Biological mechanisms for chronic fatigue in primary Sjøgren`s syndrome

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Scientific environment

The work on this thesis has been performed in the Research Group of Clinical Immunology, between 2009 and 2012. The group is lead by Professor Roald Omdal, and was started in 2003 as cooperation between the Clinical Immunology Unit, Departments of Internal Medicine, Neurology, Neuropsychology, Radiology and Biochemistry at Stavanger University Hospital (SUS). The group has expanded during the four years I have been a member, and research partners now include the Norwegian-Swedish Sjögren`s network, International Research Institute of Stavanger (IRIS), University of Stavanger (UiS), The Norwegian Veterinary Institute, Broegelmans Research Laboratory at the University of Bergen (UiB) and the Department of Rheumatology at Haukeland University Hospital, amongst others.

I have been associated as a fellow to the Department of Internal Medicine, SUS, and Institute of Medicine, UiB. My supervisor, Roald Omdal, is professor at the Institute of Medicine, UiB.

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Abbreviations

ANCOVA	Analysis of co-variance
AECG	American-European Consensus Group
AOPP	Advanced oxidation protein products
BBB	Blood brain barrier
BDI	Beck Depression Inventory
CFS	Chronic Fatigue Syndrome
CNS	Central nervous system
DNA	Deoxyribonucleic acid
FSS	Fatigue Severity Scale
GRR	Genotype relative risk
GWAS	Genome wide association study
HC	Healthy control
HLA	Human leukocyte antigen
HR-QOL	Health related quality of life
HUS	Haukeland University Hospital
IFN	Interferon
IL	Interleukin
IL-1β	Interleukin-1 beta
i.e.	Id est
IL-1Ra	Interleukin-1 receptor antagonist
IL-1sRII	Interleukin-1 soluble receptor type II
LPS	Lipopolysaccharide
MAF	Minor allele frequency
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MSG	Minor salivary gland
PAMP	Pathogen associated molecular pattern
PC	Protein carbonyl
PRR	Pattern recognition receptor
PSS	Primary Sjøgren`s syndrome
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RTX	Rituximab
SD	Standard deviation
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Secondary Sjøgren`s syndrome
SSA/Ro	Sjøgren`s syndrome A/Ro antigen
SSB/La	Sjøgren`s syndrome B/La antigen
SUS	Stavanger University Hospital
UiB	University of Bergen
TNF	Tumor necrosis factor

TLR	Toll-like receptor
VAS	Visual analogue scale

Abstract

Background:

Primary Sjøgren`s syndrome (pSS) is a chronic autoimmune disease, characterised by lymphocytic infiltration of exocrine glands and autoantibody production. Fatigue is a frequent phenomenon in pSS, associated with reduced health-related quality of life. Fatigue is influenced by depressed mood, sleep disorder and autonomic dysfunction, but also occurs without these co-factors. Evidence from animal and human studies indicates that immune activation may directly influence fatigue in chronic inflammatory disorders.

Sickness behaviour in animals is characterized by decreased activity, social withdrawal and a reduction in the intake of food and water. This behaviour is hypothesized to increase survival by shielding the sick animal from predators, and occurs automatically as a response to infection and inflammation. The pro-inflammatory cytokine interleukin (IL)-1β is crucial for this behaviour. Fatigue in humans can be considered an element of sickness behaviour, and we hypothesized that inhibition of IL-1 would lead to a reduction in fatigue in pSS. We tested this in a randomized clinical trial by giving anakinra, a recombinant IL-1 receptor antagonist (IL-1Ra) - or placebo - to pSS patients with a high level of fatigue.

Inflammation is closely connected with oxidative stress, and generation of reactive oxygen species is an important mechanism for killing of pathogens. Increased oxidative stress has been reported in relation to fatigue in human diseases, but has never been investigated in relation to fatigue in pSS. We hypothesized that pSS patients would have higher levels of oxidative stress than healthy controls, and that oxidative stress would be associated with fatigue.

Taking oxidative stress and pro-inflammatory cytokines into consideration, some pSS patients are still more affected by fatigue than otherwise comparable individuals. Part of the explanation for this might be found in the genetic makeup of each individual

patient, and several recent studies point to both genetic and epigenetic factors that may be important for fatigue generation. Based on this, we aimed to investigate genetic variation in relation to fatigue in pSS.

Main objectives:

- Write a review article of the current knowledge of biological mechanisms of fatigue in inflammatory and non-inflammatory conditions.
- Investigate the efficacy and safety of IL-1 inhibition on fatigue in pSS.
- Investigate the plasma levels of oxidative stress markers in pSS as compared to healthy individuals, and further explore any association of oxidative stress with fatigue in pSS.
- Investigate genetic variation, i.e., single nucleotide polymorphisms (SNP) in relation to fatigue in pSS.

Subjects and methods:

All patients included in this dissertation were recruited from the pSS patient pools at Stavanger University Hospital (SUS) and Haukeland University Hospital (HUS). SUS is the only hospital in the southern part of Rogaland County and HUS is the main hospital in Hordaland County, Norway.

For the double-blind, randomised treatment trial all pSS patients in the southern part of Rogaland County were identified and invited to the study, and a total of 26 patients were eligible and agreed to participate. The patients were randomly allocated to treatment with either an IL-1Ra or placebo (0.9% NaCl in identical syringes), and self-administered the drug or the placebo by a daily subcutaneous injection. Neither patients nor investigators were aware of the treatment allocation. The study ran over four weeks. Blood was sampled and a visual analogue scale (VAS) and the Fatigue Severity Scale (FSS) were used to asses fatigue at the start of the study (week 0), at week 2, at the end of the study (week 4) and at week 5.

The same 26 patients were included for the plasma measures of oxidative stress. Two markers of protein oxidation; advanced oxidation protein products (AOPP) and protein carbonyl (PC), were measured in blood samples collected at week 0, before any interventions took place.

For the genetic analysis we used whole blood samples from 207 pSS patients and 376 healthy controls. We investigated the associations of fatigue and minor allele frequencies in 85 SNPs in 12 genes, half of which are related to mitochondrial function. The genes were selected based on previous studies of gene expression in the chronic fatigue syndrome.

Results:

We found that:

- IL-1 inhibition influences fatigue in pSS as compared to placebo. We were not able to show this in the primary endpoint, but ad hoc analysis points to a strong positive effect of IL-1 inhibition on fatigue.
- IL-1 inhibition appears to be safe in pSS.
- Markers of protein oxidation are increased in pSS as compared to healthy controls. There is no association between fatigue and plasma protein oxidation in pSS.
- Genetic variation in *SLC25A40* and *PKN1* show signals of association with fatigue in pSS.

Conclusions:

This dissertation strengthens the view that at least some part of fatigue has a biological fundament, related to inflammation. The IL-1 system is crucial in the development of fatigue in this setting, and IL-inhibition seems to reduce fatigue generation. There is a trend for association between genetic variation and fatigue in pSS. Fatigue is not associated with the amount of oxidised plasma proteins in pSS.

List of publications

1. **Norheim KB**, Harboe E, Gøransson LG, Omdal R. Interleukin-1 inhibition and fatigue in primary Sjögren`s syndrome – a double blind, randomised clinical trial. PLoS ONE. 2012;7:e30123.

2. **Norheim KB**, Jonsson G, Harboe E, Hanasand M, Gøransson L, Omdal R. Oxidative stress, as measured by protein oxidation, is increased in primary Sjøgren`s syndrome. Free Radical Research. 2012;46:141-6.

3. **Norheim KB**, Le Hellard S, Nordmark G, Gøransson LG, Brun JG, Wahren-Herlenius M, Jonsson R, Omdal R. A possible genetic association with chronic fatigue in primary Sjögren`s syndrome: a candidate gene study. Submitted.

4. **Norheim KB**, Jonsson G, Omdal R. Biological mechanisms of chronic fatigue. Rheumatology (Oxford) 2011;50:1009-18.

Contents

Table 1: Revised International Classification Criteria for Sjøgren`s Syndrome

1. Background

1.1 Primary Sjøgren`s syndrome

Primary Sjøgren`s syndrome (pSS) is a systemic autoimmune exocrinopathy affecting 0.05-0.5% of the population, women nine times more often than men (1, 2). The mean age for disease onset is in the fifth decade of life. The main symptoms are dry mouth and dry eyes – xerostomia and keratoconjunctivitis sicca – caused by a chronic inflammation of the salivary and lachrymal glands. Mononuclear cell infiltration in exocrine glands is the histopathological hallmark of pSS, and ectopic germinal centre formation in minor salivary glands (MSG) is seen in about one fourth of the patients (1, 3-5). PSS is immunologically characterised by B cell activation, hypergammaglobulinemia and autoantibody production against SSA/Ro- and SSB/La ribonucleoprotein particles. There is a small, but increased risk of B-cell lymphoma (3, 6). Most patients experience systemic features like fatigue, arthralgia and myalgia in addition to the dryness of mucous membranes. Other systemic manifestations, such as arthritis, Raynaud phenomena, cutaneous vasculitis, central nervous system (CNS) involvement and serositis, are also seen (5). Hypothyroidism is a common co-morbidity, observed in 20-30% of the patients (5). Fatigue is an important and often disabling phenomenon reported by 70-80% of the patients (7, 8). It is a leading cause of inability to work and may lead to a reduced health related quality of life (HR-QOL) in this patient group (9).

The American-European consensus group (AECG) criteria for primary and secondary Sjøgren`s syndrome were published in 2002 (Supplementary Table 1) (10), and are now widely accepted and used. It is based on two subjective and four objective criteria. The diagnosis of pSS requires four out of six criteria including a positive focus score (>50 mononuclear cells/4mm^2) in MSG tissue sections and/or positive antibodies to anti-SSA/Ro and/or anti-SSB/La, or three out of four objective criteria. Secondary Sjøgren`s (sSS) syndrome requires one subjective criteria and two objective criteria (with the exception of autoantibodies) in a patient with an

established autoimmune rheumatic disease, such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE).

PSS is named after the Swedish ophthalmologist Henrik Sjögren, who first described the association between dry mouth, dry eyes and arthritis in 1933 (11). The lack of a disease activity measure has been a limitation to research in pSS. Results from different studies are difficult to compare due to the variety of methods used to assess disease severity and improvement after treatment. In this respect, the recently developed European League Against Rheumatism (EULAR) Sjøgren`s Syndrome Disease Activity Index (ESSDAI) (12) and EULAR Sjøgren`s Syndrome Patient Reported Index (ESSPRI) (13) hold promise for future treatment trials.

1.2 Fatigue

Fatigue can be defined in several ways. We use the definition "*an overwhelming sense of tiredness, lack of energy, and feeling of exhaustion*"(14). Fatigue is fundamentally different from normal experiences of tiredness or sleepiness, and patients often describe fatigue as a persistent, deep, thorough exhaustion. Fatigue is non-specific and highly subjective (15), which can make it difficult to evaluate and quantify. It is a common symptom in inflammatory and autoimmune diseases, and also in neurological, psychiatric and malignant disease. Fatigue affects the patients' way of living, and a high level of fatigue often leads to social withdrawal, family conflicts and work disability. PSS and SLE patients with significant fatigue have a lower HR-QOL than patients without fatigue (7, 16), and fatigue interferes with emotional, social and physical functions. Fatigue, as other chronic disabilities, is costly for the society, with medical expenses, sick leave, and loss to the work force as the main expenses (17).

Fatigue can be split into sub-categories such as peripheral, physical and central. Peripheral fatigue implies muscle fatigability due to disorders of the muscle and neuromuscular junction transmission (18). Physical fatigue is the somatic experience of exhaustion following physical efforts and is distinguishable from central fatigue.

Central or mental fatigue is by definition a subjective self-reported experience and this is the phenomenon patients usually present for evaluation (19). It is generally agreed that central fatigue is a complex phenomenon, difficult to describe, but there is no universal agreement upon whether it is appropriate to subdivide it further into distinct dimensions, such as emotional, cognitive and intellectual fatigue. This disunity becomes obvious when considering the fatigue measuring instruments; some are uni-dimensional, constructed to capture the "core feelings" of fatigue, while others are multidimensional, including items related to the intensity, variability and impact of fatigue.

Recent years much attention has been paid to the chronic fatigue syndrome (CFS). This is a condition of unknown aetiology, characterised by unexplainable severe chronic fatigue and subjective complaints from several organ systems (20). It has been proposed that CFS is an autoimmune disease, and probable benefit from treatment with a B-cell depleting agent was recently shown in a small study (21). However, the study did not achieve the primary endpoint, and the results require replication and extension before a final conclusion can be drawn.

1.2.1 Fatigue in pSS and other autoimmune diseases

Fatigue is prevalent in pSS, and was recently reported in 85% of pSS patients, of whom forty percent described fatigue as their most severe symptom (7). Another study found "high fatigue" defined by Fatigue Severity Scale (FSS) score > 4 in 70.7% of the pSS patients investigated (22). Mood disorders are consistently reported to influence fatigue, but fatigue also occurs in pSS patients who are not depressed (8, 23). Pain, sleep disorders and learned helplessness may influence the experience of fatigue, in some cases accompanied by neuroendocrine disturbances and autonomic dysfunction (24). It is likely, but still unclear, whether there are common biological pathways for fatigue generation across different inflammatory somatic diseases. So far it has not been possible to establish a link between various "disease activity" markers, for example lymphocyte counts, immunoglobulin-levels or autoantibodies and fatigue in pSS (24, 25) or SLE (26). It is not unlikely that these markers have

been too broad in regard to fatigue, and that future research, mapping specific signalling molecules of the immune system, may detect this "missing link".

Several studies have reported an effect of biological drugs on fatigue, also in pSS. Treatment with rituximab (RTX), a chimeric murine/human anti-CD20 monoclonal antibody, led to a reduction in fatigue in pSS (27, 28) and CFS (21). SLE and RA patients who received an IL-6-blocking agent reported significant relief from fatigue (29, 30), and a similar effect on fatigue has been described using other biological agents. Examples include benefit from tumor necrosis factor (TNF)-α blocking agents (31) and the T-cell co-stimulation inhibitor abatacept in RA patients with fatigue (32). When taken together, these observations point to a strong association between pro-inflammatory mediators and fatigue.

1.3 The immune system

The immune system can be considered to consist of two functionally different parts - the nonspecific innate immune system and the antigen-specific adaptive immune system (33). This division is arbitrary, as there is an intense cooperation and crosstalk between the two systems. The innate immune system comprises physical barriers, such as skin and mucosa, phagocytic cells, interferons, the complement system and other signalling and effector molecules. It is evolutionarily conserved in eukaryotic organisms from slime moulds to humans. Cells of the innate immune system rely on recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) such as the Toll-like receptors (TLR) for activation, and do not generate memory or persistent immunity. The innate immune system is the first line of defence against infection, and responds much faster than the adaptive immune system.

The adaptive immune system, on the other hand, is highly differentiated. It compromises the B and T cells. Receptors on these cells recognise molecules that are "non-self" rather than specific PAMPs, and the cells of the adaptive immune system have the ability to generate a long lasting memory for specific pathogens (i.e.

immunity). Activated B-cells acquire an increased lifespan, and become immunoglobulin-producing plasma-cells or memory B-cells. T-cells are able to differentiate into several sub-populations, partly depending on the circumstances of activation. B and T cells specific for the same antigen must cooperate during activation, and there are several checkpoints both in maturation and in activation of the adaptive immune system to avoid unnecessary responses and autoimmunity (34).

1.3.1 Autoimmunity

It is generally considered that at least 5% of the population suffers from an autoimmune disease. The prevalence of autoimmune diseases seems to be increasing, not only due to reclassification of diseases or increased life span in the population (35). Loss of tolerance to self is a fundamental step in autoimmunity, but it is not fully understood how this loss of tolerance is initiated and maintained (36). It is generally agreed upon that autoimmunity develops due to a complex interplay between genetic factors, environment and chance. Autoimmune diseases are considered genetically complex, meaning that the combination of several genetic variants in one individual contribute to disease development (polygenic disease). This contrasts the Mendelian disorders, in which a single gene variant (mutation) is the cause of a specific disease. The major histocompatibility complex (MHC) class I and II are antigen-presenting molecules present on the surface of all nucleated cells and on antigen presenting cells, respectively. The human version is called human leukocyte antigen (HLA), and some subtypes of genes (alleles) coding for the HLA complex predispose for several – or in some instances specific – autoimmune diseases. The strong association between HLA-B27 and ankylosing spondylitis is currently the best example of this (37). There is an association between HLA-type and pSS, in particular the HLA-types DR2 and DR3 (38).

Several studies have reported associations between specific single nucleotide polymorphisms (SNP) and autoimmune diseases. Some SNPs are related to one disease only, while most are found to be associated with several autoimmune diseases. SNPs in *PTPN22* increase the risk of developing RA, SLE, type 1 diabetes and

autoimmune thyroid disease (39), and SNPs in *NOD2* leads to an increased risk for inflammatory bowel disease and chronic obstructive pulmonary disease (40, 41). However, the association between a certain SNP or a combination of SNPs and susceptibility to a disease is usually not very strong, most commonly with only a small increment in risk (1.1–1.5-fold) (42). Hence, factors additional to genetic variation are necessary for disease to develop. Epigenetic modifications that result in altered gene expression may be one link between genetic and environmental factors (36).

1.3.2 Cytokines

Cytokines are small peptides, produced by cells of the immune system. They act in an autocrine, paracrine and/or endocrine fashion, and influence cell growth and differentiation; however their main function is signalling between cells. Some cytokines are pro-inflammatory and others anti-inflammatory, although this view is a simplification. Cytokines are highly pleiotropic, and the effects of a certain cytokine are dependent on the local inflammatory environment and the target cells, among other factors (43). Most cytokines have a short half-life, act locally, and are found in concentrations of ng/mL or lower, challenging conditions that influence the measurement and analysis of these peptides. High levels and/or increased production of pro-inflammatory cytokines are found in patients with chronic inflammatory diseases, such as RA (44), SLE (45) and multiple sclerosis (MS) (46). Amongst others, the secretion of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and interferon (IFN)-γ is increased in pSS patients (47, 48).

1.3.3 The IL-1 system and sickness behaviour

The interplay of the cytokines controls the direction, amplitude and duration of the immune-response. IL-1 is a pro-inflammatory cytokine, released mainly by monocytes and macrophages early in the inflammatory cascade. It exists in two biologically active forms – IL-1α is found on cell membranes and inside cells, while IL-1β is soluble and secreted from the cells. IL-1 has two receptors; IL-1 receptor I (IL-1RI) induces signal transduction while IL-1 receptor II (IL-1RII) is a decoy receptor (49).

IL-1RAcP is a co-receptor for IL-1RI, necessary for the IL-1 signal to induce pro-inflammatory gene expression. However, a structurally different variant of this co-receptor is found on neurons. IL-1RAcPb arrests most, but not all of the IL-1 signalling, and does not induce pro-inflammatory gene expression. This is proposed to be a neuro-protective mechanism; brain inflammation is avoided whereas signals are transferred to the CNS (50). Multiple mechanisms to regulate IL-1 activity have evolved, and further balance of the IL-1 system is assured by a naturally occurring IL-1RI antagonist, IL-1Ra, which down-regulates the IL-1 effect by blocking IL-1RI (51). Under normal conditions and in healthy subjects, IL-1Ra is found in the range of 100-300 ng/mL in blood, while IL-1β is in the pg/mL range, and not easily measured (52). The concentration of IL-1Ra increases a few hours after an increase in IL-1β. As IL-1β is difficult to measure, IL-1Ra can be utilized as a surrogate marker of increased activity in the IL-1 system (23).

