 (3.4)	8.0 (6.3 – 12.8)
8	7.0 (4.2)	7.0 (3.5 – 9.8)	7.3 (2.4)	8.0 (4.5 – 8.8)
16	6.1 (4.4)	5.0 (3.3 – 8.8)	9.1 (4.5)	8.0 (6.0 – 13.0)
24	6.6 (3.9)	5.0 (4.5 – 9.0)	10.4 (4.4)	10.0 (6.0 – 14.0)
ESSPRI				
0	6.0 (2.4)	6.7 (5.3 – 7.7)	6.8 (1.2)	6.7 (5.9 – 7.9)
8	5.1 (2.2)	5.5 (3.4 – 6.9)	6.7 (1.3)	6.7 (5.3 – 7.7)
16	4.7 (2.5)	4.8 (3.0 – 6.6)	6.8 (1.3)	7.7 (5.7 – 8.0)
24	4.9 (2.4)	6.0 (3.2 – 7.0)	6.4 (1.3)	7.0 (5.0 – 7.7)
ESSPRI fatigue				
0	6.7 (2.9)	8.0 (5.5 – 8.5)	8.3 (1.3)	8.0 (7.0 – 9.8)
8	5.7 (2.7)	6.0 (3.0 – 8.0)	7.8 (0.9)	7.5 (7.0 – 8.8)
16	4.8 (3.0)	5.0 (3.0 – 7.5)	8.0 (1.3)	8.0 (7.0 – 9.0)
24	5.2 (3.0)	6.0 (3.5 – 8.0)	7.7 (0.5)	8.0 (7.0 – 8.0)
ESSPRI pain				
0	5.0 (2.9)	6.0 (2.0 – 7.0)	5.6 (2.8)	6.0 (4.3 – 7.8)
8	4.0 (2.2)	4.0 (2.0 – 6.0)	6.1 (2.7)	7.0 (5.3 –8.0)
16	4.0 (3.1)	3.0 (1.3 – 6.8)	6.1 (3.0)	7.0 (5.0 – 8.0)
24	3.9 (2.9)	4.0 (1.0 – 6.5)	5.4 (2.7)	6.0 (4.0 – 7.0)
ESSPRI dryness				
0	6.2 (2.7)	7.0 (4.0 – 8.5)	6.6 (2.6)	7.5 (5.5 – 8.0)
8	5.7 (2.6)	6.5 (4.0 – 7.8)	6.3 (2.4)	6.5 (3.8 – 7.8)
16	5.3 (3.0)	6.0 (2.3 – 8.0)	6.3 (3.5)	8.0 (3.0 – 9.0)
24	5.7 (2.7)	6.0 (30 – 8.0)	6.1 (2.7)	7.0 (4.0 – 8.0)
UWS				
0	331 (347)	300 (10.0 – 500)	587 (688)	300 (75 – 1275)
8	507 (548)	350 (100 – 650)	556 (635)	325 (50 – 975)
16	618 (776)	375 (105 – 763)	668 (551)	600 (50 – 1200)
24	574 (612)	400 (100 – 850)	407 (391)	450 (0.0 – 800)
SWS				
0	824 (1026)	400 (200 – 1075)	1125 (1178)	700 (150 – 2250)
8	1080 (1049)	600 (400 – 1900)	1150 (1098)	950 (175 – 2250)
16	1755 (1913)	1225 (350 – 2100)	1143 (1003)	1200 (200 – 2200)
24	1367 (1380)	800 (500 – 2000)	964 (633)	1000 (450 – 1500)

Schirmer's test				
0	5.6 (6.6)	3.0 (0.0 – 11.5)	5.3 (7.4)	3.0 (0.0 – 7.5)
8	7.2 (8.7)	5.5 (0.5 – 8.8)	6.0 (6.7)	3.8 (1.4 – 7.4)
16	7.1 (11.1)	3.3 (0.1 – 9.0)	2.0 (1.3)	2.0 (1.0 – 3.0)
24	6.8 (11.2)	2.0 (0.0 – 7.5)	3.7 (5.1)	3.0 (0.0 – 6.0)
VAS oral				
0	56.0 (29.0)	66.0 (32.0 – 76.0)	62.5 (25.5)	68.5 (45.3 – 76.3)
8	46.2 (30.3)	41.5 (21.8 – 79)	59.0 (27.5)	63.5 (34.8 – 80.0)
16	42.4 (28.6)	40.0 (13.8 – 70.8)	55.4 (29.1)	62.0 (39.0 – 83.0)
24	46.2 (28.9)	47.0 (19.5 – 72.0)	52.7 (27.2)	54.0 (30.0 – 75.0)
VAS ocular				
0	58.2 (31.5)	69.0 (36.0 – 84.5)	56.9 (25.9)	67.5 (28.0 – 78.0)
8	50.4 (30.8)	52.5 (26.5 – 73.0)	51.5 (24.7	59.5 (24.5 – 67.0)
16	44.8 (34.5)	48.0 (8.0 – 75.8)	50.0 (31.6)	58.0 (19.0 – 78.0)
24	48.6 (35.6)	46.0 (14.0 – 85.5)	55.6 (30.8)	64.0 (22.0 – 83.0)
GA patient				
0	65.0 (27.9)	71.0 (55.5 – 84.5)	72.9 (15.3)	75.5 (67.5 – 82.5)
8	46.5 (28.4)	48.5 (24.5 – 73.3)	68.8 (14.0)	69.5 (54.8 – 82.3)
16	48.6 (30.9)	58.5 (20.0 – 74.8)	66.3 (20.6)	72.0 (58.0 – 79.0)
24	45.9 (31.7)	54.0 (12.5 – 74.0)	67.4 (12.7)	63.0 (59.0 – 84.0)
GA physician				
0	69.0 (13.4)	70.0 (60.0 – 78.0)	69.6 (12.5)	68.0 (60.8 – 79.0)
8	52.1 (19.4)	52.5 (39.8 – 71.3)	66.8 (14.9)	65.0 (51.3 – 81.8)
16	52.0 (20.3)	57.0 (36.5 – 71.5)	64.6 (10.5)	63.0 (58.0 – 76.0)
24	53.4 (21.5)	43.0 (37.5 – 74.5)	67.7 (8.5)	67.0 (62.0 – 76.0)
sIgG				
0	19.4 (6.9)	18.4 (13.1 – 24.2)	13.8 (5.0)	13.8 (9.8 – 18.7)
8	17.3 (5.4)	17.2 (13.1 – 21.8)	14.0 (4.9)	14.5 (10.4 – 17.0)
16	14.7 (3.9)	15.1 (10.9 – 18.5)	14.6 (4.4)	15.6 (10.4 – 16.9)
24	13.7 (3.7)	14.4 (10.9 –16.2)	14.5 (4.5)	12.9 (10.6 – 19.6)
IgM RF				
0	53.1 (59.2)	18.0 (3.40 – 91.5)	8.3 (11.2)	3.90 (0.60 – 16.4)
8	n.a.	n.a.	n.a.	n.a.
16	n.a.	n.a.	n.a.	n.a.
24	26.8 (35.3)	15.0 (1.65 – 32.5)	12.5 (18.4)	4.60 (1.40 – 21.0)
C3				
0	1.06 (0.15)	1.03 (0.97 –1.14)	1.09 (0.17)	1.00 (0.95 – 1.26)
8	n.a.	n.a.	n.a.	n.a.

16	n.a.	n.a.	n.a.	n.a.
24	1.16 (0.19)	1.12 (1.00 – 1.33)	1.08 (0.16)	1.03 (0.95 –1.28)
C4				
0	0.19 (0.06)	0.21 (0.15 –0.24)	0.28 (0.08)	0.30 (0.19 – 0.34)
8	n.a.	n.a.	n.a.	n.a.
16	n.a.	n.a.	n.a.	n.a.
24	0.22 (0.06)	0.21 (0.18 – 0.26)	0.26 (0.09)	0.24 (0.18 – 0.34)
MFI general fatigue				
0	14.9 (4.8)	15.5 (13.0 – 19.8)	16.4 (3.5)	17.0 (13.0 – 19.8)
8	14.6 (4.9)	16.0 (11.0 – 19.0)	16.6 (3.1)	18.0 (13.0 – 19.0)
16	12.3 (5.5)	12.0 (7.3 – 19.5)	16.6 (2.8)	17.0 (14.0 – 19.0)
24	13.8 (5.2)	14.0 (9.0 – 18.0)	17.6 (2.8)	18.0 (17.0 – 20.0)
MFI physical fatigue				
0	12.6 (4.0)	14.0 (11.3 – 15.0)	14.6 (2.7)	14.0 (12.3 – 16.5)
8	13.1 (4.3)	13.0 (9.00 – 16.0)	13.6 (3.1)	13.0 (12.0 – 15.0)
16	11.8 (4.0)	11.5 (9.0 – 15.0)	14.7 (3.5)	15.0 (11.0 – 17.0)
24	13.6 (4.9)	13.0 (8.0 – 17.0)	15.1 (3.2)	16.0 (13.0 – 17.0)
MFI mental fatigue				
0	8.6 (3.9)	8.5 (4.0 – 12.0)	10.1 (4.3)	10.5 (5.8 – 14.5)
8	9.2 (4.3)	8.0 (6.0 – 12.0)	11.3 (6.8)	13.0 (4.0 – 19.0)
16	8.7 (4.1)	8.0 (4.3 – 12.0)	11.6 (4.4)	12.0 (9.0 – 16.0)
24	9.4 (4.7)	8.0 (5.0 – 12.5)	11.7 (6.4)	9.0 (6.0 – 18.0)
MFI reduced motivation				
0	9.5 (4.2)	9.0 (5.5 – 11.8)	9.6 (3.9)	9.0 (7.3 – 10.8)
8	9.1 (4.0)	9.0 (6.0 – 12.0)	10.4 (4.7)	10.0 (6.0 – 13.0)
16	9.2 (4.7)	9.0 (4.0 – 11.8)	12.4 (4.6)	11.0 (10.0 – 18.0)
24	9.2 (4.3)	9.0 (5.0 – 11.5)	11.9 (3.8)	12.0 (9.0 – 16.0)
MFI reduced activity				
0	10.9 (4.8)	10.0 (6.5 – 14.8)	11.3 (3.9)	11.5 (7.5 – 14.0)
8	11.5 (4.6)	12.0 (8.0 – 15.0)	12.0 (3.0)	14.0 (11.0 – 14.0)
16	10.0 (4.6)	10.5 (6.0 – 13.0)	12.7 (4.6)	13.0 (7.0 – 15.0)
24	11.2 (5.2)	13.0 (5.5 – 15.0)	13.6 (3.4)	13.0 (11.0 – 16.0)
SF36 physical function				
0	64.5 (23.6)	70.0 (50.00 – 83.8)	70.6 (22.0)	80.0 (57.5 – 85.0)
8	63.2 (25.1)	55.0 (40.00 – 90.0)	68.6 (22.7)	70.0 (60.0 – 85.0)
16	71.5 (21.1)	77.5 (55.0 – 88.8)	56.4 (21.5)	45.0 (40.0 – 85.0)

24	67.1 (26.0)	75.0 (50.0 – 85.0)	62.5 (24.0)	72.5 (42.5 – 80.0)
SF36 social function				
0	55.3 (21.4)	50.0 (38.0 – 81.8)	64.4 (22.6)	62.5 (41.0 – 88.0)
8	54.8 (25.5)	50.0 (38.0 – 75.0)	57.4 (25.8)	63.0 (38.0 – 75.0)
16	53.5 (28.2)	44.0 (28.3 – 88.0)	53.9 (24.6)	50.0 (38.0 – 75.0)
24	56.8 (24.6)	50.0 (38.0 – 81.5)	48.6 (25.3)	38.0 (38.0 – 75.0)
SF36 role physical				
0	35.0 (42.5)	12.5 (0.0 – 87.5)	21.9 (36.4)	0.0 (0.0 – 43.8)
8	42.1 (45.7)	25.0 (0.0 – 100.0)	53.6 (44.3)	75.0 (0.0 – 100.0)
16	51.3 (46.9)	62.5 (0.0 – 100.0)	14.3 (28.3)	0.0 (0.0 – 25.0)
24	45.2 (43.0)	50.0 (0.0 – 100.0)	17.9 (37.4)	0.0 (0.0 – 25.0)
SF36 role emotional				
0	68.3 (45.2)	100.0 (8.3 – 100.0)	70.9 (45.2)	100.0 (16.5 – 100.0)
8	66.6 (43.1)	100.0 (33.0 – 100.0)	85.7 (37.8)	100.0 (100.0 – 100.0)
16	63.4 (45.8)	100.0 (0.0 – 100.0)	66.6 (43.1)	100.0 (33.0 – 100.0)
24	71.4 (41.2)	100.0 (33.0 – 100.0)	57.1 (53.5)	100.0 (0.0 – 100.0)
SF36 mental health				
0	72.6 (17.6)	72.0 (58.0 – 86.0)	77.0 (17.2)	84.0 (59.0 – 88.0)
8	76.0 (16.3)	72.0 (64.0 – 92.0)	77.7 (26.1)	88.0 (76.0 – 92.0)
16	75.8 (18.1)	80.0 (58.0 – 91.0)	69.1 (23.6)	76.0 (64.0 – 84.0)
24	75.6 (15.3)	76.0 (62.0 – 90.0)	64.5 (29.2)	74.0 (42.0 – 83.8)
SF36 vitality				
0	51.5 (26.1)	47.5 (30.0 – 76.3)	40.0 (14.6)	40.0 (28.8 – 52.5)
8	53.2 (24.6)	45.0 (35.0 – 75.0)	41.4 (19.3)	45.0 (30.0 – 60.0)
16	56.8 (23.7)	57.5 (35.0 – 78.8)	35.7 (17.4)	35.0 (20.0 – 50.0)
24	53.1 (26.2)	50.0 (30.0 -75.0)	30.8 (20.8)	27.5 (12.5 – 52.5)
SF36 pain				
0	61.4 (24.3)	56.5 (41.0 – 81.5)	50.3 (26.3)	41.5 (41.0 – 68.3)
8	61.2 (23.4)	62.0 (41.0 - 74.0)	57.1 (23.9)	62.0 (41.0 – 62.0)
16	64.8 (22.1)	62.0 (43.5 – 84.0)	52.7 (25.8)	51.0 (32.0 – 62.0)
24	60.3 (27.7)	62.0 (41.0 – 79.5)	52.8 (25.9)	46.0 (31.8 – 71.5)
SF 36 general health				
0	41.9 (24.4)	39.5 (25.0 – 63.0)	44.9 (16.2)	40.0 (32.5 – 60.5)
8	45.5 (22.6)	40.0 (25.0 – 62.0)	49.1 (17.1)	45.0 (35.0 – 67.0)
16	48.8 (21.0)	40.0 (35.0 – 65.8)	44.4 (17.3)	47.0 (25.0 – 57.0)
24	49.9 (21.3)	47.0 (32.0 – 68.5)	41.9 (18.1)	47.0 (25.0 – 52.0)
SF36 general health change				

0	37.5 (22.2)	25.0 (25.0 – 50.0)	34.4 (18.6)	37.5 (25.0 – 50.0)
8	46.1 (28.0)	50.0 (25.0 – 75.0)	35.7 (13.4)	25.0 (25.0 – 50.0)
16	51.3 (27.5)	50.0 (25.0 – 68.8)	32.1 (20.8)	25.0 (25.0 – 50.0)
24	47.6 (23.6)	50.0 (25.0 – 62.5)	32.1 (18.9)	25.0 (25.0 – 50.0)

Table S1. Mean (SD) and median (IQR) values of all endpoints at 0, 8, 16 and 24 weeks, divided by treatment arm.

Abbreviations: ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index ; UWS, unstimulated whole saliva flow; SWS, stimulated whole saliva flow; VAS oral, Visual Analogue Scale for oral dryness; VAS ocular; Visual Analogue Scale for ocular dryness; GA patient, global assessment from patient's point of view; GA physician, global assessment from physician's point of view; sIgG, serum IgG level; IgM RF, IgM rheumatoid factor; C3, complement factor C3; C4, complement factor C4; MFI, multidimensional fatigue inventory; SF-36, short form-36 health survey; n.a., not applicable.

Table S2. Mean difference of health-related questionnaires MFI and SF-36

	8 weeks			**16 weeks**			**24 weeks**		
	Effect size	**95% CI**	**p-value**	**Effect size**	**95% CI**	**p-value**	**Effect size**	**95% CI**	**p-value**
MFI									
General fatigue	0.21	-2.19 to 2.61	0.86	-3.32	-6.69 to 0.043	0.053	-2.86	-5.78 to 0.061	0.055
Physical fatigue	1.24	-1.85 to 4.34	0.83	-1.64	-4.36 to 1.078	0.22	-1.10	-4.39 to 2.19	0.50
Mental fatigue	-0.78	-5.16 to 3.61	0.72	-1.17	-4.26 to 1.93	0.44	-0.30	-4.35 to 3.75	0.88
Reduced motivation	-0.47	-3.42 to 2.48	0.74	-2.26	-4.38 to -0.15	0.037	-1.84	-4.04 to 0.36	0.097
Reduced activity	0.46	-2.75 to 3.67	0.77	-1.46	-4.25 to 1.32	0.29	-1.75	-5.10 to 1.61	0.29
SF-36									
Physical function	0.80	-14.26 to 15.86	0.91	20.20	5.12 to 35.28	0.011	13.90	-1.01 to 28.81	0.066
Social function	5.13	-16.03 to 26.29	0.62	5.30	-10.73 to 21.31	0.501	14.30	1.91 to 26.70	0.026
Role physical	-22.32	-54.85 to 10.20	0.17	29.48	-3.68 to 62.65	0.079	18.63	-8.84 to 46.09	0.17
Role emotional	-17.06	-54.53 to 20.41	0.36	-5.89	-44.14 to 32.36	0.75	12.22	-25.64 to 50.08	0.51
Mental health	4.79	-5.19 to 14.76	0.33	9.37	-1.48 to 20.22	0.087	17.99	5.43 to 30.55	0.0070
Vitality	1.55	-15.24 to 18.33	0.85	12.15	-2.58 to 26.87	0.10	11.36	-3.94 to 26.66	0.14

Pain	-2.84	-20.48 to 14.79	0.74	4.91	-10.63 to 20.45	0.52	-0.29	-16.84 to 16.26	0.97
General health	-1.53	-10.97 to 7.92	0.74	5.35	-6.97 to 17.66	0.38	8.57	-3.47 to 20.60	0.16
General health change	5.84	-17.02 to 28.69	0.60	18.83	-6.44 to 44.09	0.14	15.06	-6.00 to 36.12	0.15

Table 4.S2. Effect sizes show the difference in the lefunomide-hydroxychloroquine group (n=21) compared with the placebo group (n=8). Health related questionnaires Multidimenstional Fatigue Inventory (MFI) and Short Form-36 (SF-36). Effect size with 95% confidence intervals for secondary endpoints MFI and SF-36 are given.

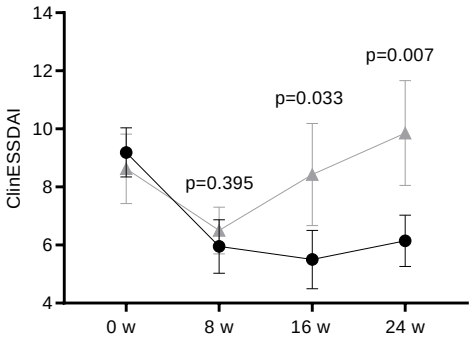
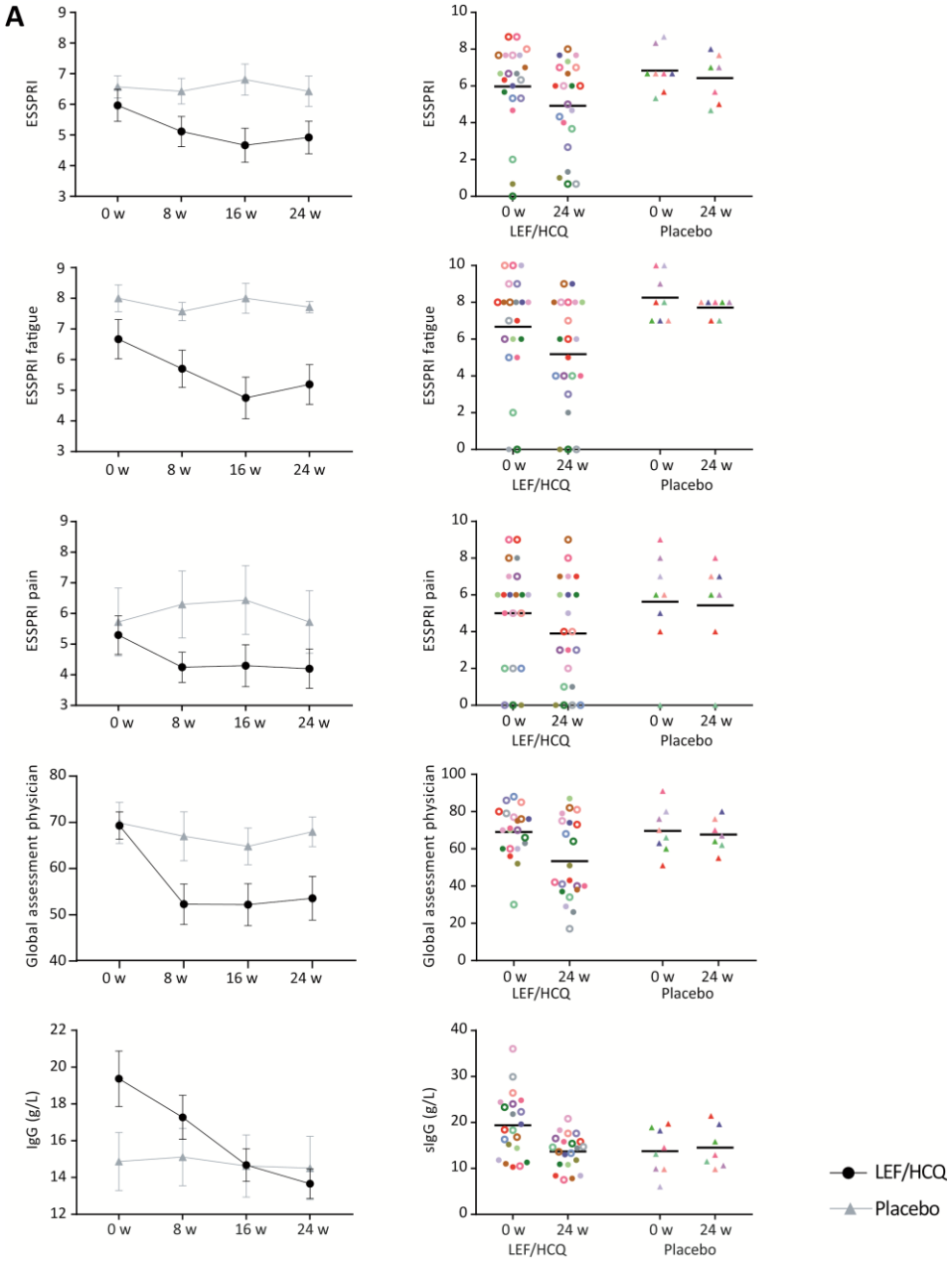


Figure S1. ClinESSDAI scores over 24 weeks treatment with either leflunomide plus hydroxychloroquine or placebo/placebo. Mean ClinESSDAI scores (± SEM) for both arms over time are shown. The p-values indicate the effect of treatment compared to placebo at 8, 16 and 24 weeks corrected for baseline values.


A
ESSPRI
ESSPRI fatigue
ESSPRI pain
Global assessment physician
IgG (g/L)
sIgG (g/L)
0 w
8 w
16 w
24 w
LEF/HCQ
Placebo
LEF/HCQ
Placebo

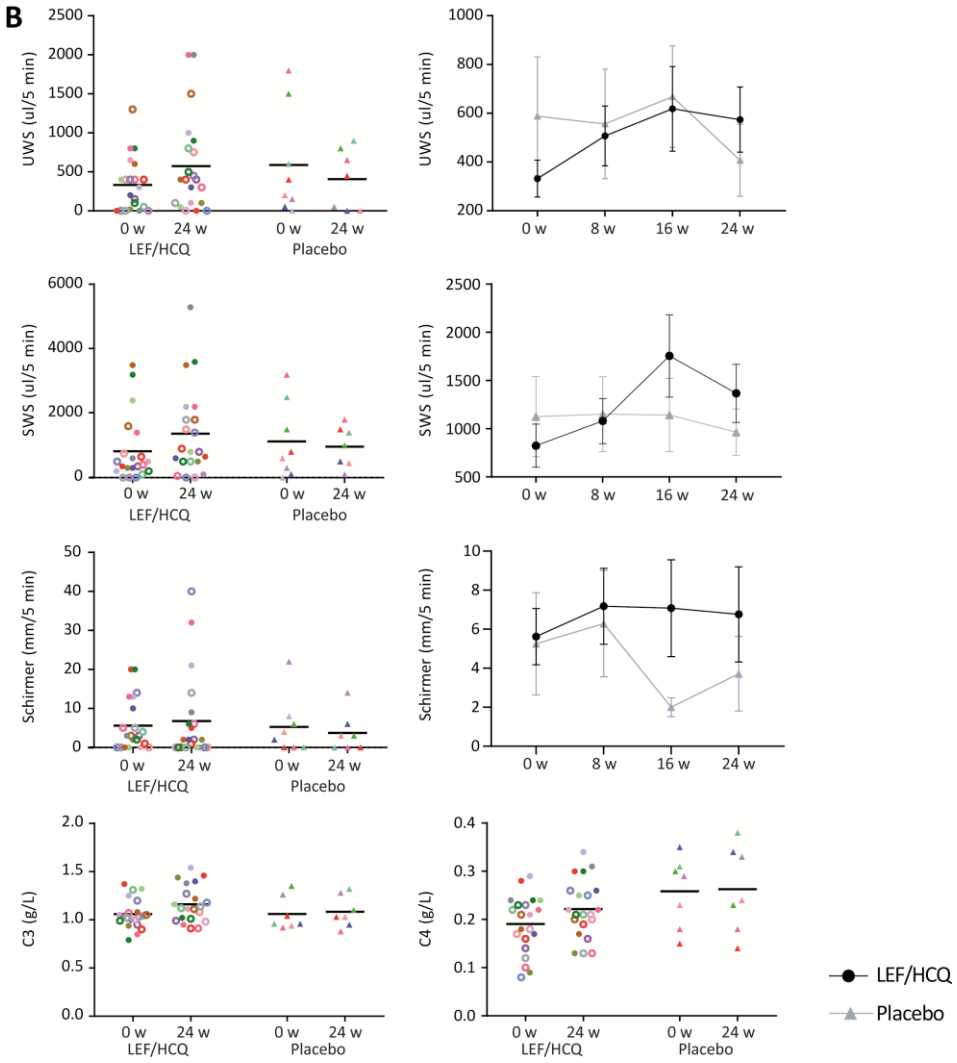


Figure S2. Treatment effect on secondary clinical endpoints in LEF/HCQ and placebo arms. Panel A shows mean scores (±SEM) at all time points for secondary endpoints ESSPRI, ESSPRI fatigue, ESSPRI pain, global assessment scored by the physician and serum IgG. In addition, scores of individual patients at baseline and 24 weeks are depicted. Patients are represented by the same colored symbol at baseline and 24 weeks. Mean values are indicated. Relevant effects on these endpoints were seen upon LEF/HCQ treatment compared to placebo treatment, when corrected for baseline values. Panel B shows mean scores (±SEM) at all time points for secondary endpoints UWS and SWS. In addition, scores of individual patients at baseline and 24 weeks are

depicted for secondary endpoints UWS, SWS and complement factors C3 and C4. Patients are represented by the same colored symbol at baseline and 24 weeks. Mean values are indicated by lines.

Abbreviations: ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index; sIgG, serum IgG; UWS, unstimulated whole saliva; SWS, stimulated whole saliva.

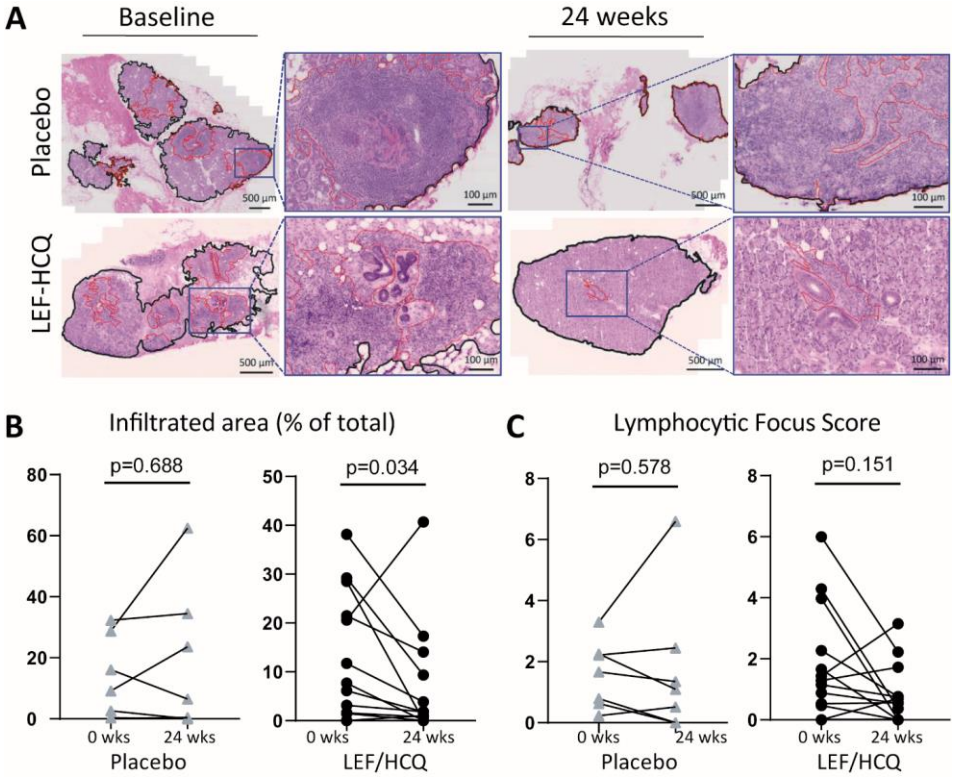


Figure S3. Parotid inflammation assessed based on haematoxylin and eosin (H&E) staining. Panel A shows representative HE staining of parotid gland biopsy specimens obtained from a patient treated with placebo (upper part) and a patient treated with LEF/HCQ (lower part) at baseline and 24 weeks.

Black lines highlight total glandular tissue considered in the analysis; red lines indicate the infiltrated areas. Blue rectangles indicate areas of image magnification illustrated on the right of each tile scan. Scale bars= 500µm for tile scans and 100µm in higher magnifications.

Parotid inflammation was quantified by inflammatory area (panel B) and number of lymphocytic foci (panel C). p-values indicate the statistical significance compared to baseline using Wilcoxon signed-rank test.

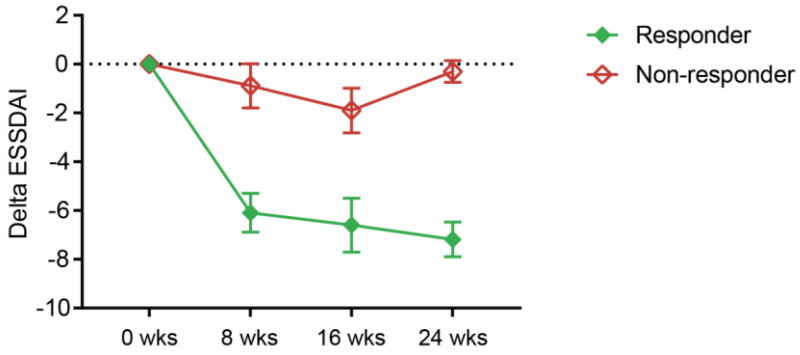


Figure S4. Change in ESSDAI in non-responders vs. responders to LEF/HCQ therapy. The figure shows mean delta ESSDAI scores (±SEM) over time for patients responding to LEF/HCQ treatment (ESSDAI decrease≥3) and patients not responding to therapy (ESSDAI decrease ≤3).

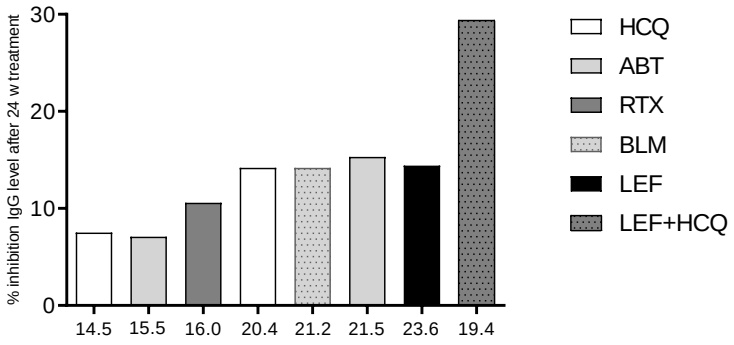


Figure S5. Serum IgG inhibition measured in RCTs investigating different treatments in pSS. The bars show the percentage of sIgG inhibition after 24 weeks of treatment, the baseline sIgG values are indicated below the bars.

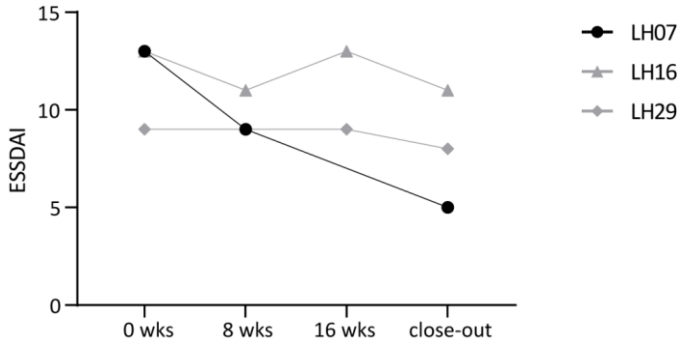


Figure S6. Patients with last observed value carried forward.

Actual measured individual ESSDAI scores of the three patients that discontinued medication and underwent a close-out visit are shown. Medication was discontinued after 10 weeks (LH07), 16 weeks (LH16) and 16 weeks (LH29). For statistical analysis results of the last visit were carried forward for the 24 weeks analysis. Statistical analysis with the actual measured endpoints or last observation carried forward of the three patients did not change the outcome. Black line indicates patient treated with LEF/HCQ, grey line indicates patients receiving placebo. Patient LH16, indicated by rectangular symbols, underwent blinded 0, 8 and 16 wks measurements, and a de-blinded close-out visit.

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Chapter 5

Serum Galectin-9 levels can be used to monitor clinical response to leflunomide/hydroxychloroquine combination therapy in patients with Sjögren's syndrome

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Submitted for publication

Chapter overview

Background. A recent clinical trial showed clinical efficacy for combination therapy with leflunomide (LEF) and hydroxychloroquine (HCQ) in patients with primary Sjögren's syndrome (RepurpSS-I). We assessed the relevance of several interferon (IFN)-signature associated biomarkers and their potential for treatment monitoring.

Methods. In the 21 patients treated with LEF/HCQ and 8 patients treated with placebo, blood was drawn at baseline, 8, 16 and 24 weeks. IFN-score was quantified in circulating immune cells and whole blood. MxA protein level was measured in whole blood, and levels of CXCL10 and Galectin-9 were quantified in serum. Differences between responders and non-responders were assessed and ROC analysis was used to determine the capacity of early changes in biomarkers to predict treatment response at the clinical endpoint

Results. IFN-signature biomarkers decreased after 24 weeks of LEF/HCQ treatment. A decrease in circulating MxA and Galectin-9 was associated with clinical response. Changes in serum Galectin-9 after 8 weeks of treatment could be used to predict clinical response at 24 weeks (AUC 0.90).

Discussion. Galectin-9 is a promising biomarker for treatment monitoring in pSS patients treated with LEF/HCQ. Our data indicate that LEF/HCQ combination therapy targets type-I IFN activity and is associated with decreased B cell hyperactivity.

Introduction

Primary Sjögren's syndrome (pSS) is characterized by mononuclear infiltration of the exocrine glands, associated with dysfunction of the glands and dryness of primarily mouth and eyes.[1] The majority of pSS patients presents with an interferon (IFN)-signature, reflecting increased expression of type-I IFN-induced genes in circulating immune cells, which is associated with increased systemic disease activity.[2] Plasmacytoid dendritic cells (pDCs) are the premier type-I IFN-producing immune cells and as pDCs from patients with pSS produce enhanced levels of type-I IFN upon activation,[3] they appear to be important IFN-producers in pSS. Type-I IFNs importantly drive hyperactivity of B cells, enhancing production of auto-antibodies and formation of immune-complexes.[4,5] As immune complexes in turn activate pDCs, pDCs and B cells form an amplification loop that is a potential therapeutic target in pSS.[6]

We recently performed a double-blind randomized placebo-controlled clinical trial to test the clinical efficacy of leflunomide (LEF) and hydroxychloroquine (HCQ) combination therapy in patients with pSS (RepurpSS-I). In this study, we demonstrated a clear clinical effect in the treated patients compared to the placebo group.[7] Seeing as both LEF and HCQ inhibit B cell-activation while HCQ also inhibits activation of pDCs via endosomal Toll-like receptors,[8,9] we hypothesized that targeting the pDC/B cell amplification loop is a mode of action of LEF/HCQ combination therapy. As such, we here evaluated the effects of LEF/HCQ on the IFN-score and three other biomarkers of type-I IFN activity. In addition, as the IFN-signature is associated with disease activity, we evaluated whether changes in these biomarkers could be of use for monitoring treatment response to LEF/HCQ.

Patients and Methods

See Supplementary Patients and Methods section for further details.

Patients and study design

Twenty-nine patients fulfilling the classification criteria for pSS[10] with clinically active disease (ESSDAI ≥5) were enrolled in a randomized, double-blind, and placebo-controlled trial investigating efficacy and safety of LEF/HCQ combination therapy. After randomization, 21 patients were assigned to LEF/HCQ treatment, 8 patients to placebo treatment (Table 1). Clinical assessment was performed after 8 and 16 weeks, and at the clinical endpoint (24 weeks). Patients showing a decrease of ≥3 ESSDAI points were considered clinical responders, according to prevailing recommendations.[11] See [7] for further details. The study protocol was reviewed and approved by the medical ethical committee of the University Medical Center Utrecht. All patients provided written informed consent.

	LEF/HCQ (n=21)	**Placebo (n=8)**
Age, mean (SD), y	54.7 (12.4)	53.5 (15.2)
Female, no. (%)	20 (95)	8 (100)
Disease duration in years, mean (SD)	7.7 (9.8)	8.9 (6.8)
ESSDAI score, mean (SD)	10.4 (3.9)	9.1 (3.4)
ESSPRI score	6.7 (5.3 – 7.7)	6.7 (5.9 – 7.9)
Serum IgG, mean (SD), g/L *	19.4 (6.9)	13.8 (5.0)
Unstimulated saliva flow, ul/5 min	300 (10 – 500)	300 (75 – 1275)
Stimulated saliva flow, ul/5 min	400 (200 – 1075)	700 (150 – 2250)
IFN-score PBMCs	4.8 (2.4 – 5.5)	5.0 (2.2 – 5.6)
IFN-score whole blood	2.4 (1.5 – 2.9)	2.4 (1.2 – 2.9)
MxA protein expression whole blood	277 (104 – 442)	226 (32.8 – 292)
Serum CXCL10, pg/ml *	620 (335 – 1181)	360 (251 – 562)
Serum Galectin-9, ng/ml *	12.9 (7.7 – 17.2)	10.1 (8.8 – 17.5)

Table 1. Baseline characteristics . Data are depicted as median (IQR) unless stated otherwise. * indicates significant difference in baseline values between the two treatment arms at $p<0.05$.

Quantification of IFN-signature biomarkers

Peripheral blood mononuclear cell (PBMCs) and whole blood (WB) IFN-scores were quantified as previously described[2] using material from 13 and 23 healthy donors for comparison, respectively. Myxovirus resistance protein A (MxA) enzyme immunoassay was done as previously described.[12] Measurement of serum CXCL10 and Galectin-9 levels was performed by multiplex immunoassay as previously described.[13]

Results

IFN-signature biomarkers decrease upon LEF/HCQ treatment

At each time point during the trial (baseline, 8 weeks, 16 weeks, 24 weeks), we measured five different biomarkers that represent the IFN-signature and are increased in patients with pSS: the IFN-score quantified in PBMCs and WB,[2] WB MxA protein,[12] serum CXCL10[14] and serum Galectin-9.[15] In addition, we included serum IgG in our analyses as a conventional laboratory parameter for B cell hyperactivity as comparison. The IFN-signature biomarkers correlate with one another and with the ESSDAI in published cross-sectional studies, but their relation to ESSDAI has not previously been analyzed in longitudinal data of patients who show clinical response to therapy. At baseline, the biomarkers generally correlated with each other and the ESSDAI (Supplementary Table 1), consistent with literature. After 24 weeks of LEF/HCQ treatment, all biomarkers except the PBMC IFN-score showed a significant decrease compared to baseline (figure 1). Similarly, when assessed over time compared to placebo and corrected for baseline, all but the PBMC IFN-score showed a significant decrease in the LEF/HCQ treatment group (Supplementary figure 1). Interestingly, changes in ESSDAI (ΔESSDAI) at 24 weeks only correlated with changes in the IFN-induced proteins MxA, Galectin-9, and CXCL10; but not with changes in the IFN-scores or serum IgG. ΔESSPRI only correlated with ΔCXCL10 (supplementary table 2).

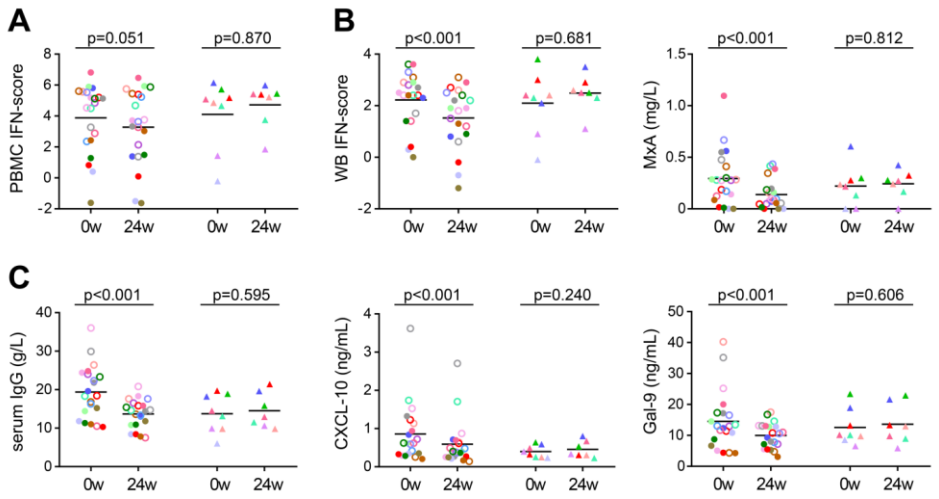

Figure 1. IFN-signature biomarkers decrease upon LEF/HCQ combination treatment. Blood was drawn from patient from the LEF/HCQ and placebo groups at baseline and 24 weeks. Six biomarkers associated with the IFN-signature were quantified in peripheral blood mononuclear cells (PBMCs) (A), whole blood (WB) (B), and serum (C). Differences between 24 weeks and baseline within the LEF/HCQ and Placebo groups were evaluated. Means are shown.

No differences in response between baseline IFN-signature positive and negative patients

Of the 21 LEF/HCQ treated patients, 11 were responders at the clinical endpoint. There was a clear difference in ΔESSDAI between the responders (mean -7.2) and non-responders (mean -0.2) after 24 weeks of treatment (supplementary figure 2). As such, finding molecular differences between these two groups could help elucidate the mode-of-action of LEF/HCQ and could be of use for biomarker discovery. We first assessed whether patients with a baseline IFN-signature (based on either IFN-score) would be more likely to respond to treatment. However, there were no differences in ΔESSDAI or in the fraction of responders between IFN-signature negative and positive patients using either method (supplementary figure 3).

MxA and Galectin-9 are promising biomarkers for monitoring of treatment response

We next evaluated the differences between responders and non-responders in the decrease of IFN-signature biomarkers over time. Consistent with the lack of correlation between ΔESSDAI and

ΔIFN-scores, there were no differences in ΔIFN-scores between responders and non-responders at any timepoint (figure 2A,B). Levels of serum CXCL10 and serum IgG decreased strongly in the responders but also went down in the non-responders, leading to significant differences at single timepoints (figure 2C). Strikingly, after only 8 weeks of treatment responders showed clear changes in both MxA and Galectin-9 levels, while non-responders showed changes comparable to those in the placebo group (figure 2B,C). As such, changes in MxA and Galectin-9 between baseline and 8 weeks could potentially be used to monitor treatment response.

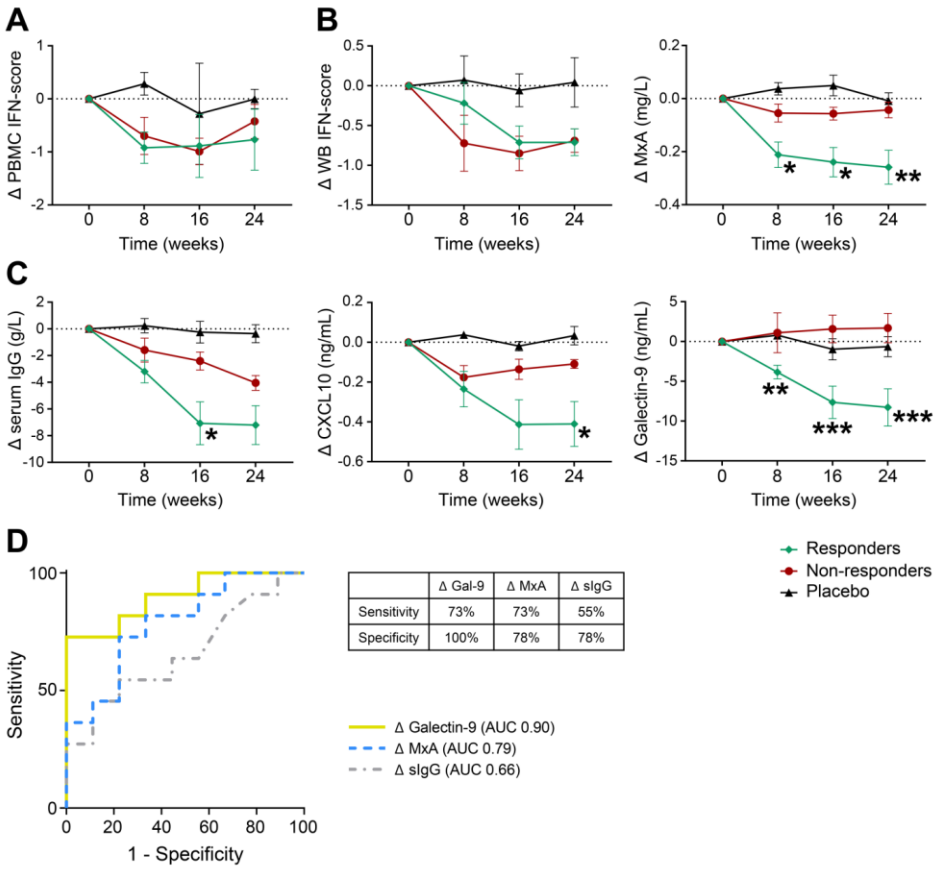

Figure 2. Early changes in Galectin-9 can be used to predict clinical response after 24 weeks. Changes over time in the biomarkers quantified in PBMCs (A), WB (B), and serum (C). Responders: LEF/HCQ-treated patients who showed a decrease in

ESSDAI of ≥3 points at 24 weeks (green diamonds). Non-responders: LEF/HCQ-treated patients who did not meet clinical response criteria (red circles). Placebo: the placebo-treated group (black triangles). Receiver Operator Characteristic analysis was used to assess whether changes in quantified biomarkers between 8 weeks and baseline could be used to predict clinical response at 24 weeks. Area under the curve (AUC), Sensitivity, and Specificity are given for serum IgG and the markers that outperformed serum IgG (D). Mean ±SEM are shown. *,**, and *** indicate p<0.05, p<0.01, and p<0.001 respectively.

To evaluate this, the changes in MxA and Galectin-9 between baseline and 8 weeks were used to discriminate responders from non-responders using ROC analysis. For comparison, changes in serum IgG at 8 weeks showed mediocre potential for predicting clinical response at 24 weeks (AUC 0.66). Strikingly, both ΔMxA (AUC 0.79) and ΔGalectin-9 (0.90) substantially outperformed Δserum IgG, and ΔGalectin-9 in particular showed promise as a biomarker with a specificity of 100% and a sensitivity of 73% for predicting clinical response at 24 weeks (figure 2D). ΔPBMC IFN-score (AUC 0.55), ΔWB IFN-score (AUC 0.64), and ΔCXCL10 (AUC 0.52) did not outperform Δserum IgG.

Discussion

Treatment with LEF/HCQ decreased systemic type-I IFN-activity in patients with pSS, in-line with literature on HCQ monotherapy.[16] Surprisingly, changes in ESSDAI were robustly associated with changes in IFN-induced proteins MxA and Galectin-9, but not with changes in either IFN-score, even though these markers typically correlate in cross-sectional studies. This discrepancy may be explained by a difference in compartments: the circulating proteins are likely produced at the site of inflammation, in-line with production of Galectin-9 in skin and muscle tissue from patients with juvenile dermatomyositis,[17] while the IFN-scores are quantified in circulating immune cells. As circulating cells are long-lived, it may take longer for changes in local inflammation to be reflected at the gene-expression level in circulating cells. Alternatively, differences in regulation between protein and RNA expression may contribute. In this respect, we found only a moderate correlation between ΔMxA and changes in expression of its gene *MX1*, which makes up part of the IFN-score, within whole blood (r=0.44, p=0.044).

Especially serum Galectin-9 seems to be an excellent biomarker for monitoring treatment response to LEF/HCQ combination therapy in patients with pSS. Changes in serum Galectin-9 after 8 weeks of treatment can robustly predict which patients respond to LEF/HCQ at 24 weeks. This is particularly relevant in light of the decrease in ESSDAI observed in the placebo group after 8 weeks of treatment (no longer present after 16 and 24 weeks)[7]; in contrast, Galectin-9 levels do not show a decrease throughout the trial in the placebo group. Combining treatment monitoring with serum Galectin-9 and the described baseline prediction-model based on the serum proteome[7] can greatly reduce the number of patients who receive inefficacious long-term treatment, adding to cost-effectiveness and preventing unnecessary side-effects. To test the applicability of these data in a larger population of pSS patients, they will be replicated in a follow-up clinical trial (RepurpSS-II).

The decreased type-I IFN activity and B cell hyperactivity in treated patients as well as the specific decrease in MxA and Galectin-9 observed in responders, suggest that LEF/HCQ combination therapy thwarts the pDC/B cell amplification loop. The lack of differences in response between IFN-positive and IFN-negative patients at baseline may be due to the low numbers of IFN-negative patients, which makes drawing definitive conclusions on this comparison difficult. Alternatively, the observed decrease in Galectin-9 levels may be in part driven via inflammatory pathways other than type-I IFN, such as IFN-γ and TNF-α which are known to induce its production.[18]

Thus, we show that serum Galectin-9 is a promising biomarker for monitoring of clinical response to LEF/HCQ combination therapy. In addition, our data indicate that the pDC/B cell amplification loop is a target of LEF/HCQ therapy. These data represent the first analysis of the relationship between IFN-activity and clinically relevant changes in ESSDAI within longitudinal data, and provide compelling evidence for a central role of IFN-activity in pSS immunopathology.

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Supplementary material

Supplementary Patients and Methods

Quantification of IFN-signature biomarkers

PBMCs were isolated from heparinized peripheral blood using Ficoll-Paque Plus (GE Healthcare) and stored at -80°C. Whole blood (WB) samples were collected in PaxGene tubes (Qiagen) and stored at -20°C. RNA was isolated using the All prep Universal Kit (Qiagen) using a Qiacube (Qiagen) according to the manufacturer's instructions. IFN-scores were quantified as previously described[1,2] based on the expression of *MX1, IFIT3, IFI44, IFI44L,* and *Ly6E* using material from 13 (PBMC) and 23 (WB) healthy donors for comparison. IFN-signature positivity was defined as an IFN-score above the mean +2SD of scores in the healthy donors.

For MxA quantification, heparinized blood (25 µl) was lysed 1:20 and stored at -80°C until analysis. Myxovirus resistance protein A (MxA) enzyme immunoassay was done as previously described.[3] Briefly, samples and biotinylated detector-monoclonal antibody (MAb) were loaded onto MAb-coated microtiter strips. After overnight incubation at 8°C, colour reaction was stopped, absorbance at 450nm was measured, and MxA concentration was read from a standard curve. The detection limit was determined as three times the standard deviation of eight negative control replicates (10 µg/l).[4]

Serum was collected in SST-II vacutainers (BD) according to manufacturer's instructions and stored at -80°C until analysis. Measurement of CXCL10 and Galectin-9 levels was performed using multiplex immunoassay as previously described.[5]

Statistical analysis

To evaluate changes in parameters between baseline and clinical endpoint within each group and the differences between LEF/HCQ and placebo arms over time, a random intercept model was applied to account for repeated measures within patients, controlling for time and baseline values. To assess differences in changes in parameters between responders and non-responders,

Mann-Whitney U-test was used. Differences in the fraction of responders and non-responders between groups were evaluated using Fisher's exact test. Receiver Operating Characteristic (ROC) curves were used to determine the capacity of changes in biomarkers after 8 weeks of treatment to predict treatment response at the clinical endpoint. Spearman's correlation coefficient was used to assess correlations. Differences were considered to be statistically significant at $p<0.05$. Analyses were performed using SPSS version 25 and GraphPad Prism version 8.

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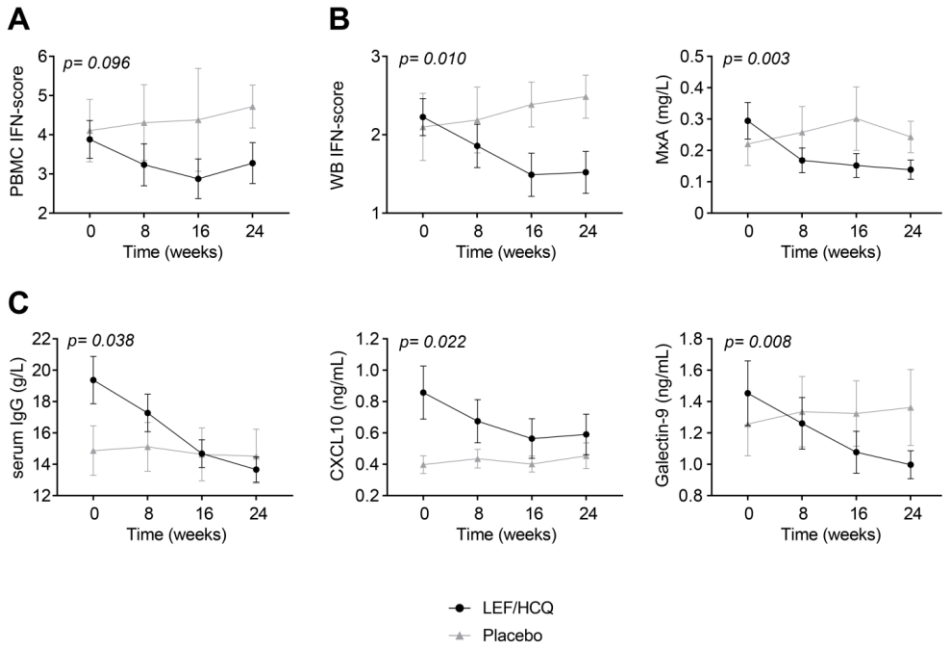
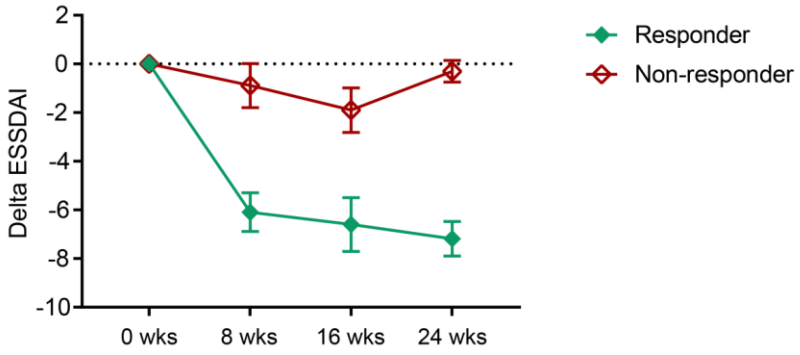
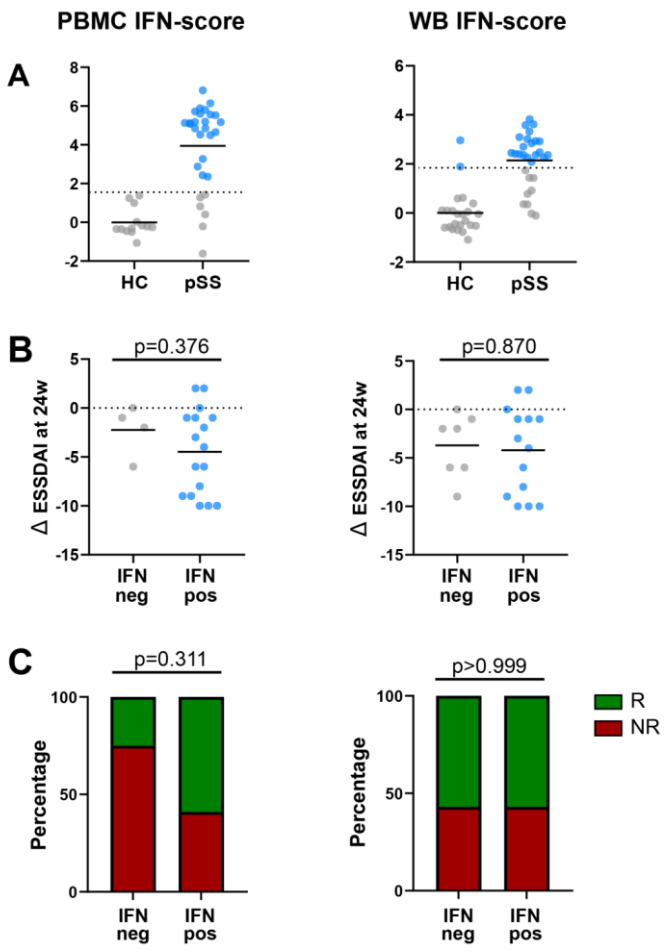


Supplementary Figure 1. Changes in IFN-signature biomarkers over time. Levels of measured biomarkers in LEF/HCQ (black dots) and placebo (grey triangles) treated patients during the trial. Biomarkers were measured in PBMCs (A), WB (B), or serum (C). Mean ± SEM are shown. Changes in LEF/HCQ-treated group compared to the Placebo group and corrected for baseline are evaluated using a random intercept model.



Supplementary Figure 2. Changes in ESSDAI in responders and non-responders over time. The figure shows mean delta ESSDAI scores (±SEM) over time for patients responding to LEF/HCQ treatment (ESSDAI decrease≥3; green) and patients not responding to therapy (ESSDAI decrease <3; red).



Supplementary Figure 3. No differences in clinical response between IFN-signature positive and negative patients. The IFN-score was quantified in PBMCs (left) and WB (right) of all 29 pSS patients included in the trial as well as a set of healthy donors. IFN-signature positive donors are shown in blue, negative donors in gray (A). Changes in ESSDAI between baseline and 24 weeks were compared between IFN-signature negative (IFN neg) and IFN-signature positive (IFN pos) patients within the LEF/HCQ treated group (n=21) (B). Differences in the fraction of responders and non-responders within the IFN neg and IFN pos groups were evaluated using Fisher's exact test (C). Means are shown.

	PBMC IFN-score	WB IFN-score	MxA	CXCL10	Gal-9	serum IgG	ESSDAI	ESSPRI	UWS	SWS
PBMC IFN-score	1.000	**0.750***	**0.826***	0.342	**0.483***	**0.524***	**0.407***	-0.039	**0.431***	0.344
WB IFN-score	**0.750***	1.000	**0.692***	0.267	**0.453***	**0.588***	**0.410***	-.0213	**0.445***	**0.412***
MxA	**0.826***	**0.692***	1.000	**0.558***	**0.690***	**0.670***	**0.394***	-0.310	0.075	0.035
CXCL10	0.342	0.267	**0.558***	1.000	**0.786***	**0.685***	0.312	-0.099	-0.269	-0.313
Gal9	**0.483***	**0.453***	**0.690***	**0.786***	1.000	**0.700***	**0.413***	-0.214	-0.181	-0.243
serum IgG	**0.524***	**0.588***	**0.670***	**0.685***	**0.700***	1.000	0.152	-0.215	-0.024	-0.146
ESSDAI	**0.407***	**0.410***	**0.394***	0.312	**0.413***	0.152	1.000	-0.101	0.064	0.270
ESSPRI	-0.039	-.0213	-0.310	-0.099	-0.214	-0.215	-0.101	1.000	0.096	0.030
UWS	**0.431***	**0.445***	0.075	-0.269	-0.181	-0.024	0.064	0.096	1.000	**0.835***
SWS	0.344	**0.412***	0.035	-0.313	-0.243	-0.146	0.270	0.030	**0.835***	1.000

Supplementary Table 1. Correlations of IFN-signature biomarkers and clinical parameters in all patients at baseline. Spearman correlations are shown. Significant correlations are indicated in bold, * indicates $p<0.05$.

	Δ PBMC IFN-score	Δ WB IFN-score	Δ MxA	Δ CXCL10	Δ Gal-9	Δ serum IgG	Δ ESSDAI	Δ ESSPRI	Δ UWS	Δ SWS
Δ PBMC IFN-score	1.000	**0.486***	**0.463***	**0.388***	0.217	**0.455***	0.138	0.315	-0.243	**-0.476***
Δ WB IFN-score	**0.486***	1.000	0.293	-0.107	0.060	0.255	-0.052	-0.088	0.089	0.008
Δ MxA	**0.463***	0.293	1.000	**0.749***	**0.731***	**0.661***	**0.556***	0.368	-0.188	-0.312
Δ CXCL10	**0.388***	-0.107	**0.749***	1.000	**0.612***	**0.644***	**0.447***	**0.627***	-0.284	-0.415
Δ Gal9	0.217	0.060	**0.731***	**0.612***	1.000	**0.599***	**0.692***	0.392	-0.136	-0.289
Δ serum IgG	**0.455***	0.255	**0.661***	**0.644***	**0.599***	1.000	0.243	0.161	-0.403	-0.355
Δ ESSDAI	0.138	-0.052	**0.556***	**0.447***	**0.692***	0.243	1.000	**0.450***	-0.152	-0.260
Δ ESSPRI	0.315	-0.088	0.368	**0.627***	0.392	0.161	**0.450***	1.000	-0.061	**-0.436***
Δ UWS	-0.243	0.089	-0.188	-0.284	-0.136	-0.403	-0.152	-0.061	1.000	**0.804***
Δ SWS	**-0.476***	0.008	-0.312	-0.415	-0.289	-0.355	-0.260	**-0.436***	**0.804***	1.000

Supplementary Table 2. Correlations of the changes in IFN-signature biomarkers and clinical parameters in the LEF/HCQ treated patients at 24 weeks of treatment. Spearman correlations are shown. Significant correlations are indicated in bold, * indicates p<0.05.

Chapter 6

Galectin-9 reflects the interferon signature and correlates with disease activity in systemic autoimmune diseases

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With much interest, we read the comments1 of our colleagues Yavuz and Rönnblom regarding our manuscript on galectin-9 as an easy to measure biomarker to detect the interferon (IFN) signature in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS).[2] We thank them for their interest in our manuscript and their shared interest in the importance of the IFN signature in SLE and the subsequent need for more easily applicable markers to detect IFN activity. In the paper, we reported independently replicated correlations of galectin-9 with the IFN signature in patients with SLE and APS. The aim of our manuscript was to identify more easy to measure markers to detect the IFN signature.

Besides serving as a marker for the IFN signature, we found that galectin-9 was elevated in patients with SLE and APS and correlated with signs of disease activity including SLEDAI. We agree with the authors that the use of galectin-9 as a marker for IFN activity needs further confirmation before clinical implementation, and studies in which longitudinal samples and patients with different ethnic backgrounds are tested are among the next steps. Other candidates include the direct measurement of IFNs by digital ELISA,[3] although these may only capture one subtype of IFN at a time.[2] As galectin-9 expression is induced by IFNs, galectin-9 is elevated in several (viral) infectious diseases and, similar to the IFN signature, may not be helpful in distinguishing (viral) infections from SLE disease activity, as discussed by Yavuz and Rönnblom. Additionally, the authors mention a protective effect of galectin-9 administration in murine models of SLE. Indeed, recent studies in lupus-like animal models support a role for galectin-9 in the pathogenesis of SLE.[4,5] Its effects in these animal models however are 'perplexing',[6] as the effects of a LGALS9 knock-out in pristane-induced lupus has opposite effects compared with galectin-9 administration in BXSB/MpJ and NZB/W F1 mice.4-6 Importantly, until our recent study, no data were available on galectin-9 levels in patients with SLE. Therefore, further studies to investigate the pathophysiological role of galectin-9 and studies in human patients with SLE are certainly relevant. Besides SLE and APS,[7,8] the IFN signature is present in several systemic autoimmune diseases including primary Sjögren's syndrome (pSS), systemic sclerosis and (juvenile) dermatomyositis (JDM).[9-11] In this regard, elevated levels of galectin-9 have also been reported in patients with JDM, correlating with

treatment-induced changes in disease activity in longitudinal samples.[12] In addition, we found elevated levels of galectin-9 in patients with pSS as compared with patients with non-Sjögren sicca, correlating with disease activity as assessed by ESSDAI (EULAR Sjögren's Syndrome Disease Activity Index) and serum IgG levels (figure 1). Notably, in a randomized placebo-controlled trial in patients with pSS,[13] we observed that successful disease inhibition by leflunomide/hydroxychloroquine combination therapy was associated with downregulation of galectin-9 levels (compared with no change in the placebo group) and changes in galectin-9 levels paralleled changes in markers of IFN activity, B-cell hyperactivity and disease activity (ESSDAI/ESSPRI) (manuscript in preparation). Therefore, serum levels of galectin-9 are a promising marker to assess the IFN signature and correlate with changes in disease activity in patients with different autoimmune diseases.

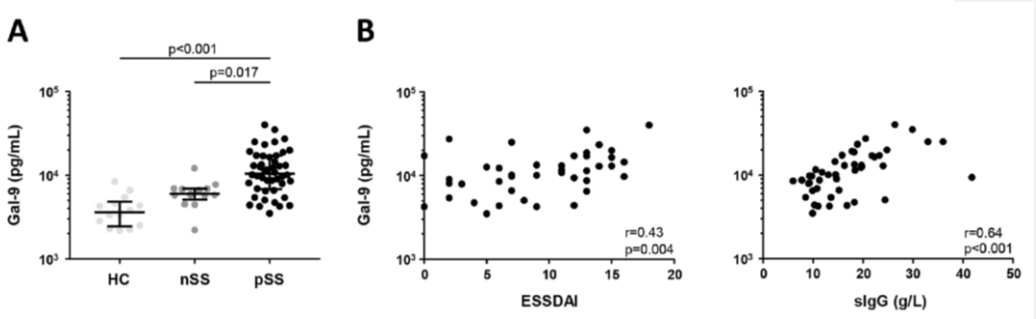

Figure 1 Increased circulating levels of galectin-9 in patients with pSS are associated with disease activity and serum IgG. Galectin-9 levels were measured in the serum of HC (n=14), patients with nSS (n=13) and patients with pSS (n=46) by Luminex multianalyte measurements. Patients with pSS had clearly increased circulating levels of Gal-9 compared with nSS and HC donors (A). In patients with pSS, serum Gal-9 levels correlated with ESSDAI and serum IgG (B). Differences between the groups were assessed using Kruskal-Wallis test with posthoc Dunn's multiple comparisons test, correlations were assessed using Spearman's correlation coefficient. ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; HC, healthy controls; nSS, non-Sjögren's sicca; pSS, primary Sjögren's syndrome.

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Chapter 7

Summary and discussion

Summary

Central to the immune-pathology of pSS is broad immune-activation involving a wide variety of immune cells, ultimately resulting in B cell hyperactivity and in part of the patients formation of ectopic lymphoid structures. In the absence of an effective therapeutic option, symptoms can only be (insufficiently) relieved by symptomatic measures. Considering the complex interplay of the involved immune cells and their effector molecules, the central hypothesis to this thesis is that successful inhibition of immune-activation in pSS requires a combination of drugs targeting both overlapping and distinct immune-pathological pathways, jointly resulting in broad immune inhibition.

Based on their properties, the combination of the established cDMARDs leflunomide en hydroxychloroquine holds great promise considering the above mentioned hypothesis. In this thesis, the efficacy (in vitro and in vivo) and safety of leflunomide and hydroxychloroquine combination therapy in pSS patients was investigated.

A detailed overview of the current knowledge on pSS immunopathology is provided in **Chapter 2**, and the place of cDMARDs in its treatment is described. A great deal of information on immunopathology of pSS remains to be elucidated, but significant progress has been made in recent years. Central to the process is focal infiltration with mononuclear cells in target organs, interacting in a complex manner and jointly stimulating B cell hyperactivity and formation of auto-antibodies. Great potency was expected from bDMARDs. However, clinical studies have failed to show clinical efficacy up to now. cDMARDs also have been investigated in pSS, but all together knowledge on cDMARDs in pSS is limited due to the lack of standardized inclusion criteria and outcome measures and lack of knowledge on safety profiles. In addition, there is little information on optimal dosing in pSS. In recent years, knowledge on molecular pathways underlying pSS immune pathology has emerged, creating new opportunities for old drugs to be repurposed. Given the upregulation of potential additive pathways in pSS, synergism between the different cell types involved is likely and combining drugs targeting several pathways underlying T-cell, B-cell and pDC (and other cell types such as NK and ILC cells) activation seems

reasonable. In this respect, a combination therapy with two complementary cDMARDs holds promise. An example of such a combination is leflunomide and hydroxychloroquine, with leflunomide targeting mainly activated T-cells and to a lesser extent B-cells and, with HCQ inhibiting mainly B-cells and pDCs that can be activated by TLR7 and 9-induced immune activation.

In **Chapter 3**, in vitro we showed that TCR/TLR9 activation of PBMCs induced strong proliferation of T and B-cells and production of CXCL13, IFN-α, IFN-γ, IgG and IgM. Leflunomide dose-dependently inhibited all measured parameters, where HCQ potently and dose-dependently decreased B cell proliferation, CXCL13, IFN-α, IgG and IgM production. At different concentration combinations leflunomide and hydroxychloroquine inhibited several immune hallmark features more potently than each single compound. Clear additive inhibition of T- and B-cell proliferation and CXCL13 production was seen using suboptimal dosages of leflunomide and hydroxychloroquine. IFN-α and B-cell activity, reflected by immunoglobulin production, were already potently inhibited by this concentration of hydroxychloroquine alone, therefore the assumed additive effect could not be seen. (Additive) inhibition of IFN-γ required higher dosages of leflunomide and hydroxychloroquine, dosages that might be achieved less easy in vivo. Thus a combination of leflunomide and hydroxychloroquine at clinically applicable concentrations additively inhibits immune activation, supported a potential implementation of this drug combination in pSS treatment.

Given the well-described mechanisms of action and anti-inflammatory activities of leflunomide and hydroxychloroquine in literature and the promising results of leflunomide and hydroxychloroquine combination therapy in vitro, a placebo-controlled, double-blinded randomized trial investigating this therapy in pSS patients was performed (**Chapter 4**). The study included 29 pSS patients with active disease, reflected by an ESSDAI score of 5 or greater. 21 patients were treated with leflunomide/hydroxychloroquine combination therapy, 8 patients were assigned to placebo treatment. Disease activity reflected by ESSDAI score, the primary endpoint, significantly decreased in the leflunomide/hydroxychloroquine arm compared to the placebo arm. Also other clinical parameters such as the patient reported outcome ESSPRI score

including its constituents ESSDAI pain and ESSDAI fatigue, and saliva output improved. Histological assessment by parotid biopsies supported these positive findings. Leflunomide/hydroxychloroquine combination therapy proved to be safe and well-tolerated. In addition to these results, response to therapy could be predicted with high accuracy by a model comprising ten circulating proteins.

In **Chapter 5**, five different IFN- associated biomarkers (IFN-score in whole blood and in PBMCs, MxA in whole blood, CXCL10 and Galectin-9 in serum) in patients treated with either leflunomide/hydroxychloroquine or placebo were assessed and their potential for treatment monitoring was investigated. At baseline, these biomarkers correlated with each other and also modestly with the ESSDAI scores. At the end of the study, all biomarkers with the exception of IFN-score in PBMC were significantly decreased compared to the baseline value in the leflunomide/hydroxychloroquine treated group. Only for protein biomarkers MxA, CXCL10 and Galectin-9 a correlation with changes (delta) in ESSDAI values at 24 weeks was seen. The presence of an IFN-signature at baseline, based on the IFN-score, was not predictive of clinical response. In contrast, patients responding to the therapy showed strong early decreases of MxA and Galectin-9 levels from week 8 onwards, whereas non-responding patients showed changes comparable to those of placebo-treated patients. Subsequent ROC analysis revealed good predictive values for early changes in (delta) MxA and Galactin-9 levels after 8 weeks, the latter outperforming the former.

In **Chapter 6** we focused on Galectin-9 as a biomarker that reflects the IFN signature in patients with SLE, APS and pSS. The findings of this chapter are in line with previous findings from our group showing that Galectin-9 is an easy to measure and accurate biomarker for the IFN signature in SLE and APS patients and correlates with disease activity measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Usage of Galectin-9 as a biomarker for the IFN signature needs further confirmation, testing generalizability. Data from this thesis indicate that at the least Galectin-9 could aid in monitoring of pSS immunopathology and disease activity. Higher levels of Galectin-9 were found in pSS patients compared to non-Sjögren sicca patients, correlating with ESSDAI score and serum IgG levels. Leflunomide/hydroxychloroquine treatment

induced a decline in Galectin-9 levels, parallel with changes in B cell hyperactivity, disease activity measured by ESSDAI and ESSPRI, and markers of IFN activity. This warrants further study of Galectin-9 as a biomarker for disease monitoring in pSS.

Discussion

Despite many efforts, no effective therapy is available for pSS. Attempts to attenuate symptoms generally are insufficient, and disease burden for patients with pSS often still is significant. pSS has great impact on several areas of life including general well-being, professional and social life. In conclusion, there is a great unmet need to inhibit morbidity and to reduce risk on development of severe extraglandular manifestations and B cell malignancies.

Translational immunology to aid in therapeutic design

The central hypothesis was that successful inhibition of the immunopathological process in pSS requires a combination of drugs such as leflunomide and hydroxychloroquine with both overlapping and distinct properties jointly inhibiting immune-activation. The idea for repurpurposing these two anti-rheumatic drugs for pSS was based on an extensive literature survey and data from our previous clinical trials with leflunomide and hydroxychloroquine monotherapies. This identified the unique anti-inflammatory mechanisms of action of each of the drugs and potential in treatment of rheumatic diseases. In addition, although monotherapies with leflunomide or hydroxychloroquine did not result in significant clinical improvement significant inhibition of B cell hyperactivity was achieved, despite partly different mechanisms of action. Furthermore, *ex vivo* analyses of material from patients that were treated with leflunomide monotherapy revealed that clinical response was associated with significant inhibition of Th1 activity (IFNy and TNFα reductions).[1] Thus, we have documented *in vitro* that leflunomide and hydroxychloroquine additively inhibit multiple inflammatory mechanisms, including T and B cell activity, and CXCL13 production, a marker of lymphoid neogenesis and lymphoma development in pSS. Subsequently, in the clinical study we have confirmed our hypothesis, showing that leflunomide and hydroxychloroquine combination therapy leads to a more efficient inhibition of immune activation compared to the single drugs culminating in a higher clinical efficacy. Currently a larger double blind randomized placebo-controlled clinical trial is prepared (RepurpSS-II) to validate the clinical findings from RepurpSS-I. This will also offer

options for further translation, including diagnostic biomarkers for response prediction and targeting of identified pathways that confer resistance to treatment.

While still awaiting confirmation of the clinical data the RepurpSS-I study suggests that combination therapy may set the stage for successful treatment of pSS.

Successful disease inhibition may require simultaneous targeting of multiple pathological pathways

Many strategies targeting one inflammatory cell-type (eg via depletion of B cells by iv. administration of CD20 antibody) or targeting one inflammatory mediator (anti-TNF, anti-IL1) so far have been unsuccessful.[2-4] Since many immunological pathways can induce immunopathology in Sjögren's syndrome a major drawback of targeting only one inflammatory cell type or mediator is that this may not sufficiently tackle this redundancy of the immune system. For example, in case of B cell depletion prominent activation of (autoreactive) T cells, NK cells, innate lymphoid cells (ILCs) and pDCs may continue to drive inflammation. In line with this, strategies targeting the interplay of inflammatory cells seem to represent promising treatment options (eg. a fusion molecule of IgG-Fc and cytotoxic T lymphocyte antigen 4 (CTLA4-Ig), anti-CD40 blocking co-stimulation).[5-6] However, though anti-inflammatory properties were observed, clinical efficacy needs replication and long-term effects need to be established.[7]

Upon their introduction, high expectations of biological DMARDs targeting B cells for the treatment of pSS existed. Given the pronounced immunoregulatory role and presence of B cells in the inflamed tissues in pSS patients and the observed B cell hyperactivity in pSS, biological DMARDs inhibiting B cell activity were considered promising therapies. Thus, strategies targeting B cells by antibodies directed to cell-specific receptors such as CD20 as well as antibodies targeting molecules that are crucial to B cell activity were tested (eg anti-BAFF/BAFF receptor).

Small open label studies showed positive effects for Rituximab, targeting CD20+ B-cells, and this was associated by immune inhibition (reduced B cell hyperactivity reflected by sIgG, RF) and changes in circulating Tfh cell numbers were observed.[8]

However clinical efficacy was not seen in larger placebo-controlled, double-blind, randomized trials.[9-11]

B-cell activating factor (BAFF), is a crucial factor for B cell maturation, plasma cell survival, antibody response promotion and isotype class switching in response to T cell dependent antigens. It is upregulated in pSS and BAFF levels correlate with disease activity, formation of ectopic lymphoid structures and autoantibody levels. BAFF is targeted by the monoclonal antibody Belumimab. Its efficacy and safety were investigated in an open label study. Belimumab showed a significant effect on ESSDAI score, which was not accompanied by amelioration on patient reported outcomes, salivary flow or local inflammation (Mariette et al Ann Rheum Dis 2015;74:526–531). Data from a randomized, placebo-controlled and double-blinded trial is lacking.

Recently, the monoclonal antibody Ianalumab was studied in a placebo-controlled, double-blinded, randomized trial. Ianalumab targets the BAFF receptor, thereby competitively blocking BAFF receptor-mediated signaling and downstream survival pathways of B cells, inducing apoptosis of B cells. Besides that, it eliminates circulating B cells by antibody dependent cellular toxicity. Also Ianalumab failed to meet the pre-defined decrease of ESSDAI score. Also ESSPRI score, salivary flow and serum IgG levels remained unchanged.[12]

Besides B cells, other targets were investigated. Baminercept aims to target ectopic lymphoid structures. This lymphotoxin-β receptor fusion protein targets the lymphotoxin-β receptor that is crucial in development and maintenance of ectopic lymphoid structures, possibly leading to a decrease in high endothelial venules and infiltration of lymphocytes. In a pSS mouse model, blockade of the lymphotoxin-β receptor resulted in less glandular inflammation, blockade of formation of high endothelial venules and salivary flow was partially restored. A clinical trial in RA failed to show clinical efficacy, but altered lymphocyte tracking and inhibition of whole blood IFN signature were seen. Baminercept was investigated in a placebo-controlled, randomized, double-blind trial in pSS patients. The primary endpoint, saliva production, was not met. Also ESSDAI score remained unchanged, as were sIgG and aSSA/aSSB levels. Baminercept did significantly lower CXCL13 levels.[13]

In line with the hypothesis that successful treatment should overcome the redundancy of the immune system, biological DMARDs targeting the interplay of inflammatory cells seem to

represent the most promising treatment options, for example CTLA4-Ig (Abatacept) or anti CD-40.

The CTLA4-Ig fusion protein Abatacept is directed against CD28-mediated T cell costimulation and will directly affect activation of T cells and cells that express the receptors CD80, CD86, such as dendritic cells, monocytes/macrophages and B cells. Clinical efficacy, measured by ESSDAI score, was seen in an open label, proof of concept study.[14] Also ESSPRI score (mainly fatigue and pain), global assessment of the physician and levels of Rheumatoid Factor and sIgG decreased. Salivary and lacrimal function did not change. Next, Abatacept was investigated in a randomized, placebo-controlled, double-blinded trial in which 80 pSS patients were enrolled .[7] The positive results of the open label study could not be reproduced. The primary endpoint ESSDAI score did not differ between Abatacept and placebo arms at the end of the study. Also patient reported outcomes such as ESSPRI score, global disease activity, dryness, fatigue and health-related quality of life remained unchanged. Glandular function did not improve. Abatacept modestly diminished B cell hyperactivity, reflected by significant decreases of levels of sIgG and rheumatoid factor. Also the number of circulating Tfh cells and expression of the T-cell activation marker inducible T-cell co-stimulator (ICOS) was lowered, the latter showing an association with improvement of ESSDAI score. Abatacept proved to be safe.

The CD40-CD40L costimulatory pathway is closely involved in the activation of B cells and antigen presenting cells (including B cells and myeloid cells) as well as formation of ectopic lymphoid structures (ELS). Recent studies show that CD40L is present on T cells that infiltrate salivary glands, whereas infiltrating B cells express CD40. This can point to the presence of T- and B-cell interactions that are involved in local inflammation and formation of ELS. Recently, a randomized, double-blind, placebo-controlled clinical trial investigating the biological drug anti-CD40 antibody Iscalimab[6] hypothesized that blockade of this pathway might result in clinical efficacy in patient with pSS. Indeed, a significant decrease in ESSDAI in pSS patients treated with 10 mg/kg Iscalimab was shown. A considerably proportion of patients (77%) was considered a responder to therapy, showing a decrease of 3 or more ESSDAI points. However, also more than half (55%) of the patients in the placebo arm met the definition of responder, thereby only little advantage of

Iscalimab treatment compared to placebo treatment was left. Other clinical outcomes, such as ESSPRI score and saliva production, were insensitive to the treatment.

In contrast to LEF/HCQ therapy, treatment with Iscalimab did not result in the proposed immune inhibition. It did not inhibit B cell activity, as reflected by serum free light chains levels, levels of complement factors and presence of cryoglobulins. Information on sIgG is lacking but since free light chains, that strongly reflect sIgG but with a shorter half-life, were unchanged this strongly suggests that sIgG were also unchanged. Biomarkers, with the exception of CXCL13, remained unchanged upon treatment. Information on histology on salivary gland tissue is not available. Iscalimab treatment proved to be safe, only minor side effects were reported.

Taken together, none of the cytokine-, B cell- or co-stimulation targeting therapies discussed above showed results comparable to those of leflunomide and hydroxychloroquine combination therapy, both when comparing inhibition of immune activation and clinical activity. To understand differences in mechanism of action it is still a challenge to compare changes in molecular markers between the different therapeutic strategies/clinical trails. If available, biomarkers have often been measured on different technological platforms and biological sampling has been performed on different timepoints. Nonetheless, one stably assessed biomarker that might be helpful is the assessment of concentrations of serum IgG. Analyzing this biomarker for B cell hyperactivity demonstrates superior activity of leflunomide and hydroxychloroquine combination therapy as compared to leflunomide and hydroxychloroquine monotherapies and all biological DMARDs. This also includes biological DMARDs that target the interplay between inflammatory celltypes such as abatacept and anti-CD40.

The reason for this apparent difference is not exactly clear, but could be related to the broader range of immunopathological pathways that are targeted by leflunomide and hydroxychloroquine combination therapy as compared to the other DMARDs. Targeting TLR7/9-medicated immune activation by leflunomide and hydroxychloroquine could affect a broad range of effectors cells including T cells, B cells, myeloid cells (monocytes, macrophages, classical DCs), pDCs, NK cells and innate lymphoid cells (ILCs) (figure 1). Future studies encompassing molecular monitoring will have to identify the precise mechanisms that are strongest associated with clinical response and should be compared between therapeutic strategies. This is extremely

important as optimization of therapeutic efficacy may require specific combination of targeted approaches. Also it is conceivable that drugs that achieve comparable anti-inflammatory effects but are giving less side effects could yield preferred combination strategies.

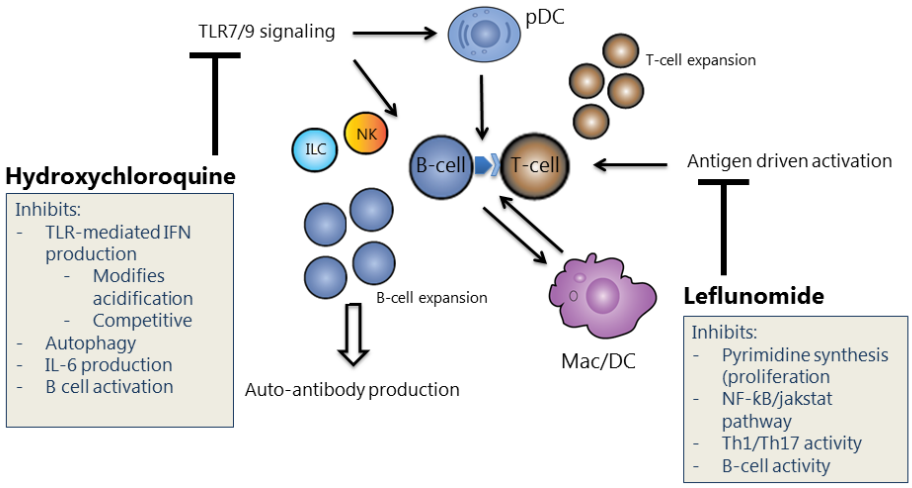

Figure 1. Conventional DMARDs leflunomide and hydroxychloroquine target both distinct and overlapping immune-pathological pathways in pSS. Main mechanisms of action of leflunomide and hydroxychloroquine targeting relevant pathways in pSS immunopathology are indicated. Hydroxychloroquine targets toll-like receptor 7 and 9 signaling that can prevent activation of plasmacytoid dendritic cells (pDCs), B cells and natural killer (NK) cells and innate lymphoid cells (ILCs). Leflunomide strongly and predominantly affects T cell function. Both drugs in vitro and in vivo have been shown to additively inhibit B cell hyperactivity.

With respect to clinical activity it seems that leflunomide and hydroxychloroquine combination therapy outperforms all the so far tested strategies using biological DMARDs. This includes cytokine-, and B cell-targeting therapies, but also strategies that target the interaction of multiple cell types such as Abatacept and anti-CD40 therapy for pSS. The observed significant biological effect of Abatacept was not accompanied by clinical improvement of both objective and patient-reported outcomes, in contrast to the broad biological effects of leflunomide and

hydroxychloroquine combination therapy that were supported by decreased disease activity and improvement in several patient reported outcomes and salivary function.

It is still unclear why the immunological effects were not associated with clinical efficacy. One reason could be that the threshold for disease inhibition as compared to placebo is higher and requires stronger immunosuppression. In this respect reduction of sIgG as a marker for successful immune inhibition (B cell hyperactivity) indicates that indeed leflunomide and hydroxychloroquine therapy results in superior inhibition.

One drawback of the abatacept trial is that there was a strong reduction of ESSDAI scores in the placebo group. This is still unexplained but could be related to subjective scoring items that are included in the ESSDAI. Other confounding factors might be insufficient washout of concomitant DMARD use such as hydroxychloroquine and MTX

Considering the above mentioned results, leflunomide and hydroxychloroquine treatment also seems to outperform anti-CD40 (Iscalimab) treatment for patients with pSS. Comparable results were seen for ESSDAI scores. However when considering other highly relevant clinical outcomes such as ESSPRI score and saliva production, leflunomide and hydroxychloroquine combination therapy showed to be effective whereas Iscalimab was not. Moreover, in contrast to Iscalimab, leflunomide and hydroxychloroquine treatment resulted in clear inhibition of immune activation, reflected by strong decrease of B cell hyperactivity, several pro-inflammatory biomarkers, and local inflammation. Taken together, these results point to superior efficacy of leflunomide/hydroxychloroquine treatment compared to Iscalimab treatment for patients with pSS, with comparable safety.

Proteomics to predict response to leflunomide/hydroxychloroquine therapy

Unique to the results of the leflunomide and hydroxychloroquine trial is the fact that using proteomic baseline data, response to therapy could be predicted with clinically meaningful accuracy based on 10 differentially expressed proteins (SLAM1, KLK10, PAG1, CXCL11, S100P, TNF-β, APEX1, CLEC6A, CCL28, β-NGF). This prediction model can not only be used to minimize long term ineffective treatment but also to identify relevant inflammatory pathways that can be targeted, possibly leading to novel strategies to successfully treat patients not responding to

leflunomide/hydroxychloroquine therapy. Several of the proteins encompassed in the prediction model are known pro-inflammatory mediators, involved in homing and activation of circulating immune cells, induction of pro inflammatory cytokines or formation of germinal centers, indicating an inflammatory endotype in patients that are likely to respond to therapy.

One of those pro-inflammatory mediators from the prediction model is the IFN induced chemokine CXCL11, involved in migration of activated T cells. Baseline measurement showed highly differential expression in responders compared to non-responders. Unfortunately, we did not measure CXCL11 levels over time. We did however measure levels of CXCL10, a chemokine with comparable function which is highly associated with CXCL11. CXCL10 expression is elevated in the salivary glands of pSS patients.[15] Leflunomide and hydroxychloroquine combination treatment resulted in early significant inhibition of CXCL10, correlating with changes in ESSDAI and ESSPRI score.

Another interesting mediator from the prediction model is kallikrein related peptidase 10 (KLK10), a serine protease abundantly expressed by epithelial cells in the salivary gland.[16] Its decreased expression at baseline in responders compared to non-responders potentially indicates increased local inflammation in responders at baseline, resulting in its downregulation. Future studies should evaluate whether KLK10 levels restore after leflunomide and hydroxychloroquine combination treatment and whether it could be a useful tool to monitor recovery of epithelial function.

IFN-associated proteins rather than gene expression might be tools to monitor disease in pSS

The immunomodulatory action of leflunomide/hydroxychloroquine therapy was also reflected by its effect on the IFN signature, which is closely involved in the stimulation of B cell hyperactivity. Five different IFN-associated biomarkers reflecting IFN activity were measured over time. Baseline levels of all five biomarkers correlated to each other and to ESSDAI score, indicating reliable reflection of the IFN signature of all methods. After 24 weeks of treatment all biomarkers except the PBMC IFN-score ($p=0.06$) were significantly decreased. However, a correlation between Δ ESSDAI score after 24 weeks and both PBMC and WB IFN-score was absent, whereas IFN-induced proteins MxA, CXCL10 and Galectin-9 did show robust correlations.

When evaluating possible differences between responders and non-responders, MxA and Galectin-9 showed clear differences after only 8 weeks of treatment between these two groups, the latter outperforming the former. These results demonstrate that early changes in IFN-associated biomarkers could help in predicting long-term responses. Since IFN-associated proteins such as CXCL11 were differentially expressed at baseline it is conceivable that combinations of these proteins may have predictive potential, but this needs to be further studied.

Cost effectiveness of leflunomide/hydroxychloroquine therapy vs. bDMARDs

Currently, the potential implementation of novel promising biologicals like anti-CD40 monoclonal antibody and CTLA4-Ig fusion protein are challenged by high costs.[5,6] In fact, if effective, many new biologicals will come at a high price (estimated ~€10000/year/pt). In addition, these medicines may not be easy to administer and safety remains to be demonstrated.

Leflunomide and hydroxychloroquine are registered drugs that are available at a fair price. The low number needed to treat (NNT) of 2 (1.92) for achieving a clinical response (ESSDAI decrease of ≥3 and higher) indicates the leflunomide/hydroxychloroquine combination as very effective. This potentially generates excellent cost-effectiveness ratios.

Data on costs for health care of pSS patients are scarce and cannot be calculated in QUALYs yet. Nevertheless, estimates can be made. In the UK the direct and indirect health care costs for pSS patients were calculated to be €2188,- and €9587,-/year/patient, respectively (15,16). The indirect health care costs were associated with strongly increased sickness absence and unemployment. Assuming equal costs in the Netherlands (total €12775,-/patient/year) and the high incidence in our country (0.5%, ~85000 patients) this clearly indicates a considerable burden to society for this chronic disease (~1 billion €/year). It is anticipated that the patients that have moderate to high disease activity will contribute the most to the socioeconomic costs. Based on our data, 52-67% of the patients (taking ESSDAI or ESSDAI plus ESSPRI responders into account, ref 17) within this group might benefit significantly, very likely inducing cost-saving assuming significant increase in socioeconomic participation. This could result in a major cost reduction of

tens of millions €/year in the Netherlands, especially when compared with a significant rise in costs when alternatively biologicals will reach the clinical arena.

Another factor possibly contributing to cost-effectiveness of leflunomide/hydroxychloroquine therapy is the potential availability of a prediction model. This could significantly prevent ineffective long-term treatment thereby reducing costs and of course prevent needless exposure of patients to ineffective drugs. This will subsequently reduce costs due to potential side effects.

Leflunomide/hydroxychloroquine combination therapy: how about patients with low disease activity?

ESSDAI is generally accepted as disease activity measure. One potential drawback of our clinical study is that patients with a high ESSDAI were included. However, a substantial amount of pSS patients present themselves with an ESSDAI lower than 5 (inclusion criterium for trials worldwide). In case clinical efficacy of leflunomide/hydroxychloroquine is confirmed in its larger follow-up trial, future trials should also focus on patients with low ESDDAI with dryness of eyes and mouth as primary outcome measures. Of interest in our study 3 leflunomide/hydroxychloroquine-treated patients with high ESSPRI scores showed a clinical response (defined as a decrease of 1 point in ESSPRI score, 15% or more[17]), while not responding in terms of ESSDAI scores.

Until now, therapies for patients with low disease activity but great disease burden are poorly studied. Given the limited costs of leflunomide/hydroxychloroquine therapy clinical efficacy could easily be tested in this patient group. It is a novelty that an affordable effective DMARD combination would be available for a pSS patient that does not have an organ- and/or life-threatening manifestation of the disease.

Final conclusion

In this thesis we confirmed the central hypothesis that given the complex interplay of involved immune-cells, successful inhibition of immune-activation in pSS can be accomplished by a combination of leflunomide and hydroxychloroquine, two drugs targeting both overlapping and distinct immune-pathological pathways, jointly resulting in broad immune inhibition. We applied

a translational approach, in which the in vitro positive results set the starting point for a placebo-controlled, randomised, double-blinded clinical trial. Clinical efficacy of leflunomide-hydroxychloroquine combination therapy in pSS patients with moderate to high disease activity was supported by improvement in several patient-reported outcome measures, dryness and broad immune-inhibition.

Being the first therapy investigated in a randomized, placebo-controlled clinical trial in pSS patients showing clinical efficacy associated with other clinical parameters, leflunomide-hydroxychloroquine combination therapy is a promising treatment option that warrants further research in larger randomized trials.

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Nederlandse samenvatting

De ziekte van Sjögren is een auto-immuun ziekte waarbij spontaan ontstekingen ontstaan. Deze ontstekingen worden voornamelijk gevonden in de speekselklieren en traanklieren, maar ook ontstekingen in andere organen zijn mogelijk. Hierdoor hebben patiënten last van droge ogen en een droge mond, en afhankelijk van het bestaan van ontstekingen in andere organen specifieke klachten behorend bij dat orgaan (bijvoorbeeld nieren, huid of longen). Daarnaast hebben de meeste patiënten veel last van vermoeidheid en pijn in spieren en gewrichten, en bestaat er een verhoogd risico op het krijgen van lymfeklierkanker. De klachten hebben vaak een grote invloed op het dagelijks leven van patiënten met Sjögren. Zo kunnen zij beperkingen ervaren in het werk, maar ook in hun sociale leven. Tot op heden is er geen behandeling die de ziekte van Sjögren kan genezen of tot rust kan brengen. Het zo goed mogelijk proberen de symptomen te verlichten is tot op heden het hoogst haalbare. Helaas zijn de huidige behandelingen om symptomen te bestrijden niet altijd afdoende om de ziektelast tot een acceptabel niveau te verlagen.

Er is de laatste jaren veel kennis vergaard over het ontstaan van de ziekte van Sjögren, maar helaas is ook nog veel onbekend. Wat duidelijk is geworden, is dat er veel verschillende cellen behorende tot het afweersysteem bij betrokken zijn. Deze immuun cellen beïnvloeden op allerlei wijzen elkaars werking, zij hebben elkaar nodig om uiteindelijk samen te resulteren in de ontsteking die gezien wordt bij de ziekte van Sjögren. De betrokkenheid van verschillende cellen die met elkaar samenwerken is een belangrijk gegeven in de zoektocht naar een effectieve behandeling. In het verleden zijn veel medicijnen onderzocht, medicijnen die zich veelal richten op één soort immuun cellen. Zoals eerder beschreven, is er tot op heden nog geen enkel medicijn gevonden wat in staat was de ziekte activiteit effectief te remmen. Wellicht is het niet voldoende om slechts één soort immuun cel te remmen, aangezien meer soorten cellen een belangrijke rol spelen in het ziekte proces, en er een combinatie van verschillende medicijnen die samen meerdere soorten immuun cellen remmen nodig is.

Een dergelijke combinatie van medicijnen zou mogelijk de combinatie van de anti-reumatische middelen leflunomide en hydroxychloroquine kunnen zijn. Deze combinatie van medicatie remt onder andere 3 belangrijke immuuncellen die betrokken zijn bij de ontsteking bij pSS, namelijk

de T-cellen (door leflunomide), de B-cellen en dendritische cellen (door hydroxychloroquine). In dit proefschrift is het effect en de veiligheid van deze combinatie bij patiënten met pSS onderzocht.

Allereerst is er gekeken of de combinatie van leflunomide en hydroxychloroquine deze cellen, welke waren verkregen uit het bloed van patiënten met pSS, kan remmen, en of de combinatie van de middelen tot sterkere remming leidt dan de middelen afzonderlijk. Hieruit bleek dat er inderdaad sterkere remming van de vermenigvuldiging van T- en B-cellen optrad wanneer leflunomide en hydroxychloroquine gecombineerd werden dan wanneer deze afzonderlijk bij de cellen werden gevoegd. Ook produceerden zij met de combinatie minder stoffen die ontsteking stimuleren.

Vervolgens werd de combinatie van leflunomide en hydroxychloroquine onderzocht in een klinische studie met patiënten met pSS. Het betrof een placebo-gecontroleerde (een deel van de patiënten kreeg de medicatie, een deel kreeg placebo medicatie), gerandomiseerde (toewijzen aan echte medicatie of placebo medicatie gebeurde willekeurig, op basis van het toeval) en dubbel-blinde (zowel de patiënten als de onderzoekers waren niet op de hoogte van tot welke behandelgroep zij waren toegewezen) studie. 29 patiënten namen deel, waarvan 21 patiënten de echte medicatie kregen, en 8 patiënten placebo medicatie. Om de effectiviteit van de medicatie te kunnen beoordelen werd primair gekeken naar de ziekte activiteit, die gescoord werd door middel van de EULAR Sjögren's syndrome disease activity index (ESSDAI) score, een score welke zowel klachten en symptomen als laboratoriumwaarden bevat. Hoe hoger de ESSDAI score, hoe hoger de activiteit van de ziekte is. De ESSDAI score daalde aanzienlijk in de groep patiënten die behandeld werd met de combinatie van leflunomide en hydroxychloroquine, in tegenstelling tot in de groep patiënten die placebo medicatie kreeg. Dit positieve effect werd ondersteund door een daling van de EULAR Sjögren's syndrome patient reported index ESSPRI sub-scores vermoeidheid en pijn, een score waarbij patiënten zelf scoren hoeveel last zij hebben van bepaalde klachten. Daarnaast nam de gemiddelde productie van speeksel toe. Het klinische effect ging gepaard met een sterke daling van ontstekingsactiviteit die te meten was in het bloed en in het klierweefsel.

Om te onderzoeken of het mogelijk is op voorhand te voorspellen welke pSS patiënten waarschijnlijk baat zullen hebben van deze behandeling en welke patiënten waarschijnlijk niet, werden bij aanvang van de studie een grote hoeveelheid verschillende eiwitten in het bloed gemeten die betrokken zijn bij ontsteking. Gevonden werd dat patiënten die een grote kans hebben om op de behandeling te reageren andere spiegels van deze eiwitten hadden dan de patiënten die een grote kans hebben om niet te reageren op de behandeling. Tien van deze eiwitten in het bloed, werden gebruikt voor een voorspellend rekenkundig model. Door in de toekomst deze eiwitten te meten in het bloed van een pSS patiënt kan met aanzienlijke nauwkeurigheid voorspeld worden of deze individuele patiënt wel of niet baat zal hebben van leflunomide en hydroxychloroquine combinatietherapie.+ ff

De combinatie van leflunomide en hydroxychloroquine bleek veilig, er werden geen ernstige bijwerkingen gezien.

Om het positieve effect wat gezien werd op de zogenaamde klinische uitkomstmaten verder te ondersteunen werden verschillende effecten op het afweersysteem verder onderzocht om te kijken of de daling van de ziekteactiviteit samenging met remming van het immuunsysteem. Zo is er gekeken naar de zogenaamde interferon-handtekening. Interferon is een eiwit dat geproduceerd wordt als een virus het lichaam binnendringt. Het interferon kan vervolgens allerlei genen in andere afweercellen activeren waardoor deze cellen actief worden en de afweerreactie op gang komt. In pSS wordt echter ook zonder de aanwezigheid van een virale infectie een verhoogde activiteit van deze genen in verschillende afweercellen gezien. Het is nog niet geheel duidelijk wat de beste manier is om deze interferon-handtekening te meten, en welke methode geschikt is om veranderingen in de interferon-handtekening tijdens behandeling goed te kunnen meten. Daarom werd onderzocht welke van de methoden het beste overeenkwam met de reactie van de patiënt op de behandeling. Aan het begin van de studie werd op verschillende manieren de hoogte van deze interferon-handtekening gemeten. Deze meting werd herhaald na 8 weken behandeling. De patiënten die na 8 weken behandeling een sterke daling hadden van het eiwit Galectin-9 in het bloed (een bekende marker voor de interferon-handtekening) bleken vaak de patiënten te zijn die na 24 weken een goede respons hadden laten zien op behandeling

met leflunomide-hydroxychloroquine. Hiermee kon geconcludeerd worden dat leflunomide en hydroxychloroquine combinatie behandeling inderdaad de interferon-handtekening remt, en dat de mate waarin dit gebeurt na 8 weken ook goed kan voorspellen of een patiënt op de lange duur, na 6 maanden, baat zal hebben van de behandeling. Als deze methode gecombineerd wordt met het voorspellingmodel met daarin de tien eiwitten in het bloed die voor aanvang van de behandeling gemeten werden, zal een groot deel van de patiënten die geen baat zal hebben van de medicatie vroegtijdig geïdentificeerd kunnen worden, waardoor zij geen onnodige behandeling hoeven te starten of langere tijd te continueren. Dit voorkomt onnodige bijwerkingen en kosten.

In conclusie: leflunomide en hydroxychloroquine combinatie behandeling lijkt een effectieve en veilige behandeling voor patiënten met pSS met matige tot hoge ziekte activiteit, waarmee de ziekte activiteit verlaagd kan worden, patiënten minder vermoeidheid ervaren en de overactiviteit van het immuunsysteem geremd wordt. Niet eerder heeft een behandeling voor pSS deze resultaten laten zien. Een groot voordeel van leflunomide en hydroxychloroquine combinatie therapie is verder dat beide medicijnen geregistreerde middelen zijn, waarvan veel informatie bestaat over de veiligheid, en welke in vergelijking met de nieuwe reumamiddelen zeer goedkoop zijn.

Grotere vervolgstudies zijn nodig om deze resultaten te bevestigen. Ook zouden er vervolgstudies kunnen plaatsvinden waarin het effect van leflunomide en hydroxychloroquine combinatie therapie onderzocht wordt in patiënten met een lagere ziekte activiteit gemeten door orgaan betrokkenheid, maar wel veel patiënt gerapporteerde klachten van de ziekte, zoals pijn en vermoeidheid.

Met opmerkingen [MO1]: Is gehonoreerd door zonmw