# Interleukin-1β, heat shock protein 90α, and hypocretin-1 in chronic fatigue

by

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# Scientific environment

This project was carried out at the Research Department and in the Clinical Immunology Research Group at Stavanger University Hospital between 2013 and 2019. The research group is led by professor Roald Omdal, who also was the main supervisor.

The work followed the PhD-program in Biological chemistry at the University of Stavanger with professor Peter Ruoff (Centre for Organelle Research (CORE), Faculty of Science and Technology) as co-supervisor.

Laboratory experiments were performed at The Research laboratory and at the Department of Medical Biochemistry at Stavanger University Hospital. Mass-spectrometry studies were performed with help from associate professor Cato Brede (Department of Chemistry, Bioscience and Environmental Engineering, University of Stavanger), who also served as co-supervisor.

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Sandnes, 2019

Kjetil Bårdsen

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# Abbreviations

| | |
|---|---|
| AECG | American-European Consensus Group |
| AIDS | Acquired immunodeficiency syndrome |
| ARE | Antioxidant response element |
| BBB | Blood-brain barrier |
| BDI | Beck depression inventory |
| CFS | Chronic fatigue syndrome |
| CID | Collision induced dissociation |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| DAMP | Danger-associated molecular patterns |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| fVAS | Fatigue visual analogue scale |
| Hcrt1 | Hypocretin-1 |
| Hcrt2 | Hypocretin-2 |
| HO-1 | Hemeoxygenase-1 |
| HSF | Heat shock factor |
| HSP | Heat shock protein |
| IFN | Interferon |
| IL-1α | Interleukin-1 alpha |
| IL-1β | Interleukin-1 beta |
| IL-1Ra | Interleukin-1 receptor antagonist |
| IL-1RAcP | Interleukin-1 receptor accessory protein |
| IL-1RAcPb | Interleukin-1 receptor accessory protein brain |
| IL-1RI | Interleukin-1 receptor type 1 |
| IL-1RII | Interleukin-1 receptor type 2 |
| ICSD | International classification of sleep disorders |
| ISTD | Internal standard |
| Keap1 | Kelch-like enoyl coenzyme A hydratase (ECH)-associated protein 1 |

| | |
|---|---|
| LC | Liquid chromatography |
| LPS | Lipopolysaccharide |
| MS | Mass spectrometry |
| MSLT | Multiple sleep latency test |
| MVB | Multivesicular body |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| NT1 | Narcolepsy type 1 |
| NT2 | Narcolepsy type 2 |
| NVU | Neurovascular unit |
| PAMP | Pathogen-associated molecular patterns |
| PCA | Principal component analysis |
| PRR | Pattern recognition receptor |
| pSS | Primary Sjögren's syndrome |
| RAGE | Receptor for advanced glycation endproducts |
| REM | Rapid eye movement |
| RIA | Radioimmunoassay |
| ROS | Reactive oxygen species |
| SLE | Systemic lupus erythematosus |
| SSA | Sjögren's syndrome related antigen A |
| SSB | Sjögren's syndrome related antigen B |
| TLR | Toll-like receptor |
| TGF | Transforming growth factor |
| TNFα | Tumor necrosis factor alpha |

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I. Bårdsen K., Nilsen M. M., Kvaløy J. T., Norheim K. B., Jonsson G., and Omdal R. Heat shock proteins and chronic fatigue in primary Sjögren's syndrome. *Innate Immunity*. 2016;22(3):162-7.

II. Bårdsen K., Gjerstad M. D., Partinen M., Kvivik I., Tjensvoll A. B., Ruoff P., Omdal R., and Brede C. Considerably lower levels of hypocretin-1in cerebrospinal fluid is revealed by a novel mass spectrometry method compared with standard radioimmunoassay. *Analytical Chemistry.* 2019;91(14):9323-9.

III. Bårdsen K., Brede C., Kvivik I., Kvaløy J. T., Jonsdottir K., Tjensvoll A. B., Ruoff P., and Omdal R. Interleukin-1 related activity and hypocretin-1 in cerebrospinal fluid contribute to fatigue in primary Sjögren's syndrome. *Journal of Neuroinflammation.* 2019;16(1):102. doi: 10.1186/s12974-019-1502-8.

# 1 Abstract

**Background:** Fatigue, defined as an overwhelming sense of tiredness, lack of energy, and feeling of exhaustion, is a phenomenon many people have experienced in connection with infections such as influenza, Epstein-Barr virus, etc. Fatigue is also common in cancer, neurological conditions like multiple sclerosis, Parkinson's disease, and in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, psoriasis, and others.

"Sickness behavior" observed in animals is a conceptual model for understanding fatigue. In this model, infection or tissue damage is followed by behavioral changes like social withdrawal, inactivity, sleepiness, fatigue, and reduced food and water intake. The pro-inflammatory cytokine interleukin (IL)-1β produced during activation of innate immune cells has a prominent role in mediating this behavior. IL-1β crosses the blood-brain barrier and in the brain IL-1β amplifies its own signaling by inducing microglia to produce IL-1β. In cerebral neurons IL-1β signals through a receptor complex including interleukin-1 receptor I (IL-1RI) and an alternative IL-1 receptor accessory protein that does not mediate inflammation but induce neuronal activation and sickness behavior.

The inflammatory response needs to be controlled and is therefore downregulated in a timely manner not to run rampant. In addition, cellular protection mechanisms are activated during inflammation and tissue damage to preserve cellular life from reactive molecules that kill pathogens. Some variants of heat shock proteins (HSPs) released into the extracellular space could represent a defense mechanism of cellular life that also influence fatigue mechanisms.

In addition, sleepiness and weariness are closely related to fatigue and part of the sickness behavior response. Inflammation can alter sleep patterns. The master regulator of sleep- and wakefulness, neuropeptide hypocretin-1 (Hcrt1), could therefore have a role in fatigue generation.

**Objectives:**
I) Investigate if mechanisms that protect cellular life and homeostasis are involved in the generation of fatigue.
II) Develop a non-radioactive, sensitive and selective method for measurement of Hcrt1 in cerebrospinal fluid (CSF).
III) Explore how IL-1β and other selected molecules interact in generation of fatigue, and to investigate a possible link between the neuropeptide Hcrt1 and fatigue.

**Methods:** To explore mechanism of fatigue, a cohort of 71 patients with primary Sjögren's syndrome were investigated. CSF samples where available from 49 patients. A method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was developed for measurements of Hcrt1. Hcrt1 was measured in CSF samples from 22 healthy subjects and 9 patients with narcolepsy type 1.

The clinical variables fatigue, depression and pain were scored using the fatigue Visual Analogue Scale (fVAS), Beck Depression Inventory, and the pain item of the Medical Outcome Survey short form 36, respectively. ELISAs were used to measure HSP32, -60, -72, and -90α in plasma, and in CSF to measure concentrations of IL-1Ra, IL-1RII, IL-6, and the calcium binding protein S100B. Hcrt1 in CSF was measured using a radioimmunoassay (RIA) method, in addition to a non-radioactive method based on liquid chromatography coupled with tandem mass spectrometry. Results were analyzed by non-parametric group comparisons, logistic regression, univariate- and multiple regression, and principal component analysis (PCA).

**Results:** Measures of HSP32, -60, -72, and -90α in plasma revealed that the concentrations of HSP90α were significantly higher in pSS patients with high fatigue versus low fatigue. A tendency toward higher concentrations of HSP72 was observed in patients with high fatigue compared to patients with low fatigue.

The LC-MS/MS method for Hcrt1 in CSF revealed much lower concentrations in healthy subjects than what has previously been published. Patients with narcolepsy type 1, a sleep disorder characterized by low levels of Hcrt1 in CSF, also had lower levels of Hcrt1 in CSF compared to previous published studies. The LC-MS/MS method was compared to the commonly used RIA method. A Bland-Altman plot showed agreement between the two methods.

Analysis of IL-1β related proteins (IL-1Ra, IL-1RII, and S100B), IL-6, and Hcrt1 in CSF demonstrated that IL-1Ra showed significant association with fVAS scores together with the clinical variables BDI scores and pain scores. The relationship of the biochemical variables was explored in PCA, and two significant components appeared: Variables related to IL-1β activity dominated the first component while in the second component there was a negative association between IL-6 and Hcrt1. Fatigue was introduced as an additional variable in a second model. In this PCA, fVAS scores were associated with the first component as was the IL-1β related variables. In addition, the second PCA model revealed a third component that showed a negative relationship between Hcrt1 and fatigue.

**Conclusions:**
I) HSP90α and to a lesser degree HSP72 in blood may possibly be parts of a fatigue inducing mechanism.

II) The LC-MS/MS method with high selectivity and accuracy revealed considerably lower levels of Hcrt1 in CSF than previously reported.

III) IL-1β signaling is a primary driver in fatigue. Several other proteins and molecules interact with IL-1β in a complex network, in which several cell types (neurons, microglia, and astrocytes) probably participate.

IV) Hcrt1 also influences fatigue, but probably through another pathway than the IL-1β route.

# 2 Introduction

## 2.1 Fatigue and sickness behavior

Fatigue is often defined as “an overwhelming sense of tiredness, lack of energy and a feeling of exhaustion“[1]. Most people have experienced fatigue in relation with infectious diseases like influenza, but fatigue is also a common phenomenon in cancer, in chronic inflammatory autoimmune diseases, and in chronic neurological conditions like multiple sclerosis, and Parkinson’s disease. In an early study of the autoimmune disease systemic lupus erythematosus (SLE) 80 % of the patients reported fatigue to be a phenomenon affecting daily living and quality of life [2].

When fatigue occurs without an underlying condition or disease, the term chronic fatigue syndrome (CFS) or myalgic encephalomyelitis (ME) is often used. However, this condition represents only a few percent of cases suffering from chronic fatigue, the vast majority having fatigue as part of an underlying disease [3].

Fatigue has gained increased attention over the last years. New drug trials in various diseases now frequently include measures of fatigue as one of the trial endpoints. Today we are, however, still faced with the fact that there exists no specific and effective treatment for chronic fatigue, and the mechanisms that induce and maintain fatigue are largely unknown [4]. To explore and uncover the pathophysiology of fatigue, the signaling pathways and the possible genetic background, is therefore a great challenge.

A concept for understanding fatigue is the “sickness behavior” model in animals [5]. A number of studies have shown that infection, injection of inflammation inducing agents like lipopolysaccharide (LPS) or tissue damage is accompanied by behavioral changes like social

withdrawal, inactivity, sleepiness, fatigue, and reduced food and water intake. Fatigue is a dominant part of this behavior. The sickness response is deeply based in evolution and shares many similarities with fatigue in humans [6, 7]. The concept of the sickness behavior model involves innate immunity driven activity with pro-inflammatory cytokines acting on the brain resulting in altered behavior. The pro-inflammatory cytokine interleukin-1β (IL-1β) has a central role in this response [8].

## 2.2 The immune system

The immune system has the ability to kill invading pathogens, and to protect the body in conditions with tissue or organ damage. Functionally the immune system is divided into the innate- and the adaptive immune systems.

The innate immune system immediately recognizes, destroys and eliminates foreign and dangerous pathogens and substances that threaten the body. It communicates with the adaptive immune system, which is important for more specific killing of pathogens, but which also is slower acting and relies on production of antibodies and development of activated T-cells. The innate immune system in mammals includes physical-, biological-, and biochemical barriers, innate immune cells such as macrophages, granulocytes, and dendritic cells, antimicrobial peptides, acute phase proteins, the complement system, and cytokines. The system is activated when the innate immune cells encounter pathogens or substances and molecules that are perceived as foreign or represent danger to cellular life. These cells express pattern recognition receptors (PRR) that recognize specific parts of pathogens known as “pathogen-associated molecular patterns” (PAMPs) [9, 10]. In addition, endogenous molecules can under various conditions also activate innate immunity cells, for example when endogenous molecules occur out of their ordinary location or setting, such as during bodily damage. In such “danger-conditions” these

endogenous molecules are called “danger-associated molecular pattern” molecules” (DAMPs) [11]. PAMPs and DAMPs are recognized by a number of different PRRs, such as Toll-like receptors (TLRs) [12, 13], retinoic acid inducible gene-I-like receptors (RLRs) [14, 15], C-type lectin receptors (CLRs) [16, 17], and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors/NLRs) [18, 19]. The different PRRs have during evolution evolved to discern a wide area of characteristic molecular structures present on bacteria, helminths, protozoa, vira, and other substances that represent dangers to life. LPS which is part of the cell membrane of gram-negative bacteria is an example of a PAMP molecule that is recognized by TLR4.

The adaptive immune system evolved phylogenetically later in vertebrates, and is characterized by T- and B-lymphocytes, antibody production and immunological memory. T-cells (that develop in the thymus) express T-cell receptors that recognize specific foreign antigens. T-cells also express glycoprotein co-receptors known as cluster of differentiation (CD). CD is a protocol used for identification of cell surface molecules, and three important subtypes of T-cells can be labelled according to this system: T helper (Th) cells (CD4+), cytotoxic T-cells (CD8+), and regulatory T-cells (Foxp3+ CD25+ CD4+) - Treg cells. Foxp3 is a transcription factor specific to Treg cells [20].

T helper cells assist other cells in the adaptive immune response. Important T helper cells are Th1, Th2, Th9 and Th17. These cells facilitate development of an optimal response against the pathogen in question, intra- or extracellular pathogens, tumors, intestinal worms, or fungi, respectively. Together with cytotoxic T-cells they kill infected cells. T helper cells drive the immune response while Treg cells downregulate and balances the process, so it does not run out of control. There are several types of Treg cells, and in general these cells are important for holding the immune system in check under normal

conditions and to prevent autoimmune reactions and disease to develop. This is demonstrated in humans carrying mutations of Foxp3 that develop *immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome* - a rare, but severe and often fatal condition early in life [21].

B-cells develop in the bone marrow and secondary lymphoid organs such as lymph nodes. They produce specific antibodies against antigens on pathogens or other foreign molecules after the B cells have been exposed to them. As the adaptive immunological response progresses, the process will become stronger and more specific as it is constantly refined as a result of clonal selection of both T-cells and B-cells. During clonal selection and lymphocyte differentiation long lived T- and B-memory cells develop. These cells form the basis of immunological memory that gives a faster and more efficient adaptive immune response upon a second time of infection with the same pathogen.

## 2.3 Cytokines

Cytokines are a family of soluble peptides and proteins that are important in cell signaling. These proteins signal between nearby (paracrine) or distant cells (endocrine), or even the same cells they are released from (autocrine). Important groups of cytokines produced in immune cells include interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factors (TGFs), and interferons (IFNs). Based on their effect on immune function and inflammation it is common to divide them into pro- and anti-inflammatory cytokines. Cytokines act through receptors, and one cytokine can have different effect on cellular functions depending on the local environment and situation. In addition, cytokines act through complex networks where the action of one single cytokine can be difficult to discern from others.

## 2.4 Interleukin-1β and sickness behavior

The cytokine interleukin-1 (IL-1) was fully described in 1984 [22], but already ten years earlier it became evident that IL-1 was more than one protein [23]. Today the IL-1 family consists of 11 proteins including pro-, and anti-inflammatory agonists, and receptor antagonists (Table 1) [24]. IL-1α and IL-1β are regarded as the original members of the IL-1 family. These proteins have different genetic locations, shares only about 25 % similarity in protein sequence [25], but IL-1α and IL-1β have highly similar tertiary structure and bind to the same receptors [26]. Both proteins are produced as precursors that are cleaved to form the final form of the protein. IL-1α is the evolutionary oldest protein in this family and is located inside cells and on cell membranes. Both the IL-1α precursor molecule and the final protein show biological activity. On the other hand, the precursor for IL-1β is not biologically active and has to be cleaved by caspase-1 into the active form of IL-1β. This process takes place in inflammasomes, intracellular multiprotein complexes [27]. IL-1β is highly inducible and is released from innate immune cells as a response to PAMPs or DAMPs and is a master inducer of innate inflammation.

Receptors in the interleukin-1 receptor (IL-1R) family consists of 10 known members and are transmembrane proteins characterized by extracellular immunoglobulin like domains with similarities to antibodies, and with intracellular domains that share similarities with intracellular domains of PRRs. IL-1R1 is the receptor for IL-1α and IL-1β. Binding of IL-1α or IL-1β induces the formation of a trimeric complex consisting of the agonist, IL-1RI, and the receptor accessory protein IL-1RAcP (in newer nomenclature known as IL-1R3). Activation of IL-1RAcP is an essential step in the IL-1 signaling chain. On the other hand, IL-1 activity can be blocked or downregulated by the endogenous antagonist IL-1 receptor antagonist (IL-1Ra) and the soluble decoy receptor IL-1RII.