IL-1 has been thoroughly investigated in relation to the concept of *sickness behaviour* in animals. Sickness behaviour is a survival-enhancing strategy, observed during infection and inflammation (53). It is characterized by sleepiness, social withdrawal and loss of appetite, and is a complex and automated behaviour believed to protect the sick individual from predators. Several animal studies have explored the pathways leading to sickness behaviour, and it seems clear that IL-1 is a fundamental actor in this concept (54). In particular, signals leading to sickness behaviour seem to be transmitted through the IL-1RI/IL-1RAcPb complex on neurons in the brain. Engagement of this receptor complex leads to neuronal signalling, but no inflammation (50, 52). This allows behavioural adaptations due to inflammation to be initiated through IL-1, without the dangers of inflammation taking place in the CNS itself.

In mice, intraperitoneal or intracerebrovascular injection of IL-1β or the bacterial endotoxin lipopolysaccaride (LPS) leads to sickness behaviour within a few hours (54, 55). The behavioural effects of IL-1β can be blocked by injections of IL-Ra and is not seen in IL-1RI knockout mice (56). IL-1 acts upstream from other pro-

inflammatory cytokines, such as IL-2 and IL-6 (57, 58).

Fatigue in humans can be considered a component of sickness behaviour, and human experiments have confirmed several of the results from animal studies. In healthy males, intravenous administration of low doses of LPS was followed by increased plasma levels of IL-6, TNF-α and IL-1Ra within hours, accompanied by decreased mood in a dose-dependent fashion (59). Injection of IL-1 in human leads to chills, fever, fatigue, hypotension and nausea (60, 61). Most studies in chronic inflammatory conditions report an association between pro-inflammatory cytokines and fatigue, although the results are conflicting, see Table 1. This variation may be due to differences in patient populations, sample handling and cytokine assessment methods. We have previously reported an up-regulation of IL-1Ra in the cerebrospinal fluid (CSF) of pSS patients with fatigue, which reflects an intratechal activation of the IL-1 system (23).

1.4 Biologic agents

Biologic agents are immune modulating drugs developed by recombinant DNA technology. These drugs offer a targeted strategy in contrast to the nonspecific immunosuppressive agents traditionally used to treat most forms of autoimmune- and immune-mediated chronic inflammation. However, it is important to keep in mind that both classes of drugs merely dampen the disease process, and there is currently no definite curative pharmacological treatment for any chronic inflammatory disease.

There are several classes of biologic agents. These drugs generally inhibit specific components or pathways of the immune-system through interference with kinases, proteasome or cytokine function, inhibition of T-cell activation, depletion or inhibition of B-cells, or inhibition of the homing of immune-cells.

Table 1 Selected studies[a] of cytokines[b] and fatigue

Disease	Cytokine	Fatigue measure	No. of pts.[c]	Assoc. with fatigue
PSS (22)	25 cytokines	FACIT-F, FSS, VAS	141/0/0	IL-17 (weak)
PSS (23)	IL-1Ra[d]	VAS	54/0/53	IL-1Ra
PSS (25)	IL-1β, IL-2, IL-6, IL-10, TNF-α	MFI	60/0/139	No
SLE (62)	IFN-I system	MFI-20, VAS	58/0/20	No
SLE (26)	IL-2, IL-6, IL-10, TNF-α, IFN-α, TGF-β	FSS	57/0/0	No
MS (46)	IL-10, TNF-α, IFN-γ	FSS	15/15/0	TNF-α and IFN-γ
BC (63)	IL-6, IL-1Ra, sIL-6R, TNF-RII	SF-36 (Vitality)	50/50/0	IL-1Ra, sIL-6R
BC (64)	IL-1β, IL-1Ra, sTNF-RII	RAND, FSI	20/20/0	IL-1Ra, sTNF-RII
BC (65)	IL-1Ra, sTNF-RII	FSI	103/0/0	sTNF-RII
BC, PC (66)	IL-1β, IL-6, IL-1Ra	FSI	28+20/0/0	IL-1Ra
TrC (67)	IL-6, TNF-α	BFI-K	90/0/0	No
TC (68)	IL-6, IL-1Ra, sTNF-RI	FQ	92/191/0	IL-1Ra

BC, breast cancer; BFI-K, Brief Fatigue Inventory – Korean; FACIT-F, Functional Assessment of Chronic Illness Therapy – Fatigue; FSI, Fatigue Symptom Inventory; FSS, Fatigue Severity Scale; FQ, the Fatigue Questionnaire; IL, interleukin; IFN, interferon; MFI, Multidimensional Fatigue Inventory; MS, multiple sclerosis; PC, prostate cancer; PSS, primary Sjögren`s syndrome; RAND, RAND 36-Item Health Survey; SF-36, Short Form (36) Health Survey; SLE, systemic lupus erythematosus; TC, testicular cancer; TrC, terminal cancer; TNF, tumor necrosis factor; VAS, visual analogue scale.
[a] Longitudinal or cross-sectional studies; [b] Measured as plasma concentration, whole blood stimulatory capacity or peripheral blood gene expression; [c] Patients with/without fatigue/healthy controls; [d] Measured in cerebrospinal fluid.

The first biologic agent developed was a TNF-α inhibitor, approved by the US Food and Drug Administration for the treatment of RA in 1998 (69). The list of biological agents approved for RA has expanded, and now includes several TNF-α inhibitors, cytokine inhibitors, and other drugs interfering with the activity of the innate and adaptive immune system.

TNF-α inhibitors have proven to be very effective in several autoimmune diseases, such as RA, ankylosing spondylitis and inflammatory bowel disease (70-72). However, infliximab, a chimeric monoclonal IgG1 antibody directed against TNF-α, was without effect in pSS in a large multicenter placebo-controlled trial (73). Further, etanercept, a fusion protein consisting of the soluble human TNF receptor linked to the Fc part of human IgG1, showed no effect in pSS on a variety of disease related variables (74).

In contrast, RTX, a chimeric humanized monoclonal anti-CD20 antibody, is reported to be efficient in pSS, with improvements in dryness, tender points and fatigue (27, 28). Patients with shorter pSS disease duration appear to benefit more than patients with longer disease duration. RTX seems to have an acceptable safety profile in pSS, although several issues still need to be addressed; selection of patients for treatment, treatment intervals and duration, and long term outcome of RTX in pSS (75). Despite these promising results, no biologic agent has so far been approved for the treatment of pSS.

Currently, three biological drugs are developed to target the IL-1 system; anakinra - a recombinant IL-1Ra, canakinumab - an anti-IL-1β monoclonal antibody, and rilonacept - a fusion-protein consisting of the extracellular chains of IL-1RI and IL-1RAcP linked to the Fc portion of IgG.

1.4.1 Anakinra

Anakinra (Kineret ™, Biovitrum AB, SE-112 76 Stockholm, Sweden) is a recombinant IL-1Ra, currently approved for use in RA, adult Still's disease and auto-inflammatory diseases (52). The anti-inflammatory effect is due to inhibition of the

IL-1 system, through antagonist action at the IL-1RI. It can be self-administered by a daily subcutaneous injection in the abdomen or the thigh. Some patients may experience a local allergic reaction; otherwise the frequency of side-effects and adverse events of the drug is low (76). Anakinra has a half-life of only four to six hours, which makes withdrawal easy in case of adverse events. Both endogenous IL-1Ra and anakinra are capable of crossing the blood brain barrier (BBB), presumably through a saturable transport system (51, 77), and has a direct IL-1 inhibiting effect in the brain. However, the drug also works through a reduction in the peripheral effects of IL-1. Thus, it is still uncertain whether the influence of IL-1Ra on brain signalling is due to peripheral or central inhibition of IL-1.

1.5 Oxidative stress

Oxidative stress results from an imbalance between the physiological antioxidant defences and the production of reactive oxygen species (ROS). ROS are mainly free radicals and other highly reactive oxygen species, produced in all cells of an organism. The majority of ROS are generated by oxidative phosphorylation in the mitochondria and by activated cells of the innate immune system (78). ROS are important in bacterial defence and are released through the NADPH-oxidase-dependent respiratory burst to kill engulfed pathogens. Recent evidence also point to a role for mitochondrial ROS in elimination of bacteria in macrophage phagosomes through activation of cell surface TLRs 1, 2, and 4 (79). ROS may have a dual role in chronic inflammation. It activates the ubiquitous transcription factor NF-κB, promoting transcription of a number of pro-inflammatory genes (57), but is also reported to dampen the inflammasome activity under certain conditions (52). It has been proposed that the redox state of resting inflammatory cells influence the redox response to PRR stimulation (80).

Under normal conditions, pro-oxidant forces are balanced by the anti-oxidant systems. Circulating red blood cells contain several anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and are highly potent scavengers of ROS. Anti-oxidants and reducing enzymes are present in plasma

and ubiquitously distributed in most cells. However, infection or autoimmune disease accompanied by chronic inflammation may lead to increased production of ROS in activated phagocytes, outplaying the anti-oxidant defences (79). This disruption of the redox balance results in a state of increased oxidative stress, as reported in SLE (81), RA (82), and MS (83).

ROS are difficult to measure due to short half-life and local variations in concentration from one tissue or even one cell to another. End products of oxidative stress are more stable and can be examined in serum, blood cells, urine, cerebrospinal fluid and tissue samples. Therefore, most oxidative stress assays have been developed based on lipid-, protein- and DNA oxidation products.

Oxidative attack on macromolecules is a relatively unspecific process, but some ROS are known to cause damage to certain amino acids or lipids, resulting in more specific products. One example is the hypochlorous acid (HOCl) generated in activated neutrophils by myeloperoxidase, contributing to the formation of advanced oxidation protein products (AOPP). AOPP comprise pentosidine, proteins cross-linked by dityrosine and protein carbonyls (PC), in addition to other chromophores, all of which show absorbance at wavelength 340 nm (84). Protein oxidation probably reflects the general level of oxidative stress in an individual, although lipid peroxidation is the most widely used marker of oxidative stress.

1.5.1 Oxidative stress in pSS and fatigue

It is not unlikely that oxidative stress is involved in the pathogenesis of pSS. Oxidative stress may contribute to salivary tissue destruction (85), and increased levels of oxidative stress markers in salivary ductal cells from sSS patients have been reported (86). Moreover, pro-oxidant enzyme expression is increased in the conjunctival epithelium in sSS (87), and oxidative stress has been proposed as a treatment target in SLE (88). Further, oxidative stress may play a role in fatigue generation. Several studies point to an association between oxidative stress and fatigue in CFS (89), SLE (90) and fibromyalgia (91), Table 2. Despite the above

mentioned investigations of oxidative stress in SS (85-87), plasma levels of oxidised proteins have not been reported, and the relationship between oxidative stress and fatigue has never been explored in pSS.

Table 2 Selected studies of oxidative stress and fatigue.

Condition	Measure of OS	No.[a]	Assoc. fatigue	Comments
CFS (92)	F2-isoP	25/23	Yes	Fatigue not assessed[b], pediatric CFS
CFS (93)	MDA	40/40	No	Fatigue not assessed[b]
CFS (89)	F2-isoP	47/34	Yes	High CV risk patients excluded
CFS (94)	PC	36/16	Yes	Fatigue not assessed[b]
CFS (95)	MDA[c]	31/41	Yes	Fatigue not assessed[b]
FMS (91)	F2-isoP	48/96	Yes[c]	
SLE (90)	F2-isoP	95/103	Yes[c]	

CFS, chronic fatigue syndrome; CV, cardiovascular; FMS, fibromyalgia syndrome; F2-isoP, F2-isoprostane (urine); MDA, malondialdehyde (serum); OS, oxidative stress; PC, protein carbonyl (serum); SLE, systemic lupus erythematosus.
[a] Patients/healthy controls; [b] Investigated association with CFS/ME, no fatigue scale reported; [c] No significant difference in oxidative stress between patients and healthy controls, more oxidative stress in patients with more fatigue.

1.6 Genetics

Most diseases have a heritable component. This pattern was proposed even before the gardener Gregor Mendel published his famous paper on inheritance of certain traits in the pea plant in 1866 (96). The deoxyribonucleic acid (DNA) was first isolated by Swiss physician Friedrich Miescher in 1869, and the double helix structure of the DNA was described by James D Watson and Francis Crick in Nature in 1953 (97), a discovery rewarded with the Nobel Prize in Physiology or Medicine in 1962. The euchromatic human genome was mapped in 2003, a process that took 13 years and cost 3 billion USD (98). The abilities of the gene sequencing technologies have since

expanded exponentially, and at present time the human genome can be mapped in hours, at a cost of less than 10 000 USD (99).

1.6.1 DNA and genes

DNA consists of two long strings of nucleotides, linked to backbones made of alternating sugar (deoxyribose) and phosphate. The nucleotides are adenine (A), guanine (G), thymine (T) and cytosine (C), and these four nucleotides form hydrogen linked base-pairs between the strands; A-G and C-T. The haploid human genome consists of more than 3 billion base-pairs. The two DNA strands run in opposite directions and are wrapped around each other in the structure of a double helix. DNA is tightly packed in structural units named nucleosomes, consisting of a histone octamer with 147 base-pairs wrapped around it. The nucleosomes are packed closely together, and this structure enables large genomes to be contained in the nucleus of a single cell, Figure 1.

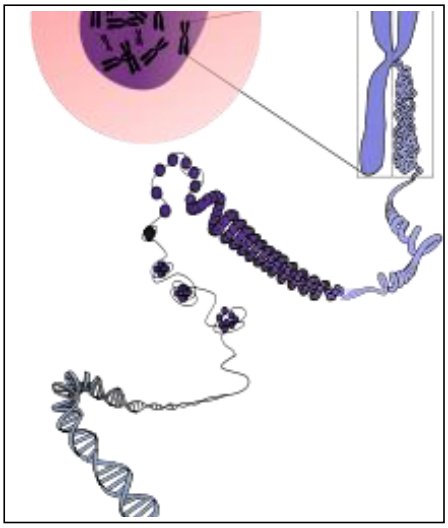


Figure 1. DNA is tightly packed into chromosomes, contained in the nucleus of the cell.

A gene is the molecular unit of heredity. It is a defined sequence of DNA that encodes a functional unit, either a protein or a RNA molecule. The sum of the genes and the

non-coding DNA sequences in an organism or cell is known as a genome. The human genome is stored on 46 chromosomes, 22 pairs of autosomes and one pair of sex chromosomes. A locus is the region of a chromosome in which a particular gene is stored (100). The human genome contains approximately 30 000 (25 000) genes, and the genetic code is about 99.8% similar across the human population (101).

1.6.2 Single nucleotide polymorphisms

The 0.2% variation in genetic code across mankind is mainly the result of copy number variation (CNV) and SNPs (101). A SNP is a variation in the DNA sequence at a specific position, due to a difference in a single nucleotide –A, T, C or G – between members of the same species, or between the paired chromosomes in one individual. The more common variant is denominated the *major allele* and the rare variant the *minor allele*. By definition SNPs occur with a minor allele frequency (MAF) of >0.5% in the population under study, otherwise the polymorphism is considered a rare or private variant (101). SNPs may fall within coding (exon) or non-coding (intron) regions of genes, or in the intergenic regions. Also, the consequences of a SNP depends on its position (locus) and nucleotide sequence; a synonymous polymorphism leaves the gene product unaltered, a mis-sense polymorphism results in a different amino acid, and a non-sense polymorphism results in a premature stop codon (100). Variations in the DNA sequence may influence a person's susceptibility to disease, sometimes through changes in the immunological response to pathogens, drugs, chemicals and vaccines. Genome-wide association studies (GWAS), testing hundreds or thousands of samples for disease associations with several hundred thousand SNPs, have successfully uncovered many genetic variants that increase the risk of complex diseases.

It was assumed that mapping the human genome would reveal the patterns of heritability in complex diseases, however, disease-associated SNPs, alone or in combination, usually account for only a small proportion of the inheritable component of disease risk (42, 102), with an overall risk of 1.1-1.5 in most cases. Genetic variation has been extensively studied in autoimmune diseases, and even when several

disease-associated SNPs are found in one individual, the clinical pattern is not fully explained.

1.6.3 Epigenetics

Epigenetics is the study of heritable and potentially reversible changes in genome function not involving alterations in the DNA nucleotide sequence. Epigenetic modifications can occur in response to environmental factors and play a fundamental role in the regulation of gene expression. The epigenome, i.e. the epigenetic state of an organism, is proposed to be just as important as the genome to normal development (103). In contrast to genetic changes, epigenetic alterations arise in a gradual manner, leading to a progressive change in expression of specific genes. The major epigenetic mechanisms, histone modifications and DNA methylation, lead to conformational changes in the chromatin structure. Hypermethylation of tDNA makes chromatin more condensed, a process that impairs binding of transcription factors and ultimately leads to decreased gene expression from these loci (104). This is important for imprinting, the final differentiation of cells, as DNA methylation patterns ensure that fully differentiated cells do not enter aberrant gene expression. Demethylation of the DNA is reported to be involved in both cancer and autoimmune disease development (105, 106). Hypomethylation of the *TNFSF7* promoter region in CD4+ T-cells, associated with increased expression of the T-cell surface molecule CD70, has been reported in pSS (107). CD70 is a B-cell co-stimulatory molecule that promotes plasma cell differentiation and immunoglobulin production; thus aberrant expression of CD70 may lead to B cell auto- and hyperactivity. Epigenetic modifications are reversible, and could represent new treatment targets in autoimmune diseases.

1.6.4 PSS, fatigue, and genetics

Genetic variation has mainly been investigated in relation to fatigue in CFS (108, 109) and in cancer related fatigue (110, 111). A recent systematic review covering SNP analyses, gene expression studies and bioinformatics data-mining studies in

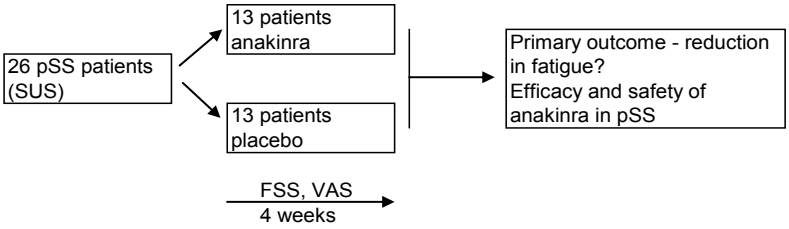
relation to fatigue identified 33 articles on CFS and 8 articles regarding conditions other than CFS. The main conclusion was that *"....there is great heterogeneity within genetic studies of fatigue in terms of sample sizes, sample descriptions and findings"* (111). Genes related to the hypothalamus-pituitary-adrenal (HPA)-axis and to the immune system were implicated in more than one study, but otherwise the complexity of the findings was more striking than the unity. So far no single gene variant that clearly increases the susceptibility to fatigue has been found. Genetic variation has never been explored in relation to fatigue in pSS, but it is not unlikely that certain gene variants or SNPs may be of importance. It is a conundrum why some pSS patients develop more fatigue than others, independent of disease activity or co-morbidity. Maybe parts of the explanation can be found in the genetic or epigenetic makeup of each individual.

It is an interesting thought that epigenetic modifications influence fatigue, and the epigenome could represent a connection between environmental factors and fatigue. This hypothesis is new and so far virtually no research has been performed in this area (112).

2. Aims of the study

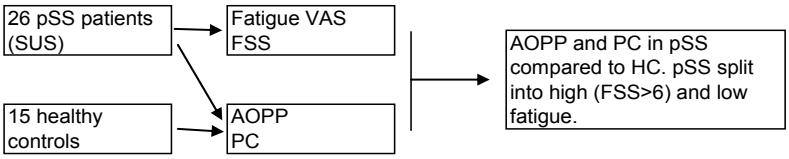
- Write a review article of the current knowledge of biological mechanisms of chronic fatigue in inflammatory and non-inflammatory conditions.
- Investigate the efficacy and safety of IL-1 inhibition on fatigue in pSS.
- Evaluate the level of plasma oxidative stress markers in pSS as compared to healthy individuals, and further explore any associations of oxidative stress with fatigue in pSS.
- Investigate SNPs in selected genes in relation to fatigue in pSS.

3. Overview over Papers I-IV



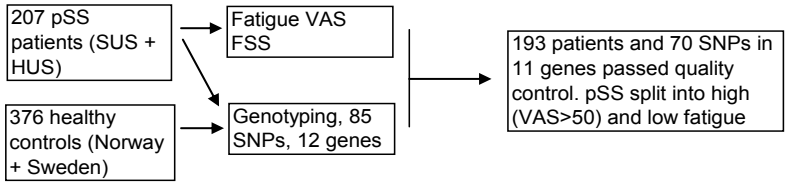
Paper I: Interleukin-1 inhibition and fatigue in primary Sjøgren`s syndrome

No effect on fatigue in primary outcome. Post hoc analysis indicate reduction in fatigue from anakinra.



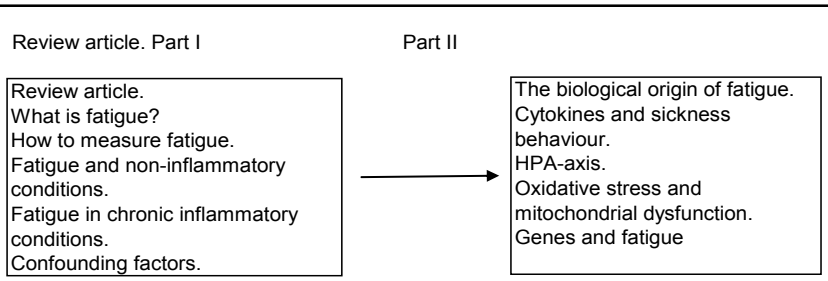
Paper II: Oxidative stress, as measured by protein oxidation is increased in primary Sjøgren`s syndrome

Increased protein oxidation in pSS. Not associated with high fatigue.



Paper III: A possible genetic association with chronic fatigue in primary Sjøgren`s syndrome: a candidate gene study

A trend for association between SNP in *SLC25A40* and pSS high fatigue vs controls, *and PKN1* and pSS high vs low fatigue.



Paper IV: Biological mechanisms of fatigue

4. Subjects and methods

4.1 Patients with pSS

The patient register used to recruit the pSS patients included in this dissertation was set up by our research group, and the work was led by professor Roald Omdal (113). The register is based on a thorough review of the medical charts of all patients with a pSS diagnosis referred to SUS in 1980 – 2005. Patients who fulfilled the AECG criteria (10) were included, and patients close to fulfilling the criteria were invited to a new screening visit. In addition, the results of all MSG biopsies performed at SUS during the same time period were identified, and medical charts of patients with a focus score ≥ 1 were reviewed. Ninety-nine patients were identified in 2005. The register was updated in 2008, including 125 pSS patients aged 18-80 years living in Rogaland County, Norway. This updated register was used to recruit patients to the clinical trial and the oxidative stress assessment (Paper I and II).

For the genetic analysis, patients from both the 2005 SUS cohort and the 2005 HUS cohort were included. The HUS cohort was identified by investigators at the Department of Rheumatology, HUS, through assessment of medical records of all patients fulfilling the ICD-10 code for pSS (M35.0) from 1999 to 2005. A total of 141 patients fulfilling the AECG criteria were identified. Seventy-two patients from SUS and 135 patients from HUS were included in the genetics study (Paper III).

All patients and healthy controls who participated in any of the studies or assessments gave written informed consent to participate, and all investigations were carried out in compliance with the principles expressed in the Declaration of Helsinki. The clinical trial was registered at ClinicalTrials.gov (number NCT00683345) and was approved by the regional ethics committee, REK-Nord, Norway.

4.2 Healthy controls

Healthy controls (HCs) were included for Paper II and Paper III. Apart from age and gender, no demographic or clinical information was registered in the healthy subjects.

None of the HCs had known neurological, immunological or active malignant disease, and the HCs were not related to the pSS patients. Fifteen HCs, recruited among employees at SUS, were included in Paper II to provide reference values for oxidative stress measures. Three hundred and seventy six HCs, both Norwegian and Swedish, were included in Paper III to give a comparison to the local genetic background. The Swedish HCs (n=236) were partly population based and partly recruited from blood donors. The Norwegian HCs (n=140) were recruited from hospital staff, friends and colleagues of the patients.

4.3 Blood and urinary samples

All pSS patients were screened at inclusion in 2005 or 2008 and once again for the clinical trial. The screening included routine haematological and biochemical tests in addition to autoantibody, complement and immunoglobulin assessment. Conventional urinary analyses with dip-stick and protein/creatinine ratio were performed. The same combination of analyses was repeated at week 0, week 2, week 4 and week 5 for the patients included in the clinical trial.

4.4 Evaluation of depression

The Beck Depression Inventory (BDI) was used to assess mood (114). BDI is a generic instrument that evaluates the current level of depression, widely used in clinical studies. A BDI score below 13 indicates no depression, while a score of 14-19 is regarded to represent mild depression. A cut-off score of ≥ 20 was used in Paper I and II to exclude moderately to seriously depressed individuals. Mood was assessed in all patients in 2005 and 2008, and the assessment was repeated for the patients included in the clinical trial.

4.5 Evaluation of fatigue

The Fatigue Severity Scale

The FSS is a generic fatigue measuring instrument (15). The FSS score is the mean of the sum of scores in 9 items, each rated from 1-7. Patients are asked to relate the

questions to the last two weeks. A FSS score of 3 is commonly applied as a cut-off value for fatigue in systemic lupus erythematosus (SLE) and was used in this dissertation. The FSS was selected because it is unidimensional, has a high sensitivity, reliability and internal consistency, and is validated and used in pSS and a number of other diseases (15, 115, 116)

The fatigue visual analogue scale

A horizontal 100 mm line with vertical anchoring lines represents the fatigue VAS used. The wording at the left end (0 mm) is "No fatigue" and at the right end (100 mm) is "Fatigue as bad as it can be". The patients are asked to rate their fatigue at present. The VAS is a single uni-dimensional measure, relatively easy for patients to understand and score, and sensitive to change (117, 118).

4.6 Paper I

Inclusion criteria were: pSS according to the AECG criteria (10), Caucasian descent and Norwegian language. Exclusion criteria were: moderate to severe depression (BDI score $\geq$ 20 (114)), untreated comorbidity that may explain the presence of fatigue (i.e. heart failure, untreated hypothyroidism), lack of fatigue (FSS $\leq$ 3) (15), neutropenia (neutrophil count < 1.5 x 10^9/L), anemia (haemoglobin < 100g/L), present or recurrent infections, pregnancy, lactation, or concurrent treatment with biologic agents.

Twenty-six patients were deemed to be ineligible based on a review of their medical record. Thus, 99 patients were sent an invitation to participate in the study, which for logistic reasons was conducted in two phases. Fifteen patients agreed to participate in the pilot trial in 2008, of whom eight were included. The remaining 84 patients were invited to participate in the main study in 2010, and 18 patients were included. Figure 2 illustrates the inclusion and reasons for non-inclusion.

The study was designed as a single centre, prospective, randomised, double-blind placebo-controlled parallel-group trial. Anakinra or placebo was given as a daily

subcutaneous injection for four weeks. All patients had a total of five study visits; baseline (inclusion), week 0 (first injection), week 2, week 4 (last injection) and week 5. Blood samples were drawn and fatigue and safety was assessed at every visit, whereas depression was assessed at baseline and week 5. All study visits took place at SUS.

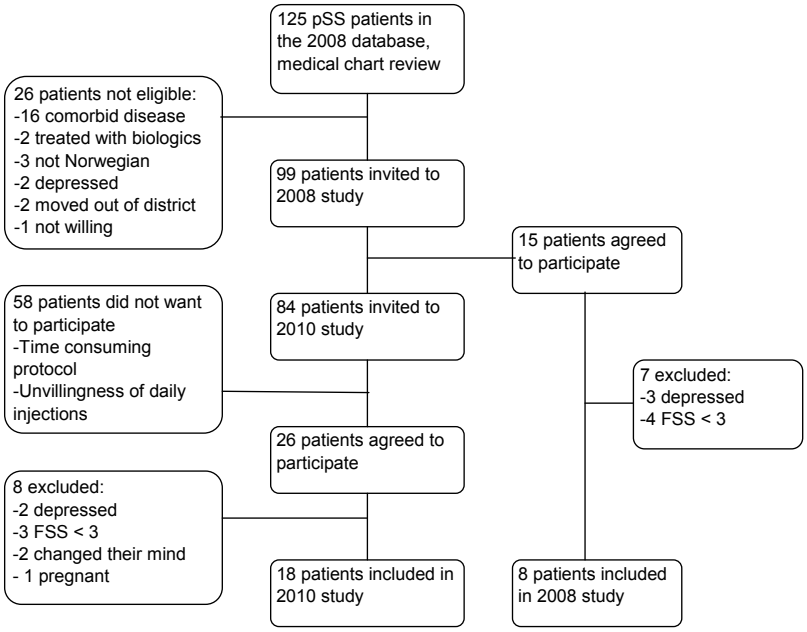


Figure 2. Flowchart of inclusion in the study. The pilot study was conducted in 2008 and the main study in 2010. All patients underwent the same procedures.
FSS, Fatigue Severity Scale; pSS, primary Sjøgren`s syndrome.

Study drug and randomisation

The 26 participants were randomly assigned to receive double-blinded therapy with either anakinra (Kineret^{TM}, BioVitrum AB, SE-112 76 Stockholm, Sweden) 100 mg/day or placebo (0.9% NaCl in an identical syringe). The treatment allocation took place after complete inclusion using a computer generated randomisation list administered by the hospital pharmacy. We used simple randomisation, with a 1:1

allocation, and neither the investigators, study nurses nor the patients were aware of the assigned treatment.

The study was conducted in two phases, as placebo could not be produced in syringes identical to the active drug in 2008. The eight patients included in the 2008 pilot trial received their daily injections at the hospital. The active drug or placebo was prepared in identical syringes by a research nurse who was unblinded and not involved in patient handling. Placebo could be produced in syringes identical to the active drug in 2010, with a durability that allowed the patients to receive a 14 days supply of the allocated treatment at week 0 and week 2. A study nurse supervised the first injection at week 0, and trained the patients to self-administer the drug or placebo. The patients registered each injection in a form, and the form and the empty syringes were collected at the visits at week 2 and week 4.

Outcome measures

The primary outcome measure was a group-wise comparison of fatigue scores at week 4, adjusted for baseline values. Secondary outcome measures were the change in fatigue score within each treatment-group, and safety issues. As a post-hoc outcome the proportion of patients who achieved a 50% reduction in fatigue in each treatment-group was compared.

4.7 Paper II

Plasma samples from the 26 patients included in the clinical trial (described in detail for Paper I) were used for Paper II, in addition to samples from 15 healthy control subjects, who voluntarily donated blood to the study. The healthy control group comprised 12 females and 3 males, with a median age of 49 [27-65] years. No other clinical information was registered, as the purpose of the control group was to provide reference values for oxidative stress measures, not to serve as case controls for the patients. Blood samples were drawn from patients at week 0, before any interventions or examinations had taken place. EDTA blood was centrifuged for 15 minutes at 2000

G at 4°C and stored in aliquots at minus 72°C until analysed. Freshly thawed samples were used for both AOPP and PC measurements.

AOPP

AOPP was assessed by a method recently developed by our group (119). Briefly, 40 µl of plasma was transferred to a 96 well microplate, and citric acid (60 µl; 0.20 mol/L) was added. The absorbance at 340 nm was read after 2 minutes on a microplate shaker. The external calibration standards used were chloramine-T diluted in citric acid with potassium iodide added, and the concentrations of AOPP are expressed as µmol/l chloramine-T equivalents.

PC

We used a modified version of a previously described method to analyse PC (120). Briefly, 2,4-dinitrophenylhydrazine was used to derivatize the carbonyl groups to form stable hydrazones, and excess reagent was separated by reversed phase high performance liquid chromatography (RP-HPLC, Waters Alliance e2695 connected to a Waters 2487 Dual Absorbance Detector). The 20 µl sample was then injected onto a Gemini C18 security guard column (5µm, 2 mm x 4 mm, Phenomenex, USA). The HPLC mobile phase consisted of (A) 0.1% trifluoroacetic acid and (B) 100% 2-propanol, with an elution profile as follows: 0–2 min, 10–90% B linear gradient; 2–3 min, isocratic 90% B, 3–6 min, isocratic 10% B. The flow rate was held at 0.6 ml/min. Eluted hydrazones and total protein were measured simultaneously at absorption wavelengths of 366 nm and 280 nm, respectively, and PC concentrations were reported as nmol/mg protein.

4.8 Paper III

A total of 207 patients were included for the candidate gene study, 72 from the SUS cohort and 135 from the HUS cohort. Matched control samples were collected from Sweden (n=236) and from Norway (n=140). DNA was extracted from the blood samples according to standard procedures. We choose a panel of SNPs in 12 genes;

the gene selection was based on previous studies of gene expression in CFS (108, 121, 122). The genes were *APOA2, EIF2B4, EIF4G1, SLC25A40, SLC25A16, CRAT, MRPL23, COX8A, ABCD4, COX11, PKN1* and *PEX16.* The GoldenGate Assay (Illumina Inc, San Diego, CA, USA) was used to genotype 85 SNPs in the 12 genes. Samples with a call rate <0.90, SNPs with Hardy-Weinberg equilibrium test p-values < 0.001 and SNPs with a minor allele frequency (MAF) < 0.01 or more than two alleles were excluded from further analysis. The MAF in the control samples from Norway and Sweden were found to be similar and the control subjects were therefore merged to one cohort. After quality control, 193 patients and 70 SNPs in 11 genes were available for analysis. The patients were dichotomised according to fatigue scores, with a VAS score < 50 (N=53) classified as "low fatigue" and a VAS score ≥ 50 as "high fatigue" (N=140).

4. 9 Paper IV - Review article

This is a non-systematic review article. The literature search was performed based on the authors' preferences, and included articles were regarded by the authors to be of high quality, relevant and/or describing interesting hypotheses. A special emphasis was put on the description of sickness behaviour and fatigue.

4.10 Statistical methods

Results are reported as mean ± standard deviation (SD) when normally distributed, otherwise as median and range. The Fisher's exact test and the Students t-test were used for the comparison of categorical and continuous data, respectively. In Paper I, Analysis of covariance (ANCOVA) was used to evaluate changes from baseline to week 4 between groups, and Friedman's test for repeated measures was used to analyse changes over time within groups. T-tests were used in Paper II to compare the levels of AOPP and PC between the patients and HC, and between patients with high and low fatigue after the group was dichotomised based on FSS scores. Further, we used "*limits of agreement*" to test the agreement between AOPP and PC after z-scores were calculated. In Paper III, logistic regression analysis was used to explore the

relationship between SNP genotype and high/low fatigue scores. A p-value of < 0.05 was considered significant in Paper I and II, and a p-value of ≤ 0.04 was considered significant in Paper III due to multiple comparisons. Analyses were performed using SPSS version 15.0 (Paper I, II, III) and SNP & Variation Suite 7 (Golden Helix, Bozeman, MT, USA) (Paper III). The Genetic Power Calculator was used for the genetic analysis (Paper III) (123).

5. Summary of results

Paper I

This double-blind, placebo-controlled parallel group study included 26 pSS patients, who were randomised to receive either the IL-1Ra anakinra or placebo. The two groups of patients were comparable in demographic and biochemical variables, and patients with moderate to serious depression were excluded. The study lasted 4 weeks, and the active drug (anakinra 100mg/day) or placebo (0.9% NaCl in identical syringe) was self administered by the patients. Study-visits were scheduled at week 0 (start of study), week 2, week 4 (end of study) and week 5 (safety follow-up). Fatigue was assessed by fatigue VAS and FSS at all study visits. One patient did not show at week 4, and was excluded from the analysis. The primary outcome, a baseline-adjusted reduction in fatigue at week 4 in the active drug group as compared to the placebo group, was not achieved (p=0.19). However, as a post-hoc outcome, the proportion of patients in each group who reached a 50% reduction in fatigue was calculated. Six out of 12 patients on active drug and 1 out of 13 patients on placebo reached this outcome (p=0.03).

Conclusion: This study did not achieve its primary outcome, a significant reduction in fatigue after treatment with an IL-1Ra. However, post hoc analysis indicates that IL-1 blockade has a strong effect on fatigue, and new studies of IL-1Ra and fatigue are warranted.

Paper II

In this paper we compared the serum level of oxidised proteins in pSS and in HC. Blood samples were drawn from 26 pSS patients (median age 55, range 18-80 years) and 15 HC (median age 49, range 27-65 years). Protein oxidation was measured using AOPP and PC. Fatigue was assessed in the pSS patients using fatigue VAS and FSS. Significantly increased levels of oxidative stress were detected in the pSS patients compared to the HC. This accounted for both AOPP ($p<0.002$) and PC ($p=0.0005$). There was no association between fatigue and protein oxidation in pSS. None of the demographic (age, gender, smoking), clinical (disease duration, cardiovascular disease, BMI, depression) or biochemical (neutrophil cell count, C-reactive protein, creatinine, complement factor C4, presence of anti-SSA/SSB antibodies) variables were associated with protein oxidation as measured by AOPP or PC.

Conclusion: Oxidative stress, as measured by protein oxidation, is significantly increased in pSS, but is not associated with fatigue.

Paper III

A total of 207 patients from HUS (N=135) and SUS (N=72) and 376 HCs were included in this paper. Fatigue was assessed with VAS and FSS. Genotyping of 85 SNPs in 12 selected genes was performed; the genes were selected based on previous gene expression studies in CFS. 193 patients and 70 SNPs in 11 genes were available for analysis after quality control. The fatigue scores were dichotomized, with a VAS score ≥ 50 representing "high fatigue" (N=140) and a score < 50 representing "low fatigue" (N=53). Logistic regression was used to explore allelic associations with pSS/high/low fatigue. In the pSS case versus control analysis, signals of association with pSS were detected for one SNP in *SLC25A40* (unadjusted p=0.007) and two SNPs in *PKN1* (both p=0.03), Table 3. The association with *SLC25A40* was stronger in the analysis of only pSS high fatigue patients versus controls (p=0.002). In the case-only analysis of pSS high fatigue versus pSS low fatigue, one SNP in *PKN1*

displayed an association (p=0.005). When all analyses were corrected for the number of genes and traits (pSS, pSS with high fatigue, pSS with low fatigue) tested, only the association between rs10276819 in *SLC25A40* in the pSS high fatigue versus healthy control analysis remained borderline significant (p=0.066). Power to detect association was 5-42%.

Conclusion: We detected a trend for association between genetic variation in the genes *SLC25A40* and *PKN1* and fatigue. The association with *SLC25A40* remained borderline significant after correcting for multiple testing, and replication studies are warranted.

Paper IV

This paper is a non-systematic review article of the biological mechanisms of fatigue. Fatigue is defined as an overwhelming sense of tiredness, lack of energy and feeling of exhaustion (15), and the challenges associated with the assessment of fatigue are described. Fatigue is a subjective experience, and self-reported instruments are necessary for fatigue evaluation. Fatigue is common in both non-inflammatory and inflammatory conditions, with Parkinson's disease and cancer related fatigue as examples of the former, and SLE and pSS examples of the latter. The article describes factors confounding to fatigue, with a special emphasis on depression. The biological origin of fatigue is the main part of the article, with a thorough description of the IL-1 system and sickness behaviour. Other biological mechanisms for fatigue are also important, in particular oxidative stress, genetic variation and alterations in the function of the HPA axis.

Conclusion: There are biological and psychological mechanisms for fatigue. In a clinical perspective it is important to distinguish these mechanisms, as it may influence the choice of treatment for the individual patient.

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Table 3. Allelic association of individual SNP by logistic regression

		pSS cases vs controls		**MAF**	
Allele	**Gene**	**OR (95% CI)**	**P**	**pSS (n=193)**	**Controls (n=376)**
rs10276819	*SLC25A40*	0.5 (0.2 to 0.8)	0.007	0.033	0.072
rs3786654	*PKN1*	1.3 (1.0 to 1.7)	0.03	0.39	0.32
rs10416904	*PKN1*	1.6 (1.1 to 2.5)	0.03	0.1	0.07
rs2241362	*PKN1*	0.9 (0.7 to 1.3)	0.63	0.2	0.21
		PSS+ vs controls		**MAF**	
Allele	**Gene**	**OR (95% CI)**	**p**	**pSS+ (n=140)**	**Controls (n=376)**
rs10276819	*SLC25A40*	0.3 (0.1 to 0.7)	0.002	0.025	0.072
rs3786654	*PKN1*	1.4 (1.0 to 1.8)	0.03	0.39	0.32
rs10416904	*PKN1*	1.5 (0.9 to 2.4)	0.11	0.1	0.07
rs2241362	*PKN1*	0.7 (0.5 to 1.0)	0.08	0.16	0.21
		PSS+ vs pSS-		**MAF**	
Allele	**Gene**	**OR (95% CI)**	**p**	**pSS+ (n=140)**	**pSS- (n=53)**
rs10276819	*SLC25A40*	0.4 (0.1 to 1.3)	0.14	0.025	0.06
rs3786654	*PKN1*	1.1 (0.7 to 1.8)	0.65	0.39	0.37
rs10416904	*PKN1*	0.8 (0.4 to 1.5)	0.46	0.1	0.12
rs2241362	*PKN1*	0.5 (0.3 to 0.8)	0.005	0.16	0.3

The table shows p-values from a logistic regression on allelic frequencies, three outcomes were tested: cases with pSS versus controls, pSS cases with high fatigue versus controls, pSS cases with high fatigue versus pSS cases with low fatigue.
CI, confidence interval; MAF, minor allele frequency; PSS+, primary Sjögren`s syndrome with high fatigue; PSS-, primary Sjögren`s syndrome with low fatigue.

6. Discussion

The research reported in this dissertation is based upon the hypothesis that there are biological mechanisms for fatigue in pSS. We have tested this hypothesis in several novel ways; through exploration of cytokines, genes and oxidative stress. We report the first clinical trial of IL-1 inhibition in pSS, and the results clearly point to a positive effect of the intervention. Further, our study is the first to show that plasma levels of oxidised proteins are significantly increased in pSS, although not associated with fatigue. Finally, we have investigated genetic variation in relation to fatigue. There were no significant signals of association between fatigue and SNPs in the 12 candidate genes selected for our study.

6.1 Evaluation of the main findings

Our clinical trial of IL-1 blockade shows that anakinra is safe for short-term use in pSS. Post hoc analysis of the results indicates a clear improvement in fatigue in the patients who received the active drug compared to patients on placebo. This clinical trial was initiated as a hypothesis-testing study, based on the concept of sickness behaviour. Sickness behaviour occurs in animals after administration of LPS or pro-inflammatory cytokines, and IL-1 is a key actor. Fatigue can be considered a human parallel to sickness behaviour, and our study offers support to this concept. Several other recent studies have also utilised biological drugs in the treatment of fatigue. RTX has shown promise in pSS (27, 28), and in secondary endpoints in CFS (21). Further, IL-6 blockade is reported to reduce fatigue in SLE (30) and RA (124), and TNF-α inhibitors reduce fatigue in RA (31). Taken together, these studies indicate that specific components of the activated immune system are important in fatigue signalling, and that inhibition of these factors reduces fatigue.

The study was not able to meet the primary endpoint, a baseline-adjusted improvement in fatigue VAS at week 4. It is not unlikely that low power due to too few patients is the main reason this endpoint was not achieved. All pSS patients in southern Rogaland were invited, but after baseline screening only 26 patients were

eligible and willing to participate. One way to include more patients would be to collaborate with other regional or national centres, and we suggest this option is considered for future treatment studies in pSS.

Oxidative stress, assessed by plasma protein oxidation, is increased in pSS compared to healthy individuals. This is not unexpected, as inflammation and oxidative stress are intimately connected. However, earlier studies have indicated that oxidative stress and mitochondrial dysfunction may contribute to fatigue in CFS (89, 94), SLE (90) and fibromyalgia (91), Table 2. The results of our study are conflicting. One possible explanation is that most studies of oxidative stress and fatigue have measured lipid peroxidation; only one study has investigated and found an association between PC and fatigue (94). However, oxidative stress is assumed to be a general process, and it is unlikely that extensive lipid peroxidation would not be accompanied by protein oxidation. A more likely reason for the conflicting results is that protein oxidation is not important for fatigue generation in pSS.

Genetic variation is known to influence most human traits and conditions. It seems intuitively appealing that genetic polymorphisms could contribute to fatigue susceptibility or protect from fatigue. We investigated SNPs in 12 candidate genes, and detected signals of association for three SNPs in *PKN1*and one SNP in *SLC25A40*, Table 3. The SNP rs10276819 in *SLC25A40* was associated with pSS, and this association was even stronger when only the pSS patients with high fatigue were analysed versus the controls (p=0.002, OR 0.3 (CI 0.1-0.7). MAF for this SNP was 0.025 in pSS with high fatigue, 0.06 in pSS with low fatigue and 0.072 in controls, Table 3. This illustrates the possible protective effect of the minor allele. The SNP rs2241362 in *PKN1* was associated to high fatigue in the case-only analysis of pSS high fatigue versus pSS low fatigue. Due to the 11 genes and three traits under investigation, a p-value cut off < 0.0015 (0.05/33) was necessary for statistical significance. When adjusting for multiple analysis, the SNP in *SLC25A40* remained borderline significant, while the association signals from *PKN1* were non-significant. Thus, we cannot conclude that there is an association, and replication studies are

warranted. *SLC25A40* encodes a mitochondrial protein, widely expressed in the brain but also in the periphery (125). *PKN1* is ubiquitously expressed, and the encoded protein is involved in apoptosis, cell-signalling and negative regulation of NF-κB (126). Polymorphisms in these two genes may influence biological pathways connected to inflammation and oxidative stress, ultimately related to fatigue generation. Obviously, our results only relate to the genes examined, and it is likely that other genes also influence the susceptibility to fatigue. An inherent challenge with genetic studies is patient recruitment, as several thousand individuals may be needed to detect genome-wide significance in GWAS. The need of a large sample size is due to the large number of significance tests performed simultaneously – one for each SNP or gene. A candidate gene approach reduces the number of tests performed, and thereby reduces the necessary sample size. We recruited pSS patients from two hospitals covering 18% of the Norwegian population, but still achieved a relatively low power of 5-42%. With a higher power we might have been able to detect other associations, and the reported associations might have been stronger. We cannot exclude a type II error due to low power. There is a dearth of genetic studies on fatigue in well-characterised patients, but with more research we believe several significant associations will be found. Future studies should focus on including even larger sample cohorts, probably through national and international collaboration. A GWAS focusing on fatigue in pSS would be of great interest.

6.2 Evaluation of methods

Fatigue

There is a multitude of fatigue measuring instruments, all based on self-report. The various instruments can be classified as either disease-specific or generic, and either multi- or uni-dimensional. For a non-exhaustive list of fatigue measuring instruments, see Table 3.

Table 3. Some of the most frequently used fatigue scales.

Name of scale	Dimensions	Comments
Chalder Fatigue Scale (127)	Physical fatigue, mental fatigue	Generic
Fatigue Assessment Instrument (128)	Fatigue severity, situation-specific fatigue, fatigue consequences, responsiveness to sleep/rest	Generic
FIS (129)	Physical fatigue, cognitive fatigue, psychosocial fatigue	Generic
FSS (15)	Uni-dimentional	Generic
MFI-20 (130)	General fatigue, physical fatigue, mental fatigue, reduced motivation, reduced activity	Generic
The Piper Fatigue Scale (131)	Behavioral/severity, affective meaning, sensory and cognitive/mood	Generic
Fatigue VAS	Uni-dimentional	Generic
Medical Outcomes Study Short Form 36 (SF-36) (132)	Vitality subscale assesses fatigue	Generic HRQOL measure
Parkinson Fatigue Scale (133)	Physical fatigue	Parkinson`s disease
Profile of Fatigue (134)	Somatic fatigue, mental fatigue, general discomfort	PSS

FIS, Fatigue Impact Scale; FSS, Fatigue Severity Scale; HRQOL, health-related quality of life, MFI, Multidimensional Fatigue Inventory ; PSS, primary Sjøgren`s syndrome.

It is currently not clear which approach is the best to measure (central) fatigue in pSS, and how well the different scales measure the core experience of fatigue, not influenced by depression or other confounders. Importantly, all scales are based on self-report questionnaires that each patient may interpreted differently. It is crucial that the instructions for filling out the scale are properly explained, and ideally the same person should administer the scale to all patients in a study. Recall bias and mood at present are possible hazards to all self-report instruments. The measured degree and prevalence of fatigue in a (patient) population depends on the fatigue measuring instrument used, and results from different scales are difficult to compare. Wolfe compared the fatigue VAS scale with three multidimensional fatigue

instruments in RA, and found that the VAS scale performed "*as well as or better than longer scales in respect to sensitivity to change, and is as least as well correlated with clinical variables as longer scales*" (118). It is debated what represents the minimal clinically important difference for the various fatigue-scales. A statistically significant reduction in fatigue score is not necessarily important or even detectable by the patient. A common approach is to predefine the numerical reduction in fatigue score that is considered clinically relevant, i.e. a 15 mm reduction in fatigue VAS. But a 15 mm reduction may not represent the same experience for a patient who starts off with a fatigue VAS score of 90 and a patient with a VAS score of 30. Dass et al used a 20% reduction in fatigue as an outcome measure in their clinical trial of rituximab and fatigue in pSS in 2008 (27). We believe this is a sensitive approach to fatigue assessment, although in our opinion a 20% reduction in fatigue may not always be clinically relevant. Fatigue fluctuates over days and months, and the limitations to self-report scales should be taken into consideration when defining a cut-off value. For this reason we applied a 50% reduction in fatigue as a post hoc outcome in the clinical trial (Paper I). The best approach to evaluate repeated measures of fatigue is currently not known, but intuitively a 50% reduction in fatigue over time (half as much fatigue as before) seems a sensible endpoint.

Protein oxidation

Several biomarkers are available to assess the degree of oxidative stress in an organism, but there are limitations regarding their utility. Ideally a biomarker of oxidative stress should be able to predict the development of disease, detect a major part of the oxidised target, be stable upon storage and not confounded by diet, and measured by validated technology in samples that are easily obtainable, such as blood and saliva (135). AOPP and PC fulfil most of the suggested criteria for an ideal biomarker. AOPP and PC are reportedly associated with the development of disease, not only autoimmune, but also cardiovascular, renal and pulmonary, Table 2 and (136-139). Most studies of AOPP and PC have been cross-sectional, and so a causative association between the biomarkers and disease has not been established.

Both AOPP and PC detect major parts of the oxidised protein and can be measured in plasma by validated analysing methods. Diet is a confounder in the assessment of protein-oxidation, as we have previously shown (119). It must be considered a limitation to Paper II that the individuals under study were not fasting when blood samples were drawn.

AOPP methods have suffered from poor reproducibility and accuracy, mainly due to precipitation of lipids. Our group has developed an improved method for AOPP detection, in which plasma lipids are solubilised (119). This method has a significantly improved reproducibility and accuracy when compared to formerly published methods. We strongly encourage the use of this method in future studies. PC is the most frequently used measure of oxidative stress (140) and is also considered a reliable marker of free radical reaction intensity (141). It has a longer half-life than lipid peroxidation products (142). By using two recognised markers of protein oxidation we have shown that oxidative stress is increased in pSS. One limitation to Paper II could be that we have not measured lipid or DNA oxidation, and not assessed the antioxidant defences. Regarding the latter, results are conflicting whether antioxidant capacity is reduced (depleted) or not during inflammation (135). We believe the chosen biomarkers of oxidative stress are valid and representative of the redox balance in the individual.

Gene analysis

We have investigated SNPs in candidate genes, based on gene expression studies in CFS (108, 121, 122). The candidate genes were selected in 2008, at a time when few studies of genetic variation and fatigue were available. In the light of recent years` extensive research in this area, gene selection for future studies should be based upon genetic associations found in expanded patient materials.

An inherent challenge in fatigue research is the use of self-report scales. The FSS and VAS instruments were administered by different persons at SUS and HUS; this might have influenced the fatigue scoring and ultimately the grouping of patients. In Paper

III, dichotomizing according to VAS scores ≥/<50 classified a patient with VAS 49 as "low fatigue" and VAS 51 as "high fatigue". Much information can be lost in dichotomising the results from continuous scales (143), however this approach was considered best for the associations analysis of genotype and fatigue. Other values of VAS or the FSS could have been used as cut-off values for dichotomisation; we cannot exclude the possibility that this might have altered the results. An alternative approach would be to exclude all patients with VAS scores between 40-60mm, to achieve more homogenous "high/low fatigue" populations. However, the power to detect an association would decrease with the smaller sample size.

Low power is a limitation to Paper III. Power calculations in gene studies are based on several assumptions made prior to the analysis. Power is presented as an interval, which reflects the influence of MAF on power. A low MAF decreases power while a high MAF increases power; common variants are more likely to be detected. In addition to MAF and sample size, power is influenced by the genotype relative risk (GRR) of the SNP or loci in question. We used a conservative approach, estimating GRR for fatigue to be 1.2 based on the current average genotype relative risk in autoimmune disorders. If we expected a higher GRR, i.e. 1.5, the power to detect association would increase, see Table 4. The best way to increase power is to increase the sample size. PSS patients included in Paper III were recruited from an area covering almost 18% of the Norwegian population – multicentre studies are thus required to increase sample size further.

These limitations are more or less specific to Paper III. However, several general concerns exist regarding genetic studies. As the DNA sequencing methods improve, it has also become evident that several rare, but important variants are not tagged by the commercial sequencing kits. Although the common variants are common, rare variants are more frequent when added together (144). Further, SNP analysis and GWAS do not account for genetic interactions. There is evidence to indicate that gene-gene (epistasis) or gene-environment interactions contribute to complex diseases (145). In addition, epigenetic influence and the impact of microDNA are not

accounted for in these analyses. Taken together, genetic variation seems to influence most biological pathways and disease processes, but also holds limitations that cannot be overlooked.

Table 4. Power calculations.

		Power interval					
	GRR	**MAF 0.02**	**MAF 0.5**	**Prevalence**	**Cases**	**Controls**	**Controls:cases ratio**
pSS vs controls	1.2	5%	12%	0.05[a]	193	376	1.95
pSS vs controls	1.5	5%	42%	0.05			
pSS+ vs controls	1.2	5%	10%	0.0375[b]	140	376	2.69
pSS+ vs controls	1.5	5%	35%	0.0375			
pSS+ vs pSS-	1.2	5%	7%	0.72[c]	140	53	0.38
pSS+ vs pSS-	1.5	5%	16%	0.72			

[a] Prevalence pSS.
[b] Prevalence pSS with high fatigue (0.05x0.75).
[c] Prevalence high fatigue in this cohort.
MAF, minor allele frequency; GRR, genotype relative risk; PSS, primary Sjøgren`s syndrome; PSS+; primary Sjøgren`s syndrome high fatigue; PSS-, primary Sjøgren`s syndrome low fatigue.

6.3 Summary

We conclude that some parts of fatigue in pSS have a biological origin. IL-1 inhibition seems to reduce fatigue, pointing to a role of IL-1 in fatigue generation and signalling. This is in accordance with experimental animal and human studies. Inflammatory mediators such as IL-1 have an impact on behaviour, signalled through the CNS, and future fatigue-related research should investigate this pathway. It is an important observation that protein oxidation is increased in pSS, and it could have therapeutic implications. The consequences of increased oxidative stress in pSS are not clear, and should be a prioritized area of research. We did not detect an

association between protein oxidation and fatigue, and it is possible that our markers of oxidative stress have been too broad to detect such an association. In future studies we will focus on the oxidative regulation of transcription and gene expression. Increased oxidative stress may activate cellular defence mechanisms through "danger" signalling, ultimately leading to sickness behaviour and fatigue. It is exciting that we detected a trend for association between genetic variation in the *SLC25A40* and *PKN1* genes and fatigue. These genes are involved in cell-signalling and mitochondrial function, and could be involved in inflammatory pathways influencing fatigue. The results will need to be replicated, ideally in a GWAS focusing on fatigue in pSS.

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8. Errata

Page 34, line 3-4 should read: “The main conclusion was that *“….there is great heterogeneity within genetic studies of fatigue in terms of sample sizes, sample descriptions and findings”* (112).”

Page 35, Paper I: “Interleukin-1 inhibition and fatigue in primary Sjögen`s syndrome – a double blind, randomised clinical trial”

Page 43, line 1-2 should read: “A p-value of < 0.05 was considered significant in Paper I and II, and a p-value of ≤ 0.0015 was considered significant in Paper III due to multiple comparisons.”

Page 50, Table 3 should read: “Table 4” and page 54, Table 4 should read: “Table 5”.

Reference 11, page 56 should read “Sjögren H. Zur Kenntnis der Keratoconjunctivitis Sicca (Keratitis Filiformis bei Hypofunktion der Tränendrüsen) Acta Ophtalmol (Copenh) 1933;11(suppl. 2):1-151.”

Page 67, line 23-30, has been removed. The lines were by mistake not deleted from the UiB setup, and read: “amples:) Brock, Gerald W (1994): Telecommunication Policy for the Information Age. From Monopoly to Competition, Cambridge Massachusetts: Harward University Pres. Borton, J. & Clay, E. (1986): "The African Food Crisis of 1982-1986", Disasters, Vol. 10: 258-72. Blix, G., Hofvander, Y., & Vahiquist, B. (eds.) (1971): Famine: A Symposium Dealing with Nutrition and Relief Operations in Times of Disaster. Uppasala: Almquist & Wiksell / Swedish Nutrition Foundation.”

8. Supplements

American-European Consensus Group Classification Criteria for Sjögren's Syndrome (10)

I. Ocular symptoms: A positive response to at least one of the following questions:

1. Have you had daily, persistent, and troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?

II. Oral symptoms: A positive response to at least one of the following questions:

1. Have you had a daily feeling of dry mouth for more than 3 months?
2. Have you had recurrent or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs, that is, objective evidence of ocular involvement, defined as a positive result for at least one of the following two tests:

1. Schirmer's I test, performed without anesthesia (<5 mm in 5 minutes)
2. Rose Bengal score or other ocular dye score (>4 according to van Bijsterveld's scoring system)

IV. Histopathology: Focal lymphocytic sialoadenitis in minor salivary glands (obtained through normal-appearing mucosa) evaluated by an expert histopathologist, with a focus score >1, defined as a number of lymphocytic foci (adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue.

V. Salivary gland involvement: Objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:

1. Unstimulated whole salivary flow (<1.5 ml in 15 minutes)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary, or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration, and/or delayed excretion of tracer

VI. Autoantibodies: Presence in the serum of the following autoantibodies:

1. Antibodies to Ro (SSA) or La (SSB) antigens, or both

For primary SS

In patients without any potentially associated disease, primary SS may be defined as follows:

a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive

b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, or VI)

c. The classification tree procedure represents a valid alternative method for classification, though it should be more properly used in a clinical-epidemiological survey

For secondary SS

In patients with a potentially associated disease (for example, another well-defined connective tissue disease), the presence of items I or II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS.

Exclusion criteria:

1. Past head and neck radiation treatment
2. Hepatitis C infection
3. Acquired immunodeficiency syndrome (AIDS)
4. Pre-existing lymphoma
5. Sarcoidosis
6. Graft versus host disease
7. Use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)