**Table 1. Members of the IL-1 family and their receptors.**

| Subfamily | Name | Receptor | Co-receptor | Function |
|---|---|---|---|---|
| IL-1 | IL-1α | IL-1R1<br>*IL-1RI* | IL-1R3<br>*IL-1RAcP* | Pro-inflammatory |
| | IL-1α | IL-1R1<br>(IL-1RI) | IL-1R3b<br>(IL-1RAcPb) | Non-inflammatory |
| | IL-1β | IL-1R1<br>(IL-1RI) | IL-1R3<br>(IL-1RAcP) | Pro-inflammatory |
| | IL-1β | IL-1R1<br>(IL-1RI) | IL-1R3b<br>(IL-1RAcPb) | Non-inflammatory |
| | IL-1β | IL-1R2<br>(IL-1RII) | IL-1R3<br>(IL-1RAcP) | Anti-inflammatory |
| | IL-1Ra | IL-1R1<br>(IL-1RI) | NA | Anti-inflammatory |
| | IL-33 | IL-1R4<br>(ST2) | IL-1R3<br>(IL-1RAcP) | Pro-inflammatory |
| IL-18 | IL-18 | IL-1R5<br>(IL-18Rα) | IL-1R7<br>(IL-18Rβ) | Pro-inflammatory |
| | IL-37 | IL-1R5<br>(IL-18Rα) | IL-1R8<br>(TIR8/SIGIRR) | Anti-inflammatory |
| IL-36 | IL-36α | IL-1R6<br>(IL-36R) | IL-1R3<br>(IL-1RAcP) | Pro-inflammatory |
| | IL-36β | IL-1R6<br>(IL-36R) | IL-1R3<br>(IL-1RAcP) | Pro-inflammatory |
| | IL-36γ | IL-1R6<br>(IL-36R) | IL-1R3<br>(IL-1RAcP) | Pro-inflammatory |
| | IL-36Ra | IL-1R6<br>(IL-36R) | NA | Anti-inflammatory |
| | IL-38 | IL-1R6<br>(IL-36R) | IL-1R9<br>(TIGIRR-2) | Anti-inflammatory |

Receptor names follow new nomenclature with old names in parentheses. NA = not applicable. Adapted from Mantovani et al. 2019 [24] and Boraschi et al. 2018 [28].

Early observations of IL-1 acting as a pyrogen to induce fever, identified IL-1 as a molecule able to transmit signals to the brain. A number of animal and human studies have demonstrated that systemic

inflammation triggered by peripheral administration of the LPS and is able to induce sickness responses. Also intraperitoneal or intraventricular (in the brain) administrations of the recombinant form of IL-1β or LPS induce sickness behavior in mice [7]. However, in genetically modified IL-1R1 knock-out mice administration of IL-1β does not induce sickness behavior [7], and a similar effect is also observed in mice that receive IL-1Ra [29] in the periphery or intrathecally.

Given the central role of IL-1β in innate immunity there was early an interest to use IL-1 for treatment of conditions were activation of the innate immune response could be beneficial. For example, some forms of cancer have been treated with recombinant human IL-1. In most cases IL-1 injection resulted in toxicities. However, many of the patients that underwent treatment reported sickness phenomena like chills, fever, pain, fatigue, somnolence, headache, and nausea [30, 31].

In diseases and conditions where fatigue is prominent, such as chronic inflammatory or autoimmune diseases, blocking IL-1β has been reported to have positive effects on fatigue. This has been demonstrated by the use of a recombinant form of IL-1Ra drug known as Anakinra (Kineret, Amgen) [32]. In patients with rheumatoid arthritis (RA) administration of IL-1Ra (Anakinra) resulted in a rapid and strong improvement of fatigue [33]. A double-blind placebo controlled trial in patients with primary Sjögren's syndrome (pSS) demonstrated a 50 % reduction in fatigue in six of the 12 patients that received Anakinra, versus one out of 13 on placebo. Although the primary endpoint in that study was not met, it strongly indicates that IL-1 plays a central role in fatigue [34].

## 2.5 Cellular protection mechanisms

Infectious or inflammatory conditions represent potential dangers to cellular life and homeostasis. Cells have therefore developed effective defense mechanisms against these challenges in order to maintain homeostasis and preserve cellular life. Different pathways involved in response to cellular stress can be activated dependent on the severity and type of stress [35]. Protection against oxygen radicals and other highly reactive and toxic molecules produced during states of infection are dealt with by induction of antioxidant defense systems. Synthesis of heat shock proteins represents mechanisms for protection of cellular life.

Reactive oxygen species (ROS) are synthesized in macrophages during infection to kill pathogens. However, generation of ROS in cells are strongly regulated as they are highly toxic and can damage nucleic acids, carbohydrates, lipids, and proteins [36]. Oxidative stress refers to the situation when ROS dominate over antioxidants and occurs whenever an acute or chronic inflammation is taking place. When ROS are produced, strong biochemical counteractions are generated to protect cellular life. These cellular defense mechanisms are fundamental processes that exist in all plants, bacteria, and animals, and are characterized by antioxidant molecules, - enzymes, and other proteins such as heat shock proteins [37, 38]. Not unexpectedly there are reports on associations between oxidative stress and fatigue [39-42].

### 2.5.1 Nuclear factor erythroid 2-related factor 2

The basic leucine zipper transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) encoded by the *NFE2L2* gene is a master transcription factor that regulate the expression of several hundred genes that promote cellular survival and defense against various stressors like ROS, electrophiles, and toxic products [43-45]. During

normal conditions Nrf2 is bound to the cytoplasmic inhibitor kelch-like ECH-associated protein 1 (Keap1) [46]. When ROS levels increase, Nrf2 dissociates from Keap1 and translocate from the cytoplasm to the nucleus were Nrf2 starts the transcription of genes containing antioxidant response element (ARE) in their promotor. Through downstream genes Nrf2 is part of a conserved and adaptive response to ROS and other stressors. During infections, chronic inflammatory diseases, cancer, neurodegeneration, and other conditions Nrf2 induces protective mechanism to maintain cellular functions [47]. Nrf2 knockout mice thus have a higher degree of inflammation when compared to wild-type mice [48].

### 2.5.2 Heat shock proteins

Heat shock proteins (HSPs) are a group of phylogenetically old proteins that have important functions in conditions with cellular stress. Genes encoding HSPs are regulated by transcription factors known as heat shock factors (HSF) and also by Nrf2 [49, 50]. HSPs are often numbered according to their molecular weight (in kilo Daltons (kDa)) and traditionally divided into five major families: small HSPs, HSP60, HSP70, HSP90, and HSP100. These proteins are products of the heat shock response, a strong cellular protection mechanism discovered in salivary gland cells from *Drosophila busckii* after exposure to heat stress [51, 52]. The heat shock response induces a global pause in translation of new proteins while HSPs and other important proteins for cellular defense are translated at a normal rate [53, 54]. IL-1β is one of the proteins paused by HSP72 and -90 during the heat shock response [55, 56].

HSPs function as chaperones and resolve problems related to misfolded proteins and serve to maintain cellular homeostasis. They are expressed in high amounts in the cytoplasm. HSP90 and HSP70 are each estimated to account for 2-3 % of total proteins in unstressed cells [57,

58]. Why so high concentration exist of these proteins is unclear, but indicates that some HSPs could serve other functions than just as chaperones [59].

Induction of a heat shock response in squid ganglia and axons revealed a glia-axon transfer of HSPs [60]. This observation demonstrated the possibility for HSPs to be released from and taken up by other cells. HSPs do not contain an N-terminus leader sequence required for the canonical protein release pathway and blocking the Golgi apparatus and vesicular transport that are required for the canonical pathway has no effect on HSP release [61]. This indicates that HSPs are released by an alternative pathway. Other proteins like IL-1β and high mobility group box 1 (HMGB1) also lack the leader sequence and are released by non-classical pathways [62, 63].

Secretion of HSPs is an inducible process. For example, during hypoxia the transcription factor hypoxia-inducible factor 1-alpha (HIF-1α) stimulates secretion of HSP90 [64]. It is possible that transport and distant cellular uptake of some HSPs occur via exosomes. Exosomes are small intracellular vesicles derived from endosomes; organelles derived from the plasma membrane. These vesicles carry a cargo of proteins and RNAs. Typical cargo proteins are HSP70 and HSP90 [65]. Exosomes are located within larger multivesicular bodies that can fuse with the cell membrane and release exosomes into the extracellular space [65]. Extracellular HSPs are involved in cell-to-cell signaling. Once released, these proteins bind to receptors on other cells. HSPs have no specific receptors, but can bind to a wide range of receptors including CD40, CD91, TLR2 and TLR4 [66].

HSP90 exists in two isoforms, HSP90α and HSP90β that share 76 % identity [67]. HSP90α is highly inducible and is produced in response to cellular stressors, while HSP90β represents the constitutive and most abundant isoform that under normal conditions maintains and secures

cellular functions [68]. HSP72 represents an inducible isoform of proteins in the HSP70 family. HSP72 is sensitive to a wide range of cellular stressors that trigger the release of this protein [69]. HSP60 together with HSP10 constitute the major mitochondrial HSPs, but are also found in cellular compartments other than mitochondria. Upon stress, HSP60 has been demonstrated in the extracellular space [70]. HSP32 is also known as the inducible form of heme oxygenase 1 (HO-1) [71]. This protein is part of the antioxidant defense system and catalyzes the breakdown of damaged hemoproteins and production of the antioxidant bilirubin [72]. Heat stress induces HSP32 in glial cells where it is observed in perisynaptic glial processes and synaptic junctions [73] which indicates extracellular release of this protein. In cell cultures HSP32 activates cytokine production via binding to TLR4 as also observed for HSP72 and HSP90α [74].

## 2.6 Barriers between the brain and the periphery

The brain has an extensive vascular network that supplies the brain with oxygen and nutrients and remove potentially toxic waste products. There are two barriers between the brain and circulation: the blood-brain barrier (BBB) and the blood-CSF barrier. In the BBB endothelial cells of the CNS constitute a continuum of non-fenestrated cells connected by tight junctions [75]. Tight junctions limit transport over the BBB and contribute to a selective barrier between blood and brain parenchyma. Barrier selectivity relies heavily on properties of endothelial cells with support from pericytes (multi-functional cells within capillary walls surrounding the endothelial cells), basement membrane, and astrocytes [76]. Together with neurons and microglia the components of the BBB form the neurovascular unit (NVU) [77]. The NVU integrates the BBB in a tightly connected dynamic system that regulates local blood flow and transport across the BBB. Integrity of the BBB is important for optimal brain function and homeostasis.

Substances are passively or actively transported across the BBB depending on their chemical size and structure. Small molecules or lipophilic molecules can diffuse freely across the BBB [78]. Glucose is transported by carrier molecules while amino acids, peptides, and proteins are actively transported by binding to transporters on endothelial cells and secreted into the brain by receptor-mediated or adsorptive transcytosis [79]. Activated T-cells are able to cross a healthy BBB and can trigger mechanism that modify the permeability of the barrier after they have entered the brain [80].

There are, however, locations where the BBB is leakier and not restricting the passage of larger molecules and proteins. These sites are the circumventricular organs (CVO) and choroid plexus. Specialized fenestrated endothelial cells that lack tight junction proteins are found at the CVO. These structures are located around the third and fourth ventricles and include organum vasculosum of the lamina terminals (OVLT), the median eminence and adjacent neurohypophysis, the subfornical organ (SFO) and the area postrema (AP) [81]. Fenestra are pores in the cell membrane that allows passage of larger molecules. The CVO is a main site for release of brain-derived peptides to blood and a site for less resistant passage of circulating molecules into the brain. Because neuronal cell bodies and dendrites of sensory parts of the CVO are not protected by the BBB they are in direct interaction with blood-derived molecules from leaky capillaries [82].

Fenestrated endothelial cells are also present in the choroid plexus (CP). The CP is present in all ventricles and consists of branched and highly vascular structures that extend into the ventricular space. Epithelial cells line the surface of the CP and surround the fenestrated endothelial cells and connective tissue. The epithelial cells are connected with tight junctions [83], and constitutes the blood-CSF barrier. Epithelial cells of the CP are polarized with microvilli on the CSF side. This gives a large cell surface facing the CSF that also have

high amounts of various transport proteins. These epithelial cells are therefore important in regulating the constituents of the CSF.

Substances that move into the CSF can modulate activity of hypothalamic neurons through interaction with cells lining the surface between the CSF and brain parenchyma. Ependymal cells are lining the surface of the ventricles and are in continuum with the epithelial cells of the choroid plexus. Tanycytes are specialized ependymal cells that create a barrier between the CSF and hypothalamic structures around the basis of the third ventricle. There are two main types of tanycytes: The α-tanycytes have cell bodies at the lower lateral surface of the ventricles with projections into the arcuate nucleus of the hypothalamus. At the base of the third ventricle are cell bodies of the β-tanycytes with projections ending in proximity with blood vessels of the hypothalamus-hypophysis portal system. Thus, α-tanycytes link CSF to neurons in the hypothalamus, while β-tanycytes link CSF to the blood [82].

### 2.6.1 Cerebrospinal fluid

CSF is mainly produced and secreted from the choroid plexus. The estimated volume of CSF in humans is 150 mL with a distribution of about 25 mL in the ventricular spaces and 125 mL in the subarachnoid spaces. On average the production rate varies between 0.3-0.6 mL/min. This results in a daily CSF production 500-600 mL and a turnover of 4-5 times a day [84]. Active secretion regulates the constituents of the CSF. Compared to blood plasma (Table 2) there are differences between these fluids, but this is not a result of ultrafiltration, but caused by active transport of molecules by epithelial cells.

**Table 2. Comparison of different substances in CSF and blood plasma.**

| | CSF | Plasma |
|---|---|---|
| Total protein (g/L) | 0.2 – 0.5 | 60 – 80 |
| Albumin (g/L) | 0.15 – 0.35 | 35 – 55 |
| IgG (g/L) | < 0.4 | 7 – 16 |
| Sodium ($Na^+$) (mM) | 138 | 138 |
| Potassium ($K^+$) (mM) | 2.8 | 4.5 |
| Calcium ($Ca^{2+}$) (mM) | 1.1 | 2.4 |
| Chloride ($Cl^-$) (mM) | 119 | 102 |
| Bicarbonate ($HCO_3^-$) (mM) | 22 | 24 |
| Glucose (mM) | 3.3 | 5.0 |
| pH | 7.3 | 7.4 |

## 2.7 Interleukin-1β in the central nervous system

From the periphery IL-1β can signal to the brain by activating afferent nerves. The vagal nerve induces sickness behavior after peripheral injections of IL-1β in the abdomen [85]. However, circulating IL-1β can be transported across the BBB via a saturable transport mechanism [86]. This indicates that IL-1β interacts with receptors or transport molecules on endothelial cells. IL-1RI is highly visualized by immunostaining at the endothelial cells and cells of the choroid plexus and neuronal populations in the rodent brain [87].

Neurons in the brain have a specific form of the IL-1RI accessory protein first described by Lu et al. [88]. The protein was later functionally characterized as to mediate non-inflammatory signals [89, 90]. Thus, in neurons IL-1β can modulate neuronal responses without generating inflammation in these cells.

Microglia can be considered as macrophages in the brain and are able to mediate an innate immune response in the CNS. Microglia actively monitor and survey their surrounding environment for pathogens and

inflammatory molecules [91]. Microglia are the primary producers of IL-1β under stress and inflammatory conditions [92]. The cells also act as central amplifiers of peripheral IL-1β signaling as they produce IL-1β in response to IL-1 β activation of themselves, or through other molecules such as HSPs or astrocyte derived S100B that act on TLR4 on microglia.

Astrocytes are an abundant cell type in the CNS and constitute an active and integrated part of synapses [93]. They have important roles in regulation of the BBB permeability as an active part of the NVU [77]. Astrocytes can respond to IL-1β through their close interaction with endothelial cells in the BBB and produce and release the calcium regulating protein S100B in response to IL-1β stimulation [94].

## 2.8 Hypocretins and narcolepsy

Sleepiness is an important part of the sickness behavior response and has considerable similarities with fatigue. The neuropeptide Hcrt1is the master regulator of sleep and wakefulness, and loss of Hcrt1 causes narcolepsy [95, 96], a condition characterized by hypersomnia, disturbed nocturnal sleep and sometimes sudden loss of muscle tone, cataplexy [97]. Narcolepsy type 1 is the term used when cataplexy is part of the disease spectrum and was earlier named narcolepsy with cataplexy. Narcolepsy is a chronic and lifelong sleep disease, limiting the patients' abilities of daily living and necessitating constant drug treatment. It occurs in 0.1% of people, with a delay in diagnosis from symptoms to diagnosis of 5 – 15 years [98]. Strong evidence supports an autoimmune genesis as 98% of subjects with type narcolepsy type 1 carry the HLA-DQB1*06:02 allele [99]. The observation of increased cases of narcolepsy in children following the Pandemrix vaccination for swine flu (H1N1) further supports an immunological genesis [100, 101].

Hcrt1 together with hypocretin-2 (Hcrt2) are produced in the dorsolateral part of the hypothalamus [102, 103], from the same prepro-hypocretin precursor [104]. Hcrt1 and Hcrt2 are located in glutamatergic neurons as co-transmitters in large dense core vesicles like other neuropeptides [102], and act as modulators of neuronal transmission through activation of G-protein coupled receptors [105].

Originally, hypocretins were demonstrated to be involved in regulation of food intake, appetite and energy-homeostasis [106, 107]. Feeding related activity is increased in rats after central administration of hypocretins [103, 108], and intra-cerebroventricular injection of Hrct2 increases food intake in sheep [109]. Both constant daytime sleepiness and reduced feeding and appetite are prominent features of sickness behavior. In search of biological mechanisms for fatigue, it is therefore tempting to hypothesize that Hrct1 could be another important actor that contribute to fatigue.

## 2.9 Primary Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is an autoimmune disease that mainly attacks exocrine glands, but other organs can also be involved. The typical symptoms are dry mouth and dry eyes due to reduced saliva and tear production [110]. The prevalence in Norway is 0.05% [111]. Patients also frequently report joint- and muscular pain and chronic fatigue. Typical laboratory findings are rheumatoid factor and antinuclear antibodies (ANA) directed against the ribonucleosides SSA/Ro and SSB/La A in 70-80% and 30-40%, respectively [112]. Histological examinations of the minor salivary glands (MSG) frequently demonstrate lymphocytic infiltrations. Different criteria for the pSS disease entity have been used, but since 2002 the American-European Consensus Group (AECG) criteria have been widely accepted [113]. The AECG criteria demand fulfilment of 4 of the 6 following items to set the pSS diagnose; Symptoms of xerostomia and

keratoconjunctivitis sicca, objective documentation of salivary or ocular gland involvement, plus obligate detection of either autoantibodies or a positive MSG histopathology test.

In this study pSS was of particular interest to study because there are no effective medical treatments of the disease, and molecular signaling pathways for fatigue could thus be considered to be undisturbed and not affected by immunosuppressant drugs.

# 3 Aims of the Study

I) Investigate if protection mechanisms of cellular life and homeostasis are involved in fatigue.

II) Develop a non-radioactive, sensitive, and selective method with high accuracy for measurement of Hcrt1 in CSF.

III) Explore how IL-1β and other selected molecules interact in fatigue, and to investigate a possible link between the neuropeptide Hcrt1 and fatigue.

# 4 Materials and Methods

## 4.1 Patients and healthy subjects

Papers I and III. pSS patients that had been recruited from a survey at Stavanger University Hospital participated. In total 99 patients from this survey fulfilled the American-European Consensus Group (AECG) criteria for pSS [113], and 72 gave informed consent to participate in the study. Fifty-five of these patients also gave consent to lumbar puncture and investigations of CSF. Patients were examined at Stavanger University Hospital for two consecutive days by a team of specialists in internal medicine, neurology, neuropsychology and radiology following a standardized protocol. All examinations were for research purposes only. A later MRI examination revealed a brain tumor in one patient. This patient was excluded. A total of 71 patients, 54 with CSF samples, were thus included in the present cohort. None of these received biological drug treatment.

Paper II dealt with patients with narcolepsy type 1 and healthy control subjects. Patients had been diagnosed at the Rinnekoti Research Centre in Helsinki (Finland) following the International Classification of Sleep Disorders (ICSD) criteria for diagnosing narcolepsy [114]. According to this classification two criteria must be met for narcolepsy type 1 diagnosis. The first criterion is based on the experience of irresistible sleep attacks. The second criterion is the presence of one or two of the following: i) positive sleep test or ii) low levels of Hcrt1 in CSF. The nine patients included in the study had Hcrt1 < 110 pg/mL in CSF, and six out of the seven available multiple sleep latency tests (MSLT) revealed sleep latency of 5 minutes or less, and at least two sudden-onset rapid eye movement (REM) periods. A mean sleep latency of 13.3 minutes and 1 sudden onset of REM was observed in one patient. HLA-DQB1*06:02 was present in all nine patients.

The Clinical Immunology Research Group has approval from the Norwegian Regional Ethics Committee to use excess CSF from routine examinations for research purposes. We reviewed patient records and identified 22 individuals investigated at the Neurological Department, and in whom examinations and observations over time revealed no signs of inflammatory-, autoimmune-, neurodegenerative-, malignant-, or any other somatic disease. CSF from these subjects was thus regarded as "normal" CSF.

## 4.2 Instruments for assessing fatigue, depression and pain

### 4.2.1 Fatigue

Fatigue Visual Analog Scale (fVAS) was used for fatigue assessment. A VAS instrument consists of a single horizontal line where the ends represent the extreme forms of the variable being measured. The fVAS used here was a 100 mm horizontal line with no numbers or scale along the line. The endpoints were marked with anchoring lines where the left end had the description "No fatigue" and the right end "Fatigue as bad as it can be". The subjects were asked to draw a vertical line at the point corresponding to the level of fatigue experienced the last week. When the distance in mm is measured from the left anchor point to the marked vertical line this gives a numerical score for fatigue. The data from the fatigue scoring are thus in the form of a continuous scale. The fVAS is sensitive to change and has been widely used [115].

### 4.2.2 Depression

Depression/mood was measured with the Beck Depression Inventory (BDI) [116]. This generic instrument consists of 21 items relating to different aspects of depression and gives a score of the depression level experienced for the last week. Maximum BDI score is 63. A score

below 13 is regarded as no depression, a score of 13–19 represents mild depression, and scores >19 indicate moderate-to-severe depression [116].

### *4.2.3 Pain*

The pain item of The Medical Outcome Survey (MOS) short form-36 (SF-36) [117] was used for pain assessment. SF-36 is a generic multi-item scale that measures eight health dimensions of which one is bodily pain. Items in SF-36 are scored using a Likert method and the two questions regarding bodily pain for the past four weeks were reported with low scores for high levels of self-reported pain and high scores for low self-reported pain. In Paper III the bodily pain scores were inverted by subtracting the calculated scores from 100 to fit with other relevant measures (higher scores - more abnormalities).

## *4.3 Biological samples*

### *4.3.1 Blood/plasma*

Paper I.
HSP32, -60, -72, and -90α were measured in plasma from 20 pSS patients with high fatigue and 20 with low fatigue. Blood was drawn by venipuncture and collected in EDTA tubes and immediately cooled on ice before centrifugation at 2,500 g at 4°C for 15 minutes. After centrifugation plasma samples were aliquoted and stored at −80°C until analysis.

### 4.3.2 CSF

Paper II.
CSF samples from nine patents with narcolepsy type 1 and 22 healthy control subjects were investigated. CSF from narcolepsy type 1 patients was obtained in the morning while CSF from healthy control subjects were collected between 9:15 am and 2:15 pm. CSF drawn into polypropylene tubes were immediately cooled on ice, centrifuged at 2500 g for 10 minutes at 4°C to remove cells and debris, and thereafter aliquoted and stored at -80°C.

Paper III.
CSF samples from 54 pSS patents were available for analysis. The number of samples was reduced to 49 because three samples were contaminated with blood and two samples had too small volumes for analysis of all proteins. CSF was collected by lumbar puncture between 01:00 and 02:00 pm. The samples were immediately transferred to cooled glass vials and kept on ice before centrifugation at 3000 g for 10 minutes at 4°C. Samples were stored in aliquots at -80°C until analyzed.

## 4.4 Immunoassays

Three different types of immunoassays were used in this study: Sandwich enzyme-linked immunosorbent assay (ELISA), antibody coated bead technology (Luminex), and radioimmunoassay (RIA). Calibration curves for quantitation of antigen in the samples were produced using dilutions of a standard of known concentration. All assays depended on antigen-antibody interaction but used different detection principles.

### 4.4.1 Sandwich ELISA

In sandwich ELISA, antigens in the samples were allowed to interact with an antibody immobilized on the surface of wells in a 96-well microtiter plate (Figure 1). The antigen was detected by adding excess of a secondary antibody conjugated to the enzyme horseradish peroixdase or alkaline phosphatase. A colorless substrate was converted by the enzyme to a colored compound and the intensity measured by light absorption. The intensity of the color was directly proportional to the antigen concentration.

Plasma concentrations of HSP32, -60, -72, and -90α, and CSF concentrations of IL-1RII and S100B were measured with commercial

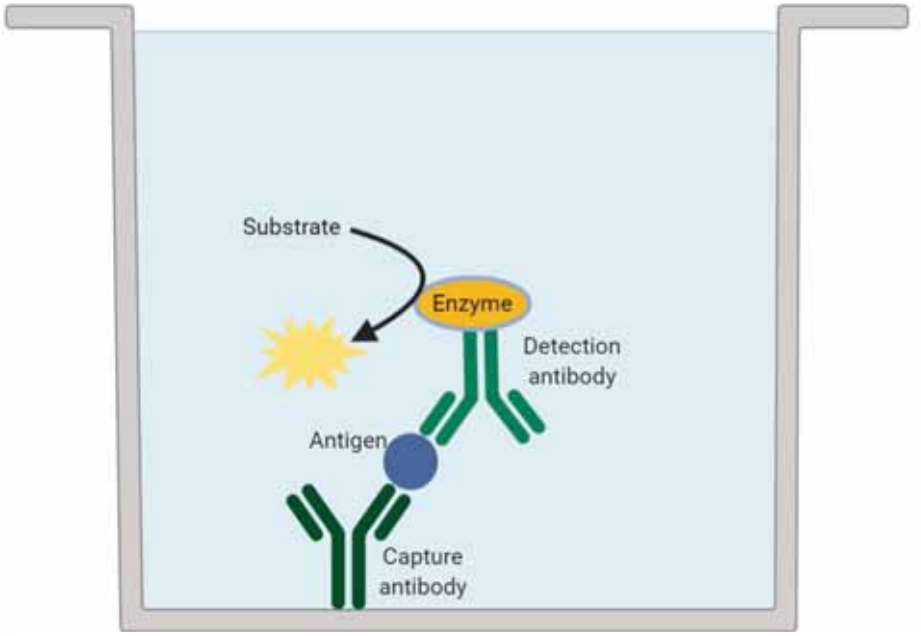


**Figure 1. Sandwich ELISA.**
In a well on a 96-well plate target antigen is "captured" between an immobilized capture antibody and a detection antibody. An enzyme coupled to the detection antibody catalyses a chromogenic reaction when a substrate is added. The intensity of colour change can be measured by passing light through the well and is proportional to the amount of antigen.

ELISA kits (HSPs: Enzo Life Sciences, Farmingdale, NY, USA; IL-1RII: R&D Systems, Minneapolis, MN; S100B: Abnova, Taipei City, Taiwan). All samples were analyzed in duplicate and according to the manufacturer's recommendations. Concentrations were read as absorbance at 450 nm.

### *4.4.2 Antibody coated microparticles*

Assays using antibody coated microspheres or beads were analyzed on a Luminex100 instrument (Luminex Corp., Austin, TX, USA). Microspheres are precoated with antigen-specific antibodies. Adding microspheres coated with different antibodies makes it possible to detect several antigens in a single sample. However, for this study all proteins were analyzed in a single setup. The principle for the assays is comparable to sandwich ELISAs. Antigen bound to the capture antibody is recognized by a detection antibody which is conjugated to a fluorescent reporter molecule (Figure 2). The Luminex 100 instrument is equipped with two lasers, one laser detects the microspheres and thus identifies the type of antigen, while the other laser detects the intensity of the fluorescent reporter. Concentrations of antigen in the samples are proportional to the fluorescent reporter intensity.

IL-1Ra and IL-6 were analyzed using commercial kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocols. All samples were measured in duplicates. For IL-6 nine samples had concentrations below the lowest calibration standard. These samples were given the value of the detection limit (1.0 pg/mL) divided by the square root of 2.

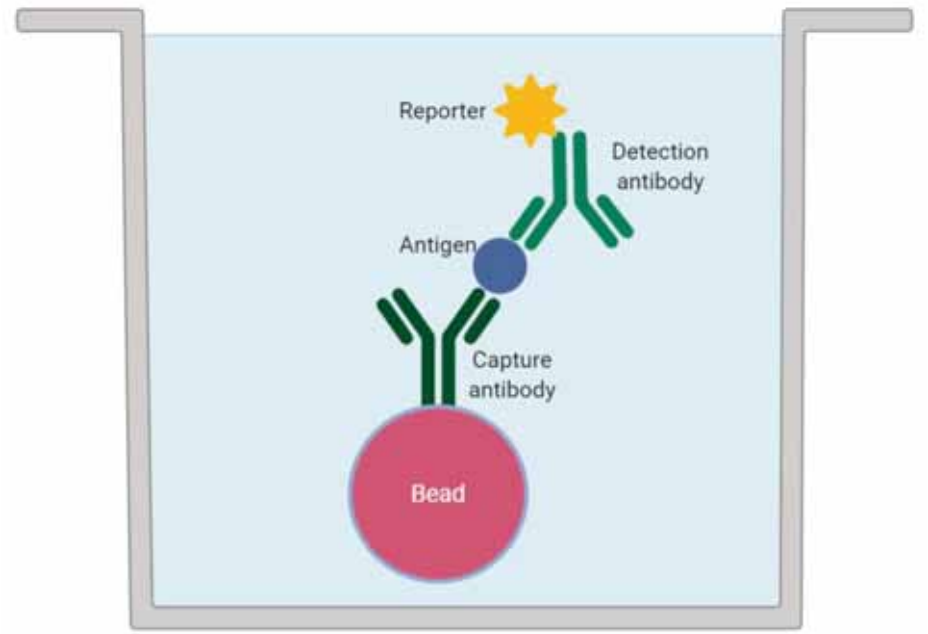


**Figure 2. Luminex principle.**
Microspheres - or beads - are coated with a capture antibody. The antigen is "captured" between the immobilised capture antibody and a detection antibody. A fluorescent reporter molecule is bound to the antigen. The instrument uses two lasers in the detection procedure.

### 4.4.3 Radioimmunoassay

Antibody based detection of Hcrt1 was performed using a competitive RIA (Phoenix Pharmaceuticals, Burlingame, CA, USA). Hcrt1 is a small neuropeptide with few antigenic epitopes Antigen in the samples competed with a fixed amount of recombinant Hcrt1 labeled with the radioactive isotope $^{125}$I in the presence of an Hcrt1 specific antibody (Figure 3). Captured Hcrt1 in the sample were pelleted by centrifugation after adding a secondary anti-immunoglobulin antibody avoiding the need for a second site for antibody binding on Hcrt1. The supernatant was discarded and the amount of radiolabeled antigen in

the pellet was estimated by γ-counting. Sample Hcrt1 concentrations were thus inverse proportional to the amount of labeled antigen and samples were measured in duplicates on a RIASTAR gamma counter (Perkin Elmer, USA).

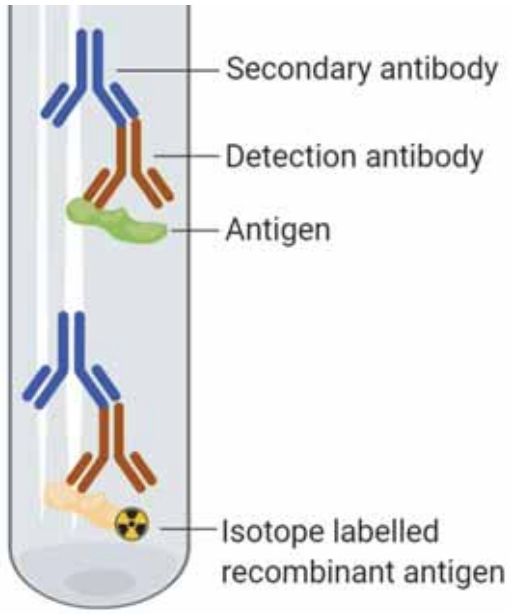


**Figure 3. Radioimmunoassay.**
Detection antibody is added and allowed to interact with target antigen in a sample. After this initial step an isotope labelled antigen is added in a fixed amount and competes with the endogenous antigen for antibody binding. A secondary antibody recognises the detection antibody. This complex is heavy enough to be isolated by centrifugation.

## 4.5 Mass Spectrometry

### 4.5.1 Reagents

Synthetic Hcrt1 (Phoenix Pharmaceuticals and Peptide Institute, Osaka, Japan) and synthetic Hcrt1 labelled with stable $^{13}C$ and $^{15}N$ isotopes (CPC Scientific, Sunnyvale, CA, USA) were used in the development of a quantitative LC-MS/MS method for Hcrt1, and later in preparation of calibration standards and as internal standard (ISTD), respectively. The sequence, mass, and nominal mass-to-charge ratio (m/z) for molecular ions are given in Table 3. Nominal mass uses the mass number of the most abundant isotope.

**Table 3. Properties of synthetic Hcrt1 and isotope labelled Hcrt1 ISTD.**

| | **Hcrt1** | **Hcrt1 ISTD** |
|---|---|---|
| Peptide sequence | Glp-P**L**PDCCRQKTCSCR**L**YELLHGAGNHAAGILTL-$NH_2$ | Glp-P-**L**(U13C6,15N)-PDCCRQKTCSCR-**L**(U13C6,15N)-YELLHGAGNHAAGILTL-$NH_2$ |
| Average mass (Da) | 3561.1102 | 3575.0088 |
| Nominal mass (Da) | 3558.7104 | 3572.7447 |
| Nominal *m/z* (z = 3) for $[M+3H]^{3+}$ | 1187.2446 | 1191.9227 |
| Nominal *m/z* (z = 4) for $[M+4H]^{4+}$ | 890.6854 | 894.1940 |
| Nominal *m/z* (z = 5) for $[M+5H]^{5+}$ | 712.7499 | 715.5568 |

Position of modified leucines (L) are marked with bold typeface. Cysteines that forms disulfide bridges are underlined.

Synthetic Hcrt1 and labeled Hcrt1 ISTD were diluted in 25 % acetonitrile (ACN) and 1 % formic acid (FA). The stock solutions (synthetic Hcrt1 = 4.4 µg/mL and Hcrt1 ISTD = 10.0 µg/mL) where stored at -80°C. A working solution for the Hcrt1 ISTD was made by diluting the stock solution to 2.0 ng/mL in 4 % acetic acid (AcOH).

### 4.5.2 Samples and calibrators

Artificial CSF was used as blank samples and for dilution of calibrators. Newborn calf serum (PN S0125, LN 1316B, Biochrom AG, Berlin, Germany) was used to make artificial CSF and was diluted to a 1 % solution in phosphate buffered saline (PBS) (PN 1890535 / 28372, Thermo Fisher Scientific, Waltham, MA, USA) to make a solution with approximate protein amount as normal CSF. For between run comparisons two quality control samples were used. One low and one high control sample were generated by making solutions of 88 pg/mL and 175 pg/mL into samples of pooled CSF. Quality control samples were aliquoted and stored at -80°C.

### 4.5.3 Sample preparation and LC- MS/MS

The whole process of sample processing to LC-MS/MS result is illustrated in Figure 4. A MiniPrep 75 pipetting robot (Tecan, Männendorf, Switzerland) was used for automating sample preparations. Solid phase extraction (SPE) was used to separate Hcrt1 from contents of CSF. The SPE was run on 96-well plates containing 33 µm Strata-X reversed phase polymer particles (Phenomenex, Værløse, Denmark). Before samples were added, the adsorbent was conditioned with a mixture of 80 % v/v methanol with 10 % v/v acetic acid and rinsing twice with water. After the conditioning step CSF samples and Hcrt1 ISTD was applied. Hcrt1 in the CSF was adsorbed to the polymer particles and rinsed twice with water.

After rinsing and air drying Hcrt1 was eluted into a 96-well polypropylene microplate (Nunc) by adding the conditioning mixture (80 % v/v methanol with 10 % acetic acid). In the last step before LC-MS/MS the samples were up concentrated by letting them spin dry in a vacuum centrifuge (miVac (Genevac Ltd., Ipswich, UK)) at 80ºC for 60 min and dissolved by adding a mixture of 80 % methanol with 10 % acetic acid.

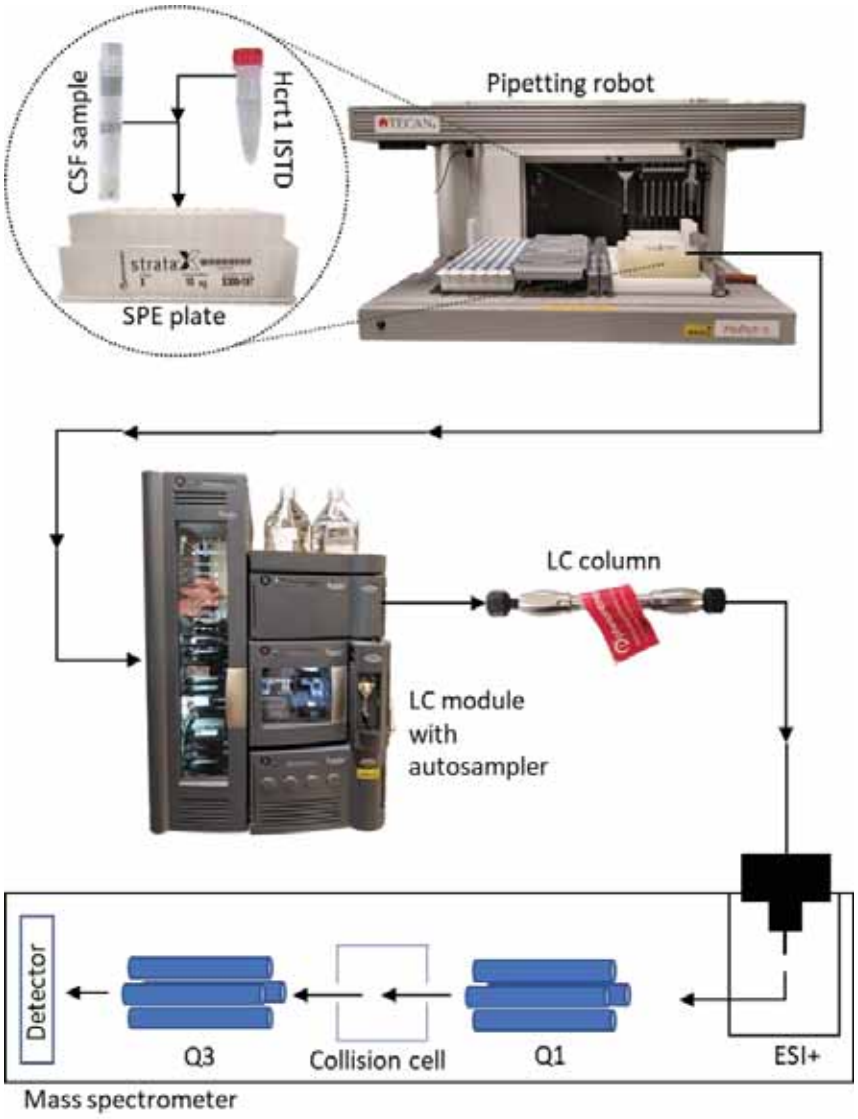


**Figure 4. Sample processing and LC-MS/MS.**
CSF sample with Hcrt1 ISTD are added to an SPE plate for enrichment and cleanup of Hcrt1 from CSF matrix. Sample extract was then eluted onto a 96-well plate, spun dry and reconstituted before being placed in an autosampler connected to a LC instrument. In the LC instrument samples pass through a reverse phase column. After chromatographic separation peptides are ionized by ESI+ in a MS instrument before travelling through the triple quadrupole and detection. In this setup the second quadrupole was a collision cell.

The LC-MS/MS instrument was an Acquity UPLC coupled with a Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). For the LC step a 2.1 mm ID and 50 mm long Kinetex C18 reversed phase column with 2.6 µm superficially porous (100 Å) particles (Phenomenex) was used. Reversed phase liquid chromatography separates compounds by different partitioning between a polar mobile phase and a non-polar stationary phase. Polar molecules have low retention and elute early from the column. Molecules with hydrophobic functional groups are retained on the column and must be eluted by increasing the concentration of solvent in the mobile phase. A sample volume of 10 µL was injected onto the column. The mobile phase, a mixture of 0.2 % formic acid and methanol, was delivered at a flow rate of 600 µL/min using gradient elution with 15 - 50 - 90 % methanol at times of 0 - 0.6 - 1 min respectively. The column temperature was 40ºC and the samples were kept at 10ºC.

A quadrupole mass analyzer consists of four parallel rods where one pair generates a continuously oscillating electric field versus the other pair. The electric field can be controlled for transmission of ions with a given m/z value, by stable oscillations. In a triple quadrupole setup, the first and third quadrupoles scans for precursor ions and fragment ions, respectively. The second quadrupole typically functions as a collision cell, where fragmentation of molecular (precursor) ions occurs by collision induced dissociation (CID). In the Xevo TQ-S instrument the second quadrupole is replaced with a ScanWave collision cell. Positive electrospray (ESI+) produces molecular ions by spraying a solution into an electric field, where large biomolecules such as peptides can have multiple protons attached and thus may be multiply charged. Here, ESI+ with 2.8 kV on the capillary and 50 V on the cone produced an Hcrt1 precursor ion predominantly with four charges. Multiple reaction monitoring (MRM) was used for detection, with the precursor to fragment ion transitions 891.2 > 1138.4 and 891.2 > 1110.9 for

hypocretin-1 and 894.7 > 1143 for the internal standard, all using 25 eV collision energy for CID.

## 4.6 Statistics

In Paper I and III not all variables were normally distributed, and clinical and biochemical variables are therefore presented as medians and ranges. For Paper I variables in the low and high fatigue groups were compared using the Mann-Whitney U-test. Logistic regression was used for analysis of associations between fatigue and HSP concentrations and relevant clinical variables. Variables reported to have $p < 0.2$ in univariable logistic regression were included in a multivariable model. The final model was based on stepwise forward and backward variable selection. The Hosmer-Lemeshow test was used to assess goodness of fit.

In Paper II descriptive statistics of the LC-MS/MS method are presented as means and standard deviations. Results from Hcrt1 measurements by LC-MS/MS and RIA were not normally distributed and were therefore presented as medians and interquartile ranges. Group comparisons between the narcolepsy and control group were done using the Mann-Whitney U-test. Agreement between LC-MS/MS and RIA methods was assessed in a Bland-Altman plot.

In Paper III associations between fatigue and potential clinical and biochemical variables were first analyzed using simple univariable linear regression. Mutual effects of these factors on fatigue were tested in a multivariable linear regression model, using backward and forward selection of variables. Criteria for inclusion of variables in the final model were variables with a significant contribution ($p < 0.05$) in the model with lowest Akaike's information criterion (AIC).

Except for logistic regression analysis in Paper I performed using SPSS version 22 (IBM, Armonk, NY, USA) all statistical modelling and analysis was performed in at the time latest available version of R (R Foundation for statistical computing, Vienna, Austria) [118] with RStudio (RStudio, Boston, MA, USA).

In Paper III principal component analysis (PCA) was applied to explore and visualize the complex interaction of multiple clinical and laboratory variables on fatigue. PCA is sensitive to differences in scale between variables. This means that variables with high numerical values will dominate over variables with lower values and influence the output of the analysis. Data were therefore centered and scaled before analysis. Reduction of the complexity while retaining as much variance in the dataset as possible is an important feature in the PCA. Only components with an eigenvalue > 1 were included. Score distance plots and orthogonal distance plots were used to detect possible outliers. PCA was performed in R with the package FactoMineR [119].

# 5 Summary of the Results

## 5.1 Paper I

HSP90α values were significantly higher in patients with high compared to low fVAS scores: 40.8 (20.0–105.0) vs. 29.9 (4.9–73.3) ng/mL, ($p = 0.02$). For HSP72 there was a tendency for higher concentrations in the group with high fVAS scores vs those with low scores: 1.14 (0.32–12.32) vs. 0.32 (0.32–15.41) ng/mL, $p=0.06$. No significant differences between high and low scores for HSP32 and HSP60 were observed.

As fatigue is known to be associated with depression, the patients were grouped into one group with mild-to-severe depression (BDI scores ≥ 13) and one group with no depression (BDI scores < 13). Plasma concentrations of HSP32, HSP60, HSP72, and HSP90α were not significantly different between these two groups.

Conclusion: Higher HSP90α concentrations in plasma are associated with higher fatigue scores, and there is a tendency to similar findings for HSP72. These extracellular HSPs could therefore be parts of a signaling mechanism for fatigue from the periphery to the CNS in conditions with cellular stress.

## 5.2 Paper II

We developed a non-radioactive method for detection of Hcrt1 using LC-MS/MS. Inspection of raw data from MS scan showed that detection of the intact peptide was possible. On the full scan mass spectrum there was a strong signal for the $[M+4H]^{4+}$ peptide at *m/z* 891.2, and a weaker signal for the $[M+5H]^{5+}$ and $[M+3H]^{3+}$ peptides. The $[M+4H]^{4+}$ precursor ion revealed strong signals for the fragment ions *m/z* 1138.4 and 1110.9. Experiments with a stable isotope standard ($^{13}C_{12}$, $^{15}N_2$) revealed similar results with a $[M4+H]^{4+}$ precursor ion at

*m/z* 894.7 and a strong fragment ion at *m/z* 1143.0. We used sample enrichment with SPE as levels of Hcrt1 in CSF are the pg/mL range. Hcrt1 is a sticky peptide and a strong eluent is needed to elute Hcrt1 from the SPE. Solvents capable of hydrogen binding were more capable than solvents without hydrogen binding. We observed higher solubility of Hcrt1 using methanol than with ethanol or ethylene glycol. A solution of 80 % methanol and 10 % acetic acid was used for elution. Repeatability, measured in 11 aliquots of a pooled CSF sample, was 11 ± 2 pg/mL with a CV% = 16. Recovery, tested at low-middle (59.4 pg/mL) and high (142 pg/mL) levels, was 142 ± 14 % and 90 ± 15 % respectively. Linearity was demonstrated trough the range of calibrators (4.8 – 155.9 pg/mL), and limit of detection and limit of quantification were 2.5 pg/mL and 3.6 pg/mL, respectively. Median CSF levels of Hcrt1 were 11 ± 3 pg/mL measured in 22 healthy subjects, while median levels were 2 ± 3 pg/mL in nine patients with NT1. These levels were significantly lower than levels measured with RIA. Conclusion: Through testing and matching of column properties for LC-MS/MS we were able to selectively detect Hcrt1 from interference on chromatograms and measure considerably lower levels of Hcrt1 in CSF than previously reported. Our findings rise questions regarding what the physiological concentration of free and intact Hcrt1 in CSF is.

## 5.3 Paper III

Clinical variables, (BDI, and SF-36 pain), and selected biochemical molecules of possible importance for fatigue (IL-1Ra, IL-1RII, IL-6, S100B, and Hcrt1) were analyzed by simple univariate regression analysis, and by multiple regression analysis with fVAS as dependent variable. In univariate regression the clinical variables BDI scores and SF-36 pain and the biochemical variable IL-1Ra were associated with fVAS scores. No significant associations were observed for the other biochemical variables. Forward and backward stepwise multiple

regression resulted in a model with scores for depression, pain, and IL-1Ra as significant independent variables explaining fVAS scores. To get a better understanding of the complex molecular interactions and pathways involved in fatigue, we applied principal component analysis (PCA). This was first performed in one model without fVAS scores to obtain an unsupervised analysis of interactions between the biochemical variables, and thereafter in one PCA model with fVAS scores to explore the association of fatigue in the data cloud together with the biochemical variables. In the first PCA model, we identified two components carrying significant information. Together these two components explained 62.77 % of the variation in the dataset. The first component was identified as *the IL-1 dimension* as it was dominated by the IL-1β related variables IL-1Ra, IL-1RII, and S100B, while the second component was identified as the Hcrt1/IL-6 dimension as it was dominated by these two components in a negative association. In the model with fatigue included, the PCA model resulted in three components carrying significant information that explained 71.63 % of the variation in the dataset. Introducing fatigue into the PCA model did not result in rearrangement of the first two components in the model without fatigue. Fatigue showed a moderate association with the first component and a strong negative association with Hcrt1 on the third component (Figure 5). The third component thus indicates that subjects with high fatigue have low Hcrt1 concentrations in CSF.

Conclusion: We found a network of molecules in CSF that were related to IL-1β driven fatigue. In addition, we confirmed that fatigue was influenced by the clinical factors BDI and pain (SF-36). The neuropeptide Hcrt1 seems to be part of a fatigue signaling network that was not directly related to IL-1β activity.

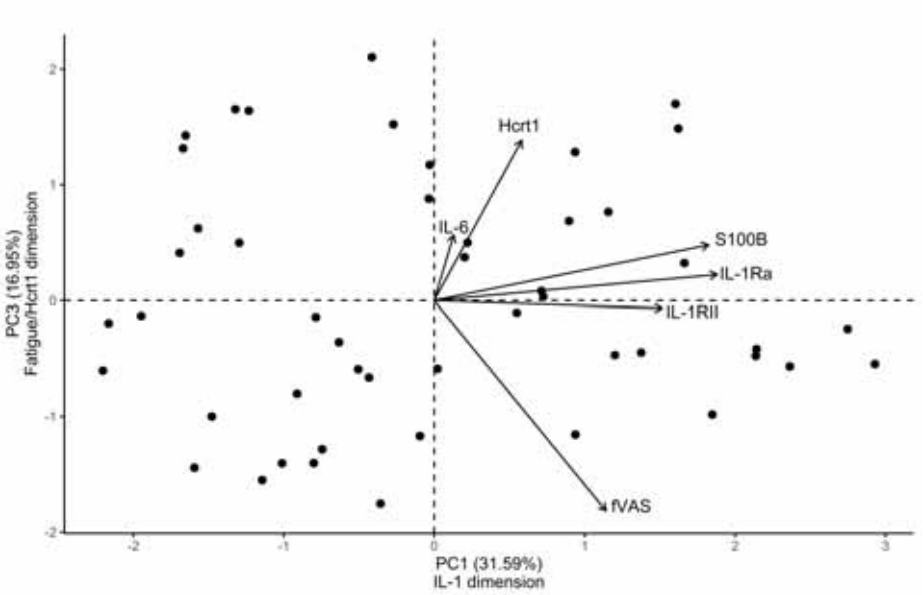


**Figure 5. PCA biplot of PC1 and PC3 in the model with fatigue.**
Dots illustrate scores of the individuals while arrows illustrate variable contribution and correlation to the components. Longer arrows mean higher correlation and arrows close to a component has a higher contribution in the generation of the component.

# 6 Discussion

## 6.1 Methods

Fatigue is a salient phenomenon in several conditions and diseases. Numerous instruments exist, mainly questionnaires, for measurement of fatigue, without any accepted gold standard. Instruments include uni- or multi-dimensional instruments, and disease-specific or generic instruments. Independent of the choice of fatigue instrument it is important that the patient fully understands the questions or statements and how to fill out and respond to the questionnaire. Incorrect interpretation can influence the result by exaggeration or minimization of scores [120]. It is therefore essential that skilled people guide on how to interpret and use instruments. It is important that questionnaires that are meant to be scored at the office under guidance of a qualified supervisor, should not be sent out by post and filled out at home. In our studies fatigue was measured with the generic and uni-dimensional fVAS. This instrument is sensitive to change and relatively easy to administer, and is widely accepted for use [115].

All patient reported outcome measures in this project was scored with guidance of skilled personnel.

Depression was assessed by the BDI [116, 121]. This tool has been used in a wide range of patient groups. Setting the cut-off levels for depression can have influence on the result and is dependent on the patient group. In general, a BDI score > 13 is accepted as a cut of for depression [116].

Self-reported bodily pain was from the quality of life questionnaire SF-36 [117]. Also, this instrument is widely used across a number of diseases and conditions.

Immunoassays, typically in the format of 96-well plates, makes it possible to measure antigens/proteins of choice in several samples in a short time and does not require advanced instrumentation. Specificity for the target protein increases in the sandwich format as two antibodies have to bind to their specific epitopes on the same antigen. Optimal assay conditions require that the sample matrix does not influence antibody binding affinity. Differences in antibody quality between production batches require application of standards and control samples and careful evaluation of assays. This also applies to non-selective antibodies as interference from nonspecific binding to other antigens affect assay properties.

RIAs are sensitive methods and require less antibody than other immunoassays like ELISAs. The risk of steric hindrance is also lower than other assays as radioactive isotopes are smaller than enzymes or fluorescent reporters. A common isotope used in RIA is $^{125}$I. This isotope has a half-life of 59.49 days. For comparison of samples in different assays it is therefore important to have a timeline for experiments in order to have the same level of radioactivity. RIAs also require that laboratories are equipped to safely handle and dispose radioactive material.

LC-MS/MS offers the possibility to measure molecules or peptides in a fast, specific, and sensitive manner. Introduction of isotope labeled peptides is an advantage in quantitative LC-MS/MS as this can be applied as internal standard early in the sample preparation and correct for analyte loss during sample preparation and instrumental analysis. Hcrt1 was analyzed as an intact peptide and thus omitting variability introduced by enzymatic digestion. The labeled internal standard also corrects for ion suppression which is a result of reduced efficiency of ion formation due to substances in the sample matrix and is typically

observed with ESI. Mass spectrometry requires expensive instrumentation and management by experienced personnel.

PCA is a powerful statistical tool for data analysis that aims to reduce the complexity of a given dataset without reducing variation in the data. The method explores the structure of a dataset rather than fitting it to a statistical model and is therefore often regarded more as a hypothesis generating tool than a hypothesis testing tool. Standardization of variables in PCA results in variables with eigenvalues =1 [122], and variables containing significant information should have eigenvalues > 1. In this respect component three in the model with fatigue had an eigenvalue = 1.02. It can however be argued that this is restrictive and in order to not exclude single variable components a cutoff of 0.7 has been proposed by some authors [123]. Of note, in datasets with high precision, variables with eigenvalues < 1 can have significant information or contribution. Finally, when inspection of the data pattern identifies a meaningful process, it is an indication of a valid component [122].

## 6.2 Results

### 6.2.1 Mechanisms that protect cellular life and the role of HSPs.

In Paper I we observed significantly higher concentrations of HSP90α in pSS patients with high fatigue compared to patients with low fatigue. A tendency toward higher levels for HSP72 in patients with high fatigue versus low fatigue was also observed. Depression was one variable that correlated with fatigue in the logistic regression model. However, there were no significant differences between severity of depression and plasma levels of HSP90α. No significant differences were observed for HSP32 and HSP60.

Use of generic and uni-dimensional fatigue instruments seldom report associations between disease activity and fatigue severity. Other factors thus have to be involved to generate fatigue. We hypothesized that downregulating mechanisms of inflammation and mechanisms involved in cellular stress defense could be involved in fatigue. Nrf2 is central in sensing different stressors and activates many cellular protectors including HSPs. In general, extracellular HSPs are anti-inflammatory [124]. In the brain extracellular HSPs are related to neuroprotective signaling. In the context of sickness behavior our observation of an association between extracellular HSP90α and fatigue indicates that some HSPs function on multiple levels and can induce an appropriate behavioral response as well as protect cellular life from stressors.

How HSPs are released into the blood is not known. To be able to generate fatigue HSPs must be able to cross, or to signal across, the BBB. Fenestrated capillaries in the CVO could be a site for entry of into the CNS. When the BBB is compromised, as in ischemia, HSP72 is observed to be able to cross the BBB [125]. Exosomes represent a secretory pathway for HSPs, and it could be that HSPs from the periphery are transported across BBB in exosomes. In addition, it has been observed that cells in the choroid plexus secrete vesicles into the CSF in response to peripheral inflammation [126].

A few studies have observed HSP60 and antibodies against HSP60 in circulation [127] and also found HSP60 epitopes in plasma from patients with myalgic encephalomyelitis [128]. Intracellular HSP60 can stimulate inflammasome mediated production of IL-1β in microglia in response to TLR4 activation [129]. We did not observe any association between fatigue and HSP32 or HSP60, indicating that these HSPs do not participate in sickness behavior signaling. This remains speculative, as higher number of samples in our study could have yielded other results.

Whether HSP90α represent a general fatigue inducing mechanism remains to be seen, but recently we showed that increasing concentrations of HSP90α in plasma were associated with increasing severity of fatigue in patients with Crohn's disease, also [130]. Similar studies in other conditions as well as functional studies are needed before a final conclusion can be made.

### 6.2.2 Hcrt1 in CSF

Sleepiness and lack of appetite are prominent features of sickness behavior, in which fatigue is a major component. Regulation of appetite and sleep are regulated by the neuropeptides Hcrt1 and Hcrt2. Probably related to peptide stability [131], Hcrt1 has been most extensively studied. The sleep disorder narcolepsy is due to lack of Hcrt1 produced by neurons in the hypothalamus [95, 132]. Based on the function of Hcrt1 we hypothesized that Hcrt1 is at some level involved in generation of fatigue. To investigate this further we wished to develop a method for Hcrt1 measurements that only needed small quantities of CSF. CSF is difficult to obtain as sampling is more invasive than blood sampling and therefore fewer patients and volunteers give consent to participate in studies. For research purposes, such a method should need only small amounts of CSF (< 200 μL) and at the same time measure the analyte with high accuracy and preferably be a non-radioactive alternative to the standard RIA.

Development and application of the LC-MS/MS method (Paper II) revealed considerably lower levels of Hrct 1in CSF than previously reported by use of other routine methods or assays [133-135], Of note, we also found lower Hrct1 concentrations than had recently been reported in another study with LC-MS/MS [136]. For healthy control subjects we observed 19 times lower levels of Hcrt1 measured with LC-MS/MS than with RIA. These results were unexpected and made it

difficult to use low sample volumes. To be within a detectable range on the instrument setup we had to increase sample volumes to 600 µL CSF.

For patients with narcolepsy type 1 we observed 22 times lower levels of Hcrt1 with the LC-MS/MS method compared to RIA. A majority of the samples from narcolepsy patients were below the detection limit. This indicate that some patients with narcolepsy type 1 have extremely low concentrations of Hcrt1 in CSF. Our findings question the true levels of Hcrt1 in CSF and further studies on this should be performed.

We applied careful testing of solvents and matching of columns in the LC step to be able to separate the Hcrt1 peak from interference. We demonstrated perfect match in retention time for the internal standard and used corresponding MRM transitions for internal standards and analytes. Together this indicate that we achieved specific and selective detection of Hcrt1 in its intact and free form. We observed no indication of the Hcrt1 ISTD being subjected to degradation or loss in other ways during sample processing and analysis as the internal standard was detected at predicted retention time in LC and at consistent level from run to run. Any degradation or sample loss because of aggregation or dimerization would thus have to occur under storage and before addition of Hcrt1 ISTD. Spiking CSF samples with Hcrt1 followed by storage at -80°C or at room temperature demonstrated good sample stability. In addition, recovery measures also indicated acceptable accuracy for the LC-MS/MS method. Finally, the RIA control was observed within the range given in the kit when this control was measured by LC-MS/MS.

Zhou et al. [137] used LC-MS/MS for detection of Hcrt1. In their study Hcrt1 was measured in microdialysis samples from the arcuate nucleus of the hypothalamus in rat brain at a level of 36 pg/mL. Sampling by microdialysis has also been performed in patients with epilepsy [138],

where a mean Hcrt1 level of 38 pg/mL was found using RIA. In these two studies the cut-off of the permeable membrane was 12 and 20 kDa, respectively. Molecules around and higher than the cut-off values will not pass through the membrane and the microdialysis procedure thus represents a fractionation step. Interestingly samples collected by microdialysis demonstrated similar levels of extracellular Hcrt1 although the samples were from different brain regions and different species and measured by different methods. In healthy controls, fractionation of CSF using reverse phase (C18 columns) showed lower Hcrt1 levels compared to crude CSF [139]. Fractionation of samples using HPLC with offline RIA detection demonstrated low levels of free and intact Hcrt1 in CSF [140], suggesting that less than 10 % of the total immunological signal is from intact Hcrt1 while the majority of the signal is due to unidentified metabolites of the neuropeptide. Compelling evidence therefore suggests that the RIA assay is subject to interference from unknown substances in CSF that to some extent can be removed during sample processing.

Compared to one other study using LC-MS/MS for analysis of Hcrt1 in CSF [136] the concentrations we observed are about 10 times lower. Although both studies measured lower levels than with RIA, procedures in sample treatment or differences in choice of LC column and -settings may explain the discrepancy in results. Further studies are needed with our LC-MS/MS method to conclude.

### 6.2.3 Interleukin-1β related molecules and Hcrt1 in fatigue

In body fluids like CSF, IL-1β occur in very low concentrations [141]. In our experience this makes measures of IL-1β difficult as many samples will have IL-1β in concentrations below the detection limits by routine immunoassays. To overcome this problem and to investigate possible interactions between IL-1β and other relevant molecules in

fatigue generation, we therefore decided to measure the IL-1β related molecules IL-1Ra, IL-1RII, and S100B in CSF from pSS patients. Paper III describes the analysis of these molecules in CSF along with IL-6 and Hcrt1. The results indicate that there exists a complex network for regulation of fatigue that also include the clinical variables depression and pain in addition to IL-1β related molecules and Hcrt1.

Depression and pain are consistently reported to be associated with fatigue. In the context of sickness behavior they may share biological mechanisms. Depression have been reported to be related to IL-1β in animals and humans [142-144] and can be regarded as an "inflammatory driven" behavioral component in sickness such as fatigue. Peripheral inflammation induced by LPS can alter pain threshold in humans [145, 146], and indicate that inflammatory signaling can regulate pain perception. Interestingly it has been observed that HSP90 might be an important co-factor for enhanced pain responses mediated by TLR4 after LPS injections in rats [147]. Taken together this suggest that during disease or bodily damage inflammatory processes can induce a range of biologically conserved behavioral responses that serve to protect the organism.

It can also be difficult to differentiate fatigue from depression as instruments used to assess these variables sometimes use similar phrasing.

Increased production of IL-1Ra and IL-1RII follow a few hours after rise of IL-1β after peripheral LPS treatment, and reflect the magnitude of the IL-1 β amount. IL-1Ra and IL-1RII are found in higher concentrations than IL-1β, are easier to measure, and therefore can be regarded as surrogate markers for IL-1β concentration [148]. The precise type and location of cells in the brain that responds to IL-1β signaling and generate sickness behavior is not known. IL-1β can influence permeability of the BBB through downregulation of tight

junction proteins through mechanism that also involve astrocytes [149]. Recent evidence suggest that peripheral IL-1β that interacts with IL-1RI receptors on vascular endothelial cells are important, and that IL-1β signaling through fenestrated endothelial cells might be central in mediating sickness behavior or fatigue [150]. IL-1β injected into the cerebral ventricles induce inflammatory responses in microglia, astrocytes, and tanycytes [150].

S100B is produced and released from astrocytes in response to IL-1β activation [94] and LPS stimulation [151], indicating that IL-1RI and TLR4 signaling is involved in regulating S100B production and release. S100B released from astrocytes can induce IL-1β production in both microglia and neurons, possibly through different mechanisms [152]. Traditionally propagation and amplification of IL-1β signaling in the CNS has been thought to be mediated by microglia. Our results from PCA indicate that astrocytes may be an alternative cellular actor that mediate IL-1β induced sickness behavior and fatigue through S100B signaling.

IL-6 showed a negative association with Hcrt1 on the second component in PCA. IL-6 is the main pro-inflammatory cytokine, and a meaningful interpretation of the second component, is that increased IL-6 activate production of TNFα. TNFα downregulates Hcrt1 mRNA and protein [153, 154]. Inflammatory processes may therefore act via IL-6 and TNFα to downregulate Hcrt1 and generate sleepiness and loss of appetite. A weakness of our study is that TNFα was not measured. In PCA each component is treated as an independent process hence variables associated with the first component are independent of variables on the second component, and so on. However, as IL-6 can induce IL-1Ra without involving IL-1β [155] we cannot exclude the possibility that IL-6 may influence the levels of IL-1Ra. Further studies on the interaction between IL-1Ra, IL-6 and TNFα are needed to identify inflammatory processes involved in regulation of Hcrt1.

The third component of the PCA model with fatigue indicate a negative association between fatigue and Hcrt1, meaning that subjects with high fatigue have lower concentrations of Hcrt1. Peripheral administration of LPS in mice show that reduced activity of Hcrt1 neurons in the hypothalamus is related to sickness behavior and lethargy [156, 157]. Also, inflammation induced by cytotoxic chemotherapy suppress activity in Hcrt1producing neurons and leads to fatigue [158]. Experimental evidence is therefore in favor of our hypothesis. Further studies are needed to confirm the observations.

The possible interaction between fatigue and Hcrt1 on the third component in the PCA and results from the PCA in total indicate that there are other processes that could explain variation in the dataset and not be linked to fatigue. However, this also indicate that there could be until now unidentified proteins and mechanisms that may contribute to fatigue.

In conclusion our results (summarized in Figure 6) add a piece to the complex and multidimensional puzzle that is fatigue.

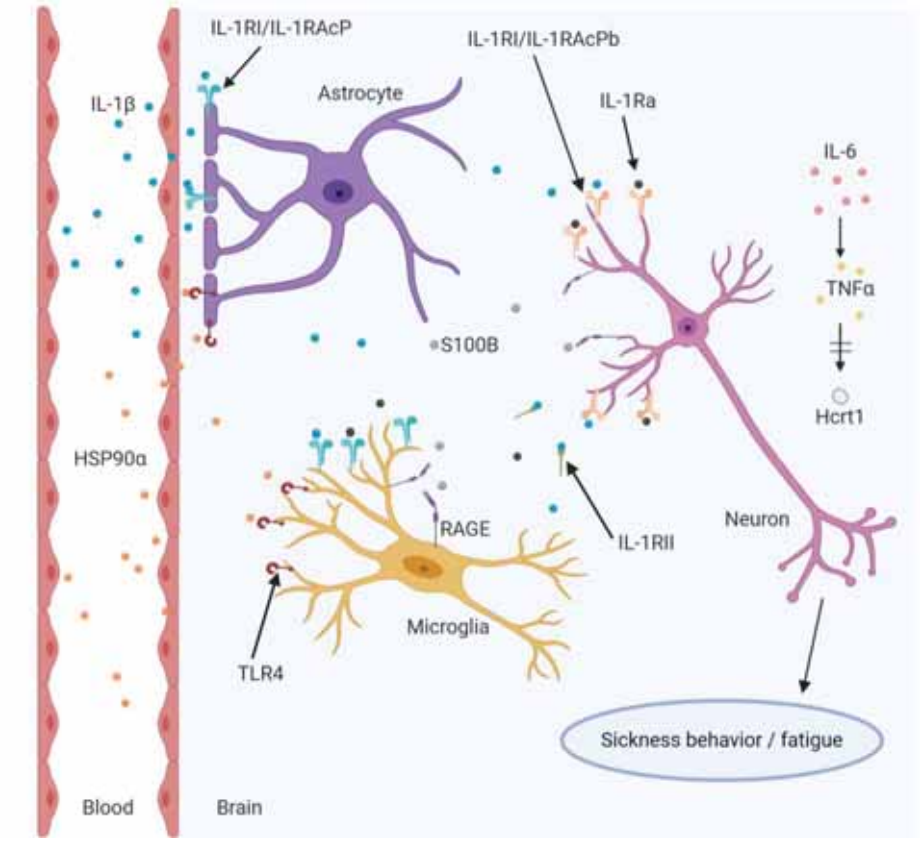


**Figure 6. A hypothetical model of fatigue signaling**
Peripheral produced IL-1β crosses the BBB and binds to the receptor complex IL-1RI/IL-1RAcP on astrocytes and microglia. IL-1β produced in glial cells propagates and amplifies inflammatory signaling in the brain. IL-1β binds to the receptor complex IL-1RI/IL-1RAcPb on neurons and does not induce inflammatory signaling, but modifies neuronal activity and leads to fatigue. The competitive antagonist IL-1Ra and the soluble IL-RII are inhibitors of IL-1β signaling.

HSP90α in blood crosses the BBB and binds to TLR4 receptors on microglia. Activation of TLR4 triggers production IL-1β that signals through the receptor complex IL-1RI/IL-1RAcPb on neurons.

Astrocytes are in close proximity with endothelial cells of the BBB and can respond to IL-1β and HSP90α. Activated astrocytes secrete S100B that can trigger production of IL-1β in microglia.

In addition, Hrct1 contributes to sickness behavior or fatigue in a parallel, but probably different mechanism: Increased concentrations of IL-6 stimulates production of TNFα, and downregulates Hcrt1 production in the hypothalamus.

# 7 Future perspectives

It would be of interest to perform functional studies of HSP90α and fatigue by administration of HSP90α blockers/antagonists in an appropriate model.

Use of the LC-MS/MS method to measure Hrct1 in other relevant patient cohorts will be important for further investigations of the role of Hcrt1 in fatigue.

Studies on possible metabolites or degradation products of Hcrt1 and also of Hcrt2 is critical in assessing the true levels of these neuropeptides in CSF and their role in sleep disorders and fatigue signaling.

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# Paper I

*Original Article*

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# Heat shock proteins and chronic fatigue in primary Sjögren's syndrome

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**Abstract**

Fatigue occurs frequently in patients with cancer, neurological diseases and chronic inflammatory diseases, but the biological mechanisms that lead to and regulate fatigue are largely unknown. When the innate immune system is activated, heat shock proteins (HSPs) are produced to protect cells. Some extracellular HSPs appear to recognize cellular targets in the brain, and we hypothesize that fatigue may be generated by specific HSPs signalling through neuronal or glial cells in the central nervous system. From a cohort of patients with primary Sjögren's syndrome, 20 patients with high and 20 patients with low fatigue were selected. Fatigue was evaluated with a fatigue visual analogue scale. Plasma concentrations of HSP32, HSP60, HSP72 and HSP90α were measured and analysed to determine if there were associations with the level of fatigue. Plasma concentrations of HSP90α were significantly higher in patients with high fatigue compared with those with low fatigue, and there was a tendency to higher concentrations of HSP72 in patients with high fatigue compared with patients with low fatigue. There were no differences in concentrations of HSP32 and HSP60 between the high- and low-fatigue groups. Thus, extracellular HSPs, particularly HSP90α, may signal fatigue in chronic inflammation. This supports the hypothesis that fatigue is generated by cellular defence mechanisms.

**Keywords**

Chronic fatigue, innate immunity, cellular stress, heat shock proteins, autoimmune diseases, Sjögren's syndrome

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## Introduction

Fatigue has been described as 'an overwhelming sense of tiredness, lack of energy and feeling of exhaustion'.[1] It is a frequent feature of chronic inflammatory and immunological diseases, cancer, and neurological disorders. Patients often have fatigue so profound that it severely interferes with activities of daily living and leads to longstanding sick leave and disability, resulting in economic burdens to society.

The mechanisms that lead to and regulate fatigue are debated. Depression and socioeconomic burden are important, but increasing evidence points to genetic and molecular mechanisms that are activated during inflammation and cellular stress conditions, and signalled via neuro-immune and oxidative/nitrosative stress pathways.[2–4]

'Sickness behaviour' is a phenomenon observed in animals during infection or 'danger', and it is highly conserved during evolution.[5] It is characterized by sleepiness; depressive mood; social withdrawal; and loss of grooming, thirst, and appetite. Sickness behaviour constitutes a complex and automated behaviour thought to protect the sick individual from predators. Fatigue in humans has some similarities to sickness behaviour in other animals. Several animal studies have explored the pathways involved in sickness behaviour and demonstrated the fundamental role of IL-1β signalling to the brain.[6] Activation of innate immunity cells such as

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macrophages and granulocytes rapidly leads to increased production of IL-1β. This activates other immune cells to destroy and eliminate the pathogen or the endogenous danger molecules. Simultaneously, IL-1β is actively transported to the brain through several mechanisms; once in the brain, it binds to specific IL-1 receptors.[7] This initiates the subconscious sickness behaviour, which persists throughout the immune response. In humans, studies indicate that IL-1β is important in the generation of fatigue.[8–10] In chronic inflammatory diseases or other conditions characterized by cellular stresses, IL-1β signalling is continuously active; thus, the fatigue phenomenon is persistent and chronic. Treatment with IL-1 blocking agents improves fatigue in humans.[9,10] This supports the hypothesis that IL-1β plays a role in fatigue signalling in humans.

When the innate immune system is activated during infections, pathogens are engulfed and destroyed by highly reactive oxygen and nitrogen species (ROS and RNS, respectively) in phagolysosomes. This is an important part of the body's first line of defence against pathogens. High levels of ROS/RNS can be detrimental, as they can cause DNA damage and cell death. To protect cellular life, cells have therefore developed strong defence systems composed of a large number of active substances and enzymes that counteract the reactive molecules, maintain redox homeostasis and protect vital cellular functions. Oxidative stress is a term used to describe the situation in which reactive molecules prevail over antioxidant and cellular defences, such as in infectious or chronic inflammatory conditions. Several studies have indicated an association between oxidative stress and fatigue.[11–15]

Heat shock proteins (HSPs) are highly conserved proteins that serve important protective functions under conditions of oxidative stress and a wide range of other cellular stresses. HSPs constitute a large family of proteins and are classified according to their molecular mass.[16] Some HSPs are known for their role in protein folding, where they serve as chaperone molecules. Other HSPs are induced upon cellular stress and are released from the cells. These extracellular HSPs take part in cell-to-cell signalling; it is tempting to speculate that extracellular HSPs are part of a signalling system that coordinates several overarching defence mechanisms, including behavioural strategies for survival. Because of the central role of HSPs in cellular defence and the presence of cellular targets for some HSPs in the brain,[17,18] we hypothesize that certain HSPs play a signalling role in fatigue.

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease that is characterized by inflammation of exocrine glands and subsequent dryness phenomena.[19] Fatigue is common in pSS patients, with a reported prevalence of 30–67%, depending on the fatigue instruments used and the patient cohorts investigated.[20,21] It poses a major impact on quality of life, described by patients as an ever-present state, unpredictable, fluctuating, and beyond their own control.[22] As observed in other diseases, the severity of fatigue is influenced by pain, depression and sleep disturbances.[20,21]

As there is no effective drug treatment for pSS, gene activity and molecular interactions are relatively undisturbed by drug treatment compared with many other diseases. Therefore, we chose to investigate the expression of HSPs in a cohort of patients with pSS. To explore the potential role of HSPs in fatigue, we included four different HSPs based on the following criteria: (1) secretion from cells upon stimulation, (2) expression in the brain and the presence of targets on cells in the brain, and (3) previous reports of possible associations with fatigue or fatigue-related mechanisms.

## Patients and methods

From a cohort of 72 pSS patients, all of whom fulfilled the American–European Consensus Group criteria for pSS,[23] we selected the 20 patients with the highest and the 20 patients with the lowest scores on a fatigue visual analogue scale (fVAS). The patients took part in a study in which they were admitted to Stavanger University Hospital for research purposes only. All examinations, testing and blood sampling were performed under strict standardized conditions, with blood sampling at fixed times during the day.

fVAS is a generic fatigue instrument that has been widely used to measure fatigue in patients with pSS and other diseases.[24] It consists of a 100-mm horizontal line with vertical anchoring lines. The description at the left end (0 mm) is 'no fatigue', and the description at the right end (100 mm) is 'fatigue as bad as it can be'. The subjects are asked to draw a vertical line at the point corresponding to their experience of fatigue the last week, and the distance from the left anchor is measured, yielding a numerical score for fatigue.

The Beck Depression Inventory (BDI) was used to assess mood. A BDI score of $<13$ is normally regarded as no depression, a score of 13–19 represents mild depression and a score of $>19$ reflects moderate-to-severe depression.

### *Blood samples*

Routine haematological and biochemical tests were performed at the hospital's routine laboratory. Antinuclear antibodies and antibodies to SSA/Ro and SSB/La were analysed with the QUANTA Lite ENA 6 kit (Inova Diagnostics, San Diego, CA, USA). Positive results were confirmed by QUANTA Lite SS-A and SS-B. The clinical characteristics of the patients are provided in Table 1.

Samples to be analysed for HSPs were collected in EDTA tubes on ice, centrifuged at 2500 *g* at 4°C for 15 min, aliquoted and stored at −80°C until analyses.

**Table 1.** Selected clinical variables for patients with pSS with high and low fVAS scores.

| Variables | High fatigue ($n = 20$) | Low fatigue ($n = 20$) | *P*-Value |
|---|---|---|---|
| Age, yr [range] | 58 [32–79] | 59 [36–87] | 1.00 |
| Duration, yr [range] | 9.1 [0.8–14.8] | 4.1 [1.8–11.0] | 0.04 |
| Female sex (%) | 16 (80) | 18 (90) | 0.82 |
| Anti-SSA/SSB (%) | 18 (90) | 14 (70) | 0.31 |
| CRP [mg/l), median [range] | 3 [0–13] | 0 [0–8] | <0.01 |
| BDI scores, median [range] | 13 [5–38] | 6 [0–18] | <0.01 |
| fVAS scores, median [range] | 88 [76–96] | 20 [3–44] | <0.01 |
| Immunosupressive drugs (%) | 9 (45) | 8 (40) | |
| Corticosteroids (%) | 2 (10) | 0 | |
| Antimalarials (%) | 5 (25) | 3 (15) | |
| Corticosteroids and antimalarials (%) | 3 (15) | 3 (15) | |
| Corticosteroids and azathioprine | 1 | 1 | |
| Cyclophosphamide | 0 | 1 | |

SSA: Sjögren's-syndrome-related antigen A (Ro); SSB: Sjögren's-syndrome-related antigen B (La); CRP: C-reactive protein.

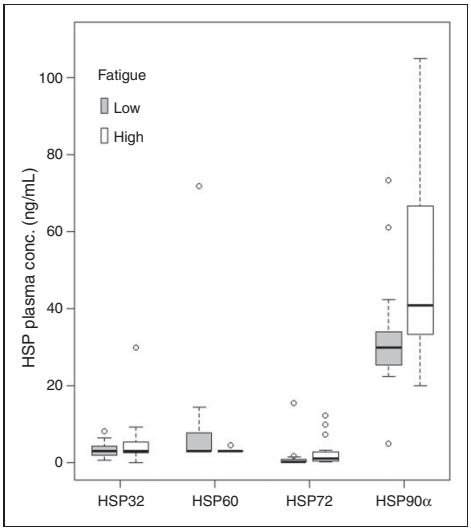


**Figure 1.** HSP plasma concentrations compared with levels of fatigue. HSP90α concentration differed between high- and low-fatigue groups ($P = 0.02$). The difference in HSP72 concentrations between high- and low-fatigue groups was close to significant ($P = 0.06$). There were no associations between fatigue level and plasma concentrations for HSP32 and HSP60. For ease of presentation, one data point for HSP60 (210 ng/ml) in a low-fatigue patient was not included in the Figure.

Plasma concentrations of HSP32, HSP60, HSP72 and HSP90α were measured with commercial ELISA kits (Enzo Life Sciences, Farmingdale, NY, USA). Samples were thawed on ice and diluted 1:3 (HSP32), 1:2 (HSP60), 1:4 (HSP72) and 1:20 (HSP90α) with assay buffer. All samples were assayed in duplicate and analysed according to the manufacturer's recommendations. HSP concentrations were read as absorbance at 450 nm for HSP32, HSP60 and HSP90α, and 495 nm for HSP72 on a Synergy H1 plate reader (BioTek, Bad Friedrichshall, Germany). Coefficients of variation (CV) between duplicates were <15% for HSP32 and HSP90α and <20% for HSP60 and HSP72. Intra-assay variability (Enzo Life Sciences) was <10% for the HSP32, HSP60 and HSP90 kits ($n = 10$). For the HSP72 kit, intra-assay variability was <10% for high concentrations (0.5 ng/ml) and 15% for low concentrations (0.14 ng/ml) ($n = 20$).

### *Statistics*

Owing to the non-normal distribution of the data, results are presented as median and range and Mann–Whitney *U*-test was used for comparisons of continuous variables in the two patient groups. Logistic regression was used to analyse associations between fatigue and HSP concentrations and other relevant patient data. Relevant variables were first tested in a univariable logistic regression model, and only variables with a *P*-value < 0.2 were added to the multivariable logistic regression model. Stepwise backward and forward model selection was used to choose the final multivariable model. Goodness of fit was examined by the Hosmer–Lemeshow test. For all analyses, significance was set to $P < 0.05$. SPSS 22 and RStudio 0.98.1102 (with R 3.1.2) were used for statistical analysis and generation of graphs.

### *Ethics approval and patients consent*

The study was performed according to the Declaration of Helsinki and approved by the Regional Ethics Committee West (2010/1455). All participants signed a legal consent form and were free to refuse any specific part of the examination.

## Results

Plasma concentrations of the four different HSPs in the high- and low-fatigue groups are illustrated in Figure 1. HSP90α plasma concentrations were considerably higher in patients with high vs. low fatigue: 40.8 (20.0–105.0) vs. 29.9 (4.9–73.3) ng/ml ($P = 0.02$). For HSP72 there was a tendency to higher concentration in the high- vs. low-fatigue group, not reaching statistical significance: 1.14 (0.32–12.32) vs. 0.32 (0.32–

**Table 2.** Logistic regression model for association of HSP90α and relevant variables with high and low fatigue.

| Variables | OR | CI (95%) | *P*-Value |
|---|---|---|---|
| HSP90α | 1.12 | 1.02–1.24 | 0.02 |
| BDI | 1.55 | 1.09–2.21 | 0.02 |

Variables not in the final model: age, duration, sex, anti-SSA/SSB, and CRP. OR: odds ratio; CI: confidence interval.

15.41) ng/ml ($P = 0.06$). For HSP32 and HSP60, there were no significant differences between the groups with high and low fatigue: HSP32, 3.02 (0.67–8.25) vs. 3.08 (0.0–7.63) ng/ml ($P = 0.48$); HSP60, 3.0 (3.0–5.0) vs. 3.0 (3.0–210.0) ng/ml ($P = 0.25$). Because of the higher number of standards needed for HSP72 and HSP90α analyses, one sample from each of these two groups ($n = 4$) were excluded owing to the limited number of wells on the plates. Twelve out of the 156 samples (7.7%) analysed had CVs that were too high for duplicates and were thus excluded from analysis (HSP72 = 6; HSP60 = 5; and HSP90α = 1).

To investigate whether relevant variables other than HSP90α influenced the fVAS scores, a multivariable logistic regression model was fitted, with high- and low-fatigue groups as the dependent variable and HSP90α, BDI, age, sex, disease duration, C-reactive protein levels and the presence of anti-SSA/SSB Abs as independent variables. In both backward and forward selection, only HSP90α and BDI remained in the final model (Table 2). The Hosmer–Lemeshow test for goodness of fit demonstrated a good model fit for the final model ($P = 0.71$).

To further investigate the influence of depression, patients were dichotomized into one group with BDI scores <13 and a second group with BDI scores ≥13. Plasma concentrations of HSP32, HSP60, HSP72 and HSP90α were then compared between these groups. There were no differences in HSP concentrations between the two BDI groups (Figure 2).

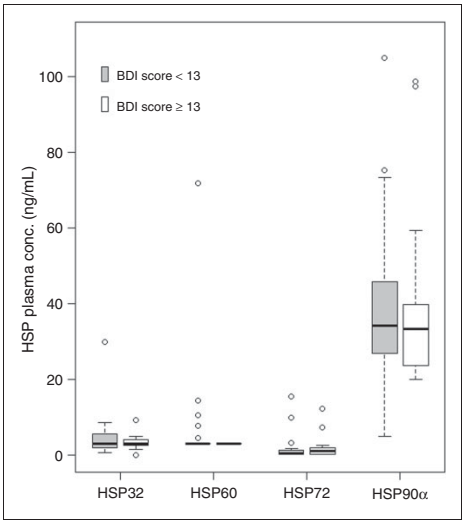


**Figure 2.** HSP plasma concentrations vs. BDI score. Patients were dichotomized into one group with no depression (BDI < 13) and one group with mild-to-severe depression (BDI ≥ 13). Plasma concentrations of HSP32, HSP60, HSP72 and HSP90α were then compared between the two depression groups. No differences in BDI score and plasma levels of the respective HSPs were observed between the two groups. For ease of presentation, one data point for HSP60 (210 ng/ml) in a low-fatigue patient was not included in the figure.

## Discussion

We found that plasma levels of HSP90α were significantly higher in pSS patients with high fatigue compared with those with low fatigue. In addition, there was a close to significant increase in HSP72 levels in patients with high vs. low fatigue. The influence of HSP90α was considerable, as the odds ratio for high vs. low fatigue was 1.12, indicating that an increase of 1 ng/ml in the HSP90α concentration increased the odds of being in the high-fatigue group by 12%. The influence of depression was even stronger than for HSP90α, as a rise of 1 in the BDI score increased the odds of being in the high-fatigue group by 55%. No differences in HSP32 and HSP60 levels were observed between the high- and low-fatigue groups.

The influence of depression on fatigue, as revealed in the multivariate model, was expected. It is well known that affective states can have a strong impact on fatigue.[25,26] However, this association is a complex issue, and it is important to realize that questionnaires used for assessing fatigue and depression often have similar wording. This may lead to circular reasoning and false conclusions regarding their relationship. Moreover, there are indications that depression and fatigue may be signalled through more or less shared molecular pathways via the IL-1 system.[27,28] Of note in this regard is the lack of difference in HSP90α concentrations between the groups with and without depression. This suggests that HSP90α itself has no direct effect on the depressive state.

Our findings point to the possibility of a mechanism in which extracellular HSP90α and, to a lesser degree, HSP72 signal to the brain and induce a state of fatigue. This implies that the HSPs have to cross the blood–brain barrier (BBB). Recombinant HSP72 has been demonstrated to cross the BBB in ischaemic brains.[29] Whether this is true for HSP90α is unknown, but it raises the question of whether HSPs can be transported across the BBB under normal conditions or only under certain conditions, such as inflammation. Specialized areas of the BBB, known as the circumventricular organs, have no

functional BBB because of fenestrated capillaries, and they permit the passage of small molecules across the BBB.[30] The circumventricular organs could represent a route for HSP trafficking and signalling into the brain.

Another possible transport option through the BBB is via exosomes. Exosomes are nanovesicles that contain RNA, microRNA and intracellular-derived proteins. Exosomes participate in intercellular signalling by being actively taken up and then releasing their contents.[31–33] Exosomes constitute a major secretory pathway for HSPs and can cross the BBB.[34–37]

A third possibility is that HSP90α and HSP72 are influenced by a common factor that more directly influences fatigue. If this is the case, levels of HSP90α and HSP72 may simply vary in response to this factor.

Finally, it could be that HSPs act as damage associated molecular proteins (DAMPs) together with redox-derived DAMPs on innate immunity cells by activating their TLRs, thus inducing the fatigue phenomenon.[38]

Specific receptors for HSPs have not been identified in neuronal or glial brain cells. However, TLR4, which is known to be activated by bacterial surface LPS, is a receptor expressed on both neurons and microglial cells. Interestingly, with respect to fatigue, administration of LPS to animals induces sickness behaviour, while simultaneous blocking of the TLR4 signalling pathway by interfering peptides prevents the behaviour.[39] In addition to LPS, HSP90α and HSP72 are among the endogenous molecules that activate TLR4.[18,40]

In cell cultures, HSP32, HSP72 and HSP90α activate microglial cells via TLR4 and lead to cytokine production by activation of p38 MAPK and NF-κB.[41] In the context of fatigue generation, it is therefore possible that HSPs in humans interact with TLR4 on microglia and produce IL-1, which is a known inducer of sickness behaviour.[6] Another possibility is a direct influence on cerebral neurons through HSP interactions with TLR4 on the surface of the neurons. The presence of TLR4 on cerebral neurons, with co-localization of HSP70, has previously been demonstrated *in vivo*.[42]

We did not find any association between fatigue and levels of HSP60 or HSP32. A recent study found antibodies against epitopes of bacterial and human HSP60 in patients with myalgic encephalomyelitis;[43] however, to our knowledge, there are no reports regarding blood levels of HSP60 protein and fatigue.

This study has some limitations. It could be argued that we should have used other fatigue measuring instruments, such as multidimensional or disease-specific instruments. However, more than 200 different fatigue instruments exist today, and it is difficult to argue the superiority of one over the other. Because it is well known that VAS show good responsiveness to change over time, and owing to of our long-term experience with the fVAS, we regarded this as the most optimal choice for this study.

Also, it has previously been documented in pSS that a number of other factors influence fatigue, such as muscular and joint pain, mental depression and poor sleep.[20,21] The severity of fatigue in the individual patient is therefore a complex phenomenon, modulated by several cofactors, and cannot be attributed to one single player.

In the HSP60 and HSP72 assays, some samples had signals close to and under the lower limit of detection, resulting in high variation between duplicate measures. Five HSP60 samples and six HSP72 samples were consequently removed from analyses. Serum might have been a better sample matrix for HSP60 and HSP72, but we choose to use plasma because we wanted to employ rapid centrifugation and aliquoting of samples at low temperature to avoid degradation of analytes.

Importantly, to be valid, our findings need to be replicated in other and even larger cohorts of pSS patients, and also in cohorts of patients with other diseases.

The strengths of our study are the relatively large groups of well-characterized patients, who were matched in age and nearly matched in sex and were not undergoing drug treatment that could potentially interfere with analysis.

In conclusion, extracellular HSP90α and, to a lesser degree, HSP72 may represent a mechanism by which fatigue is signalled to the brain under conditions characterized by cellular stress, such as chronic inflammatory diseases. These counteractive and down-regulatory processes of inflammation may explain why the severity of fatigue is seldom reported to be a function of disease activity or inflammatory markers.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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# Paper II

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# Paper III

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Journal of Neuroinflammation

RESEARCH Open Access

# Interleukin-1-related activity and hypocretin-1 in cerebrospinal fluid contribute to fatigue in primary Sjögren's syndrome

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## Abstract

**Background:** Fatigue is a common and sometimes debilitating phenomenon in primary Sjögren's syndrome (pSS) and other chronic inflammatory diseases. We aimed to investigate how IL-1 β-related molecules and the neuropeptide hypocretin-1 (Hcrt1), a regulator of wakefulness, influence fatigue.

**Methods:** Hcrt1 was measured by radioimmunoassay (RIA) in cerebrospinal fluid (CSF) from 49 patients with pSS. Interleukin-1 receptor antagonist (IL-1Ra), IL-1 receptor type 2 (IL-1RII), IL-6, and S100B protein were measured by enzyme-linked immunosorbent assay (ELISA). Fatigue was rated by the fatigue visual analog scale (fVAS).

**Results:** Simple univariate regression and multiple regression analyses with fatigue as a dependent variable revealed that depression, pain, and the biochemical variable IL-1Ra had a significant association with fatigue. In PCA, two significant components were revealed. The first component (PC1) was dominated by variables related to IL-1β activity (IL-1Ra, IL-1RII, and S100B). PC2 showed a negative association between IL-6 and Hcrt1. fVAS was then introduced as an additional variable. This new model demonstrated that fatigue had a higher association with the IL-1β-related PC1 than to PC2. Additionally, a third component (PC3) became significant between low Hcrt1 concentrations and fVAS scores.

**Conclusions:** The main findings of this study indicate a functional network in which several IL-1β-related molecules in CSF influence fatigue in addition to the classical clinical factors of depression and pain. The neuropeptide Hcrt1 seems to participate in fatigue generation, but likely not through the IL-1 pathway.

**Keywords:** Innate immunity, Cytokines, Sjögren's syndrome, Fatigue, Hypocretin

## Background

Fatigue can be defined as "an overwhelming sense of tiredness, lack of energy, and feeling of exhaustion" [1] and is a common phenomenon in infections, chronic inflammatory diseases, cancer, and neurodegeneration. Fatigue has a substantial impact on patients' lives, is sometimes debilitating, and is a major reason for using sick leave. It remains unclear whether there are different dimensions of fatigue, such as peripheral (muscle) and central (mental) fatigue, or whether fatigue is a unidimensional phenomenon that influences different aspects of human life.

While the underlying mechanisms are not completely understood, several studies have shown that pain and depression are factors that exert a heavy and consistent influence on the severity of fatigue [2, 3]. Most researchers regard fatigue as a biological and cerebral phenomenon, and increasing evidence points to a genetic and molecular basis for the generation and regulation of fatigue [4, 5].

A conceptual model for understanding fatigue is the *sickness behavior* phenomenon in animals [6, 7]. "Sickness

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behavior" is observed during infection and inflammation and is characterized by sleepiness, depressive mood, social withdrawal, and loss of grooming, thirst, appetite, and initiative [8]. Sickness behavior can be considered as a survival-enhancing strategy and is highly conserved during evolution [9]. Fatigue constitutes a substantial part of this behavior.

Several animal studies have explored the pathways involved in sickness behavior and demonstrated the fundamental role of interleukin (IL)-1β signaling to the brain for this complex and automated response [6]. In conditions with infection and/or tissue injury, activation of innate immunity cells such as macrophages will rapidly lead to increased production of IL-1β. This activates other immune cells to destroy and eliminate the pathogen or the endogenous danger molecules. In the periphery, IL-1β signals through binding the IL-1 receptor type 1 (IL-1RI) and the IL-1R accessory protein (IL-1RAcP) [7]. Activation of this signaling complex gives rise to the canonical downstream IL-1 responses (NF-κB and MAPKs) with increased inflammation and immune cell activation. Downregulation of IL-1 induced receptor activation is conducted by the IL-1 receptor type II (IL-1RII), which functions as a decoy receptor and does not signal, and by the natural IL-1 receptor antagonist (IL-1Ra), which binds to the IL-1RI and prevents activation [10]. Without these limiting steps, IL-1-driven inflammation can run rampant.

IL-1β passes through the blood-brain barrier (BBB) and reaches neuronal cells in the brain by both passive and active transport systems and can even be produced intrathecally [11]. Once in the brain, IL-1β binds to a subtype of the IL-1 receptor and to a brain isoform of the accessory protein, the IL-1RaAcPb [12]. Thus, while IL-1β in the periphery is a strong inducer of innate immunity-based inflammation, IL-1β directly modulates synaptic transmission through neuronal potassium and calcium influx (without inflammation) in the brain [12, 13] and induces sickness behavior. IL-1Ra is a robust biomarker in CSF and is in equilibrium with IL-1β in chronic conditions. High levels of IL-1Ra in CSF therefore are thought to reflect high IL-1β levels.

In humans, increased activation of IL-1 in the brain is observed in chronic inflammatory and autoimmune conditions [14, 15], and treatment with IL-1 blocking agents alleviates fatigue [16–18]. In primary Sjögren's syndrome (pSS), a chronic autoimmune disease clinically characterized by inflammation of the exocrine glands, fatigue is a dominant feature [19]. No consistently effective treatments are currently available, and pSS can be considered as an ideal disease to investigate fatigue mechanisms as gene expression and molecular pathways at large are undisturbed by immunosuppressive or cytotoxic drug treatment. To further explore models for fatigue regulation, we therefore performed measurements of selected molecules in cerebrospinal fluid (CSF) that could influence Il-1β activity (IL-1Ra, IL-1RII, IL-6, and S100B).

Animal studies show that lipopolysaccharide (LPS)-induced sickness behavior also is accompanied by reduced *c-fos* activity of lateral hypothalamic neurons that produce hypocretin-1 (Hcrt1) and also a reduction of Hrct1 levels in CSF [6, 20]. Since Hcrt1 is known as the main regulator of sleep and wakefulness, we also hypothesize that this neuropeptide could be another molecular regulator of fatigue.

## Methods

This hypothesis-generating study was designed to explore associations between the CSF concentrations of selected molecules and fatigue in a population of patients with pSS at a single institution.

All patients with systemic autoimmune diseases in the Southern part of Rogaland County are allocated to Stavanger University Hospital. We reviewed medical records and identified 99 patients that fulfilled the American-European Consensus Group (AECG) criteria for pSS [21]. None of the patients were on biological drug treatment. Exclusion criteria were past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency syndrome (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, and use of anticholinergic drugs [21]. Seventy-two patients consented to participate in the study and subjected to a 2-day stay in the hospital for research purposes only. The patients in the near population-based cohort were on no biological drug treatment. Fifty-five of the 72 patients (76%) consented to lumbar puncture. One of the 55 patients was later excluded due to a brain tumor revealed by MRI, three because of blood contamination of the CSF, and another two because of inadequate sample volumes. Thus, CSF samples from 49 out of the 72 pSS patients (68%) were available for study, 41 women (84%) and 8 men (16%).

Blood was drawn in the morning between 08.00 and 09.00 a.m, and clinical examinations and patient-reported outcome measures completed afterwards. Lumbar puncture was performed between 01:00 and 02:00 pm; samples were collected in cooled glass tubes and immediately placed on ice until centrifugation at 3000 *g* for 10 min at 4 °C. The supernatants were distributed in 200 μL aliquots and stored at − 70 °C until analysis. CSF samples from three patients were excluded because of blood contamination, and another two because of inadequate sample volumes. Thus, CSF samples from 50 pSS patients were available for study. In addition to clinical variables given in Table 1, four patients (8 %) had a BMI ≥ 30, 18 patients (36 %) were on antimalarial drugs, and the median duration of education was 12.3 years (range 7–20 years).

**Table 1** Selected clinical variables in the 49 pSS patients

| Variables | |
|---|---|
| Females (%)/males (%) | 41 (84)/8 (16) |
| Age, years, median [range] | 56.1 [34.2–78.2] |
| Duration, years, median [range] | 5.0 [0.4–16.0] |
| fVAS scores, median [range] | 64 [3.0–93.0] |
| BDI scores, median [range] | 9.0 [0.0–38.0] |
| Pain scores*, median [range] | 49.0 [0.0–90.0] |
| Presence of anti-SSA/SSB antibodies (%) | 38 (78) |
| CRP (mg/L) [range] | 1.5 [0.0–9.0] |

*Abbreviations*: *fVAS* fatigue visual analog scale, *BDI* Beck Depression Inventory, *SSA/SSB* Sjögren's syndrome-related antigen A (Ro) and B (La), *CRP* C-reactive protein
*SF-36 pain scores are reported according to the transformed scale with high scores indicating high bodily pain and low scores indicating lower bodily pain

Fatigue was assessed by the fatigue visual analog scale (fVAS), which is a generic and unidimensional fatigue instrument that has been widely used to measure fatigue in patients with various conditions [22]. It consists of a 10- mm horizontal line with vertical anchoring lines. The description at the left end (0 mm) is "no fatigue," and the description at the right end (100 mm) is "fatigue as bad as it can be." The subjects are asked to draw a vertical line at the point corresponding to their experience of fatigue the last week, and the distance from the left anchor is measured, yielding a numerical score for fatigue (Additional file 1: Figure S1).

Mood was assessed by the Beck Depression Inventory (BDI) [23]. A BDI score of < 13 is normally regarded as no depression; a score of 13–19 represents mild depression and a score of >19 reflects moderate-to-severe depression.

Pain was assessed by the pain subscale of the Medical Outcome Survey (MOS) short form-36 (SF-36) questionnaire and transformed as recommended [24].

For regression analysis and principal component analysis (PCA), the transformed pain scale was inverted by subtracting the transformed score from 100. This was performed to orient the pain scale in the same direction as scales for other variables included in the analyses.

IL-1Ra and IL-6 were analyzed on a Luminex$^{100}$ instrument (Luminex Corp., Austin, TX). IL-1Ra was measured using a Fluorokine MAP human IL-1Ra kit (R&D Systems, Minneapolis, MN) and a Fluorokine MAP human base kit (R&D Systems) according to manufacturer's protocols. Intra-assay CV% was 2.8–4.4, and inter-assay CV% was 6.6–10.9. IL-6 was measured using a human IL-6 ultrasensitive AB bead kit with the human extracellular buffer kit (Biosource, Invitrogen Corp., Carlsbad, CA). Intra-assay CV% was 7.59 and inter-assay CV% was 9.9. For both IL-1Ra and IL-6, acquired data were studied using the StarStation software v2.3 (Applied Cytometry, Sheffield, UK). In the IL-6 assay, nine samples had values below the standard curve and were given the value of the detection limit (1.0 pg/mL) divided by the square root of 2.

Because IL-1β is difficult to measure in CSF due to low concentrations, these results were not included.

S100B and IL-1RII concentrations were measured by sandwich ELISA kits according to the manufacturer's protocol (S100B: Abnova, Taipei City, Taiwan; IL-1RII: R&D Systems, Minneapolis, MN). ELISA plates were read on a Multiskan Ascent microplate reader (Thermo Scientific, Waltham, MA). For S100B ELISA kits, the intra-assay and inter-assay CV% were 1.9–2.1 and 4.7–7.1, respectively. For IL-1RII, the intra-assay CV% was 2.0–3.4 and the inter-assay CV% was 3.9–5.9.

The concentration of Hcrt1 in CSF was measured by $I^{125}$ radioimmunoassay (RIA) (Phoenix Pharmaceuticals, Burlingame, CA, USA) per manufacturer's protocol. Samples were measured in duplicate and assay tubes were counted on a RIASTAR gamma counter (Perkin Elmer, USA). Briefly, 100 μL of standard dilution, assay controls, and CSF samples were added to assay tubes together with 100 μL primary antibody and incubated for 20 h at 4 °C before adding 100 μL of $^{125}$I-peptide (tracer solution) and a new incubation for 20 h at 4 °C. After the second incubation, 100 μL of goat anti-rabbit serum and 100 μL normal goat serum was added. Following incubation for 90 min at room temperature, the assay tubes were centrifuged at 300 rpm for 20 min at 4 °C and incubated for 90 min at room temperature. The supernatant was aspirated before counts per million was counted from the remaining pellet. The average CSF Hcrt1 concentration was 239.3 ± 26.8 pg/mL. Intra-assay variation was 9.9% based on a sample assayed as ten individual samples.

### Statistics

Some clinical variables were not normally distributed, and all continuous data are therefore reported as medians and ranges. Categorical data are reported as counts and percentages. Simple univariate linear regression was first used to examine associations between fatigue and each of the potential influential factors, BDI, pain scores, IL-1Ra, IL-1RII, IL-6, S100B, and Hcrt1. Thereafter, a multiple regression analysis with forward and backward selection was performed to investigate the mutual effect of these factors on fatigue. Variables selection in the final model was based on variables with a significant contribution ($p < 0.05$) and the lowest Akaike's information criterion (AIC) value. AIC evaluates the multiple regression model by favorizing higher explained variance and penalizing the number of variables in the model. Thus, the model with the lowest AIC was selected.

To further explore and visualize the complex interaction of multiple clinical and laboratory variables on fatigue, we applied principal component analysis (PCA).

Centering and standardizing of the data were performed before analysis to avoid effects due to differences in units of the variables. The components retained in PCA were those with eigenvalues > 1. Score distance plots and orthogonal distance plots were used to detect possible outliers in PCA. Samples outside a critical boundary, the 97.5 % quantile in these plots, were declared as outliers.

All analyses were performed in R version 3.3.3 using RStudio version 1.0.144. PCA was performed using the R package FactoMinerR.

## Results

Patient characteristics are summarized in Table 1. There was no significant difference in routine hematological or biochemical variables between patients with high or low fatigue. Diagnostic evaluation of the PCA revealed that one sample was classified as an orthogonal outlier. This sample was removed and PCA was thus performed on 48 samples.

The clinical variables of depression and pain and the biochemical variable IL-1Ra were significantly associated with fatigue in simple univariate linear regression, while no significant associations were observed for Il-1RII, IL-6, S100B, or Hcrt1 (Table 2). In multiple linear regression analysis with fVAS as the dependent variable, both forward and backward stepwise selection resulted in a model with depression, pain, and IL-1Ra as significant independent variables ($R^2 = 0.37$; $p < 0.001$, Table 3). To obtain an unsupervised impression of the complex molecular interactions, we first performed PCA on a model containing only the biochemical variables. Data from the biochemical variables were centered and scaled before analysis and PCA was performed on the correlation matrix. Two components demonstrated eigenvalues > 1 in a Scree plot and were retained (Additional file 2: Figure S2a). These two components explained 62.77 % of the variation in the dataset.

**Table 2** Associations (simple regression analysis) between fatigue (fVAS scores) and selected variables

| Independent variables | Estimate | Std. error | $R^2$ | *P* |
|---|---|---|---|---|
| Depression (BDI) scores | 1.42 | 0.42 | 0.20 | 0.001 |
| Pain (SF-36) scores* | 0.48 | 0.13 | 0.23 | < 0.001 |
| IL-1Ra | 0.52 | 0.24 | 0.09 | 0.04 |
| IL-1RII | 0.13 | 0.16 | 0.01 | 0.41 |
| IL-6 | − 3.47 | 2.43 | 0.04 | 0.16 |
| S100B | 0.05 | 0.04 | 0.03 | 0.22 |
| Hcrt1 | − 0.04 | 0.14 | 0.002 | 0.76 |

*Abbreviations*: *BDI* Beck Depression Inventory, *SF-36* 36-item short form survey instrument, *IL-1Ra* interleukin 1 receptor antagonist, *IL-1RII* interleukin 1 receptor type 2, *IL-6* interleukin 6, *S100B* S100 calcium binding-protein B, *Hcrt1* hypocretin 1
*SF-36 pain scores are reported according to the transformed scale with high scores indicating high bodily pain and low scores indicating low bodily pain

**Table 3** Multiple regression model of fatigue (fVAS scores) and selected variables

| Variables | Estimate | Std. error | *P* |
|---|---|---|---|
| Depression (BDI) scores | 0.97 | 0.40 | 0.019 |
| Pain (SF-36) scores* | 0.38 | 0.13 | 0.004 |
| IL-1Ra | 0.54 | 0.19 | 0.009 |

Statistics for the final model: adjusted $R^2$ 0.37, *P* value < 0.001
*Abbreviations*: *BDI* Beck Depression Inventory, *SF-36* 36-item short form survey instrument, *IL-1Ra* interleukin 1 receptor antagonist
*SF-36 pain scores are reported according to the transformed scale with high scores indicating high bodily pain and low scores indicating low bodily pain

In the PCA bi-plot (Fig. 1a, b), patients are illustrated as dots and the variables as arrows. The length of an arrow is a function of its magnitude, and variables with longer arrows in the direction of a principal component will contribute most to the generation of this specific component.

The first component (PC1) explained most of the variance (35.66 %) (Fig. 1a), and the variable with the highest correlation with the first dimension was S100B (0.79), followed by IL-1Ra (0.78) and IL-1RII (0.68). The fatigue-related variable IL-1Ra revealed in simple and multiple regression analyses was thus highly correlated with S100B and IL-1RII. These results indicate that the first dimension was dominated by variables related to IL-1β activity, *the IL-1 dimension*.

The second component (PC2)—*the Hcrt1/IL-6 dimension*—explained 27.11 % of the variance in the dataset (Fig. 1a). Hcrt1 and IL-6 were the variables with the highest correlation, 0.82 and 0.78, respectively. The negative correlation of Hcrt1 in the second dimension indicates that individuals with low CSF concentrations of Hcrt1 also had high IL-6 concentrations.

In a second PCA model (Fig. 1b), fVAS scores were introduced as an additional variable to explore how fatigue contributed to the data cloud together with the biochemical components. In this model, PCA resulted in three components with eigenvalues > 1 explaining 71.63 % of the variance in the dataset (Additional file 2: Figure S2b). IL-1Ra showed the highest correlation (0.79) with PC1, followed by S100B (0.76), IL-1RII (0.63), and fVAS (0.48). IL-6 had the highest correlation (0.84) with PC2, followed by a negative Hcrt1 correlation (− 0.70). In the third component (data not shown), the variables with the highest correlation were fatigue (− 0.76) and Hcrt1 (0.58).

Adding fatigue-scores to the PCA resulted in a new data cloud in which the composition of the two first components was similar to the components in the PCA model without fatigue (Fig. 1a). Thus, rather than generating new dimensions or changing the composition of the components in the data cloud, fatigue showed a

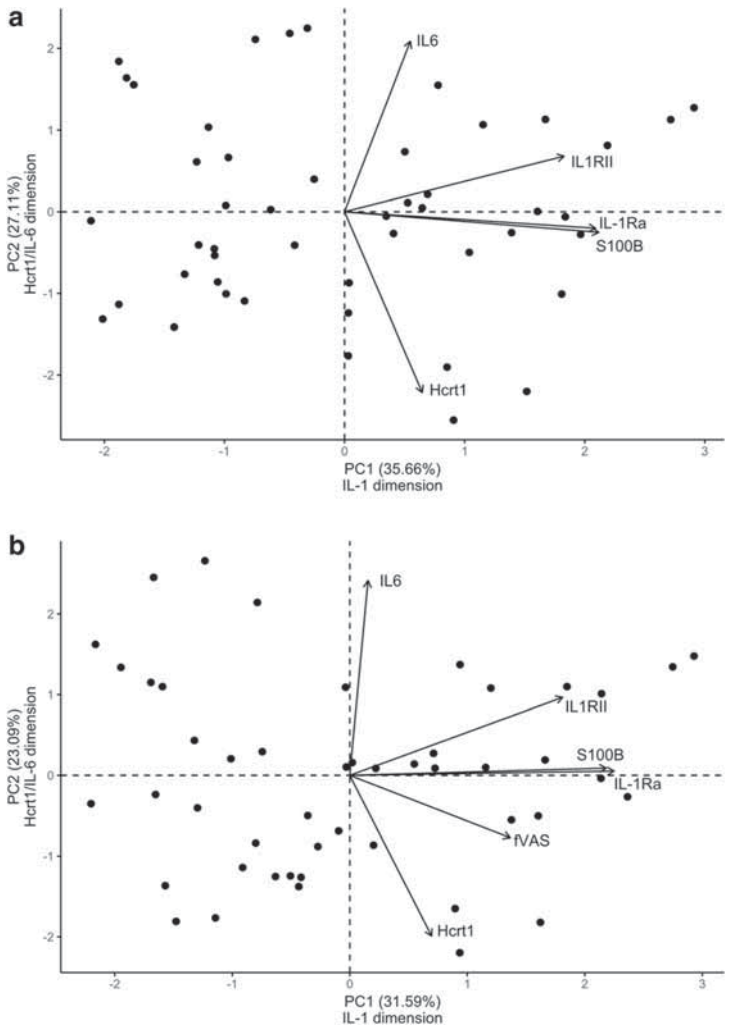


**Fig. 1** **a** PCA of the biochemical variables only. **b** PCA of the biochemical variables and fatigue variable (fVAS). Bi-plot shows scores of the individuals and variable correlations for PC1 and PC2. Individual scores are illustrated by dots. Arrows illustrate the correlations of the variables to the components. Longer arrows mean higher correlation and arrows close to a component has a higher contribution in the generation of the component

moderate association with variables on PC1 and a strong negative association with Hcrt1 on PC3.

## Discussion

The main findings in this study indicate a complex functional network in which clinical phenomena (pain and depression) together with several IL-1-related molecules in CSF influence fatigue in the context of sickness behavior. In addition, a role for the neuropeptide Hcrt1 as a fatigue-inducing molecule emerges.

Regarding molecules that relate to the IL-1 network, it is difficult to note the specific actions and relative importance of different molecules, but a common denominator seems to be a final IL-1β signaling of fatigue in the brain. The influence of the other biomarkers measured in CSF "disappear" in the complex biological

network interactions and only become evident in more advanced statistical models. In addition, the neuropeptide Hrct1—the main regulator of sleep and wakefulness—could represent a parallel, alternative, or redundant fatigue mechanism operative in inflammatory conditions and possibly driven by IL-6/TNF-α modulation of Hcrt1 (Fig. 2).

As shown in a number of previous studies, the clinical factors of pain and depression have a strong influence on fatigue. Whether these factors modulate the fatigue experience on a more psychological basis, whether pain generates fatigue through neuropeptide signaling, or if fatigue and depression are both signaled by IL-1 is not understood. Both animal and human studies have documented IL-1 signaling as one of the mechanisms underlying depression [25, 26]. Depression is also an important component of the sickness behavior response, in which fatigue is such a dominant phenomenon. The close association between fatigue and depression observed in so many studies may therefore have a biological explanation.

Pain is also a well-known associate of fatigue; many different explanations have been proposed to explain this constellation. Patients with chronic pain syndromes such as fibromyalgia consistently report fatigue. Recently, we revealed that fatigue in patients with pSS was associated with variance in a gene coding for an opioid transporter protein in which the more common allele had more severe fatigue [27]. One could therefore hypothesize that pain in an evolutionary perspective is a "danger signal" that induces sickness behavior. Regarding depression, pain and fatigue may therefore also have a biological basis.

In regard to biochemical factors, animal and human studies in acute and chronic inflammation show that IL-1 signaling is crucial for the sickness behavior response, in which fatigue is a major element. Most human studies using peripheral blood have not been able to demonstrate an influence of any cytokine on fatigue. Peripherally produced cytokines and other molecules have to cross the blood-brain barrier to act on the brain, where fatigue is generated. However, cytokines act in complex networks and the effect of one single cytokine upon a dependent factor such as fatigue can be difficult to evaluate. In the CSF, this can be even more problematic, as, for example, IL-1β appears in very low concentrations. Traditional statistical approaches may not detect effects unless they are extremely dominating. To decipher complex interactions in systems-biology research, it may therefore be of benefit to use alternative statistical approaches such as PCA. In this study, the initial regression analyses only found IL-1Ra of the biochemical factors to be a significant contributor, while in the PCA analyses, a logical pattern of molecular interactions for fatigue regulation became evident. A caveat in the deduction of processes that drive PCA dimensions is that other unmeasured covariates could be operative. It is for example known that IL-6 can

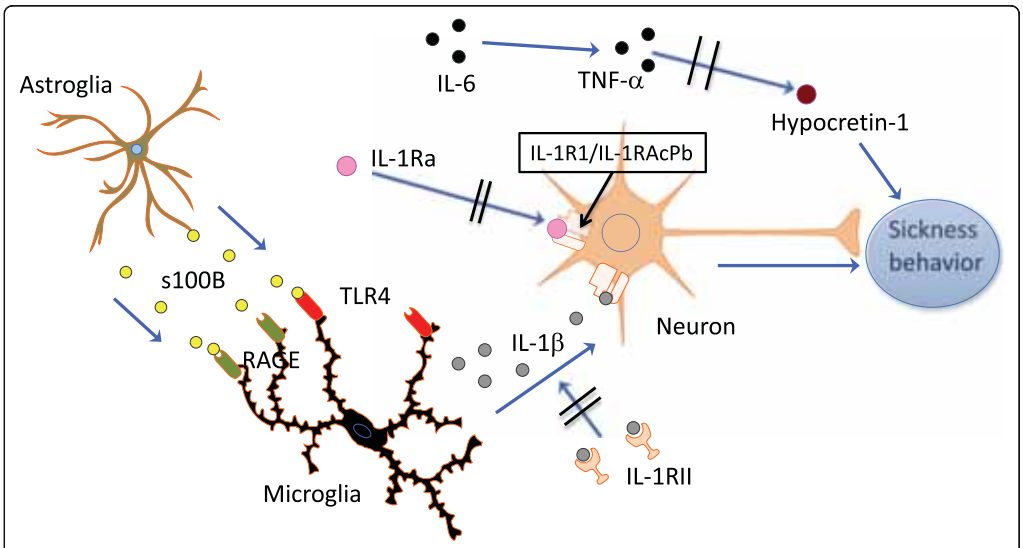


**Fig. 2** Hypothetical model for fatigue. Danger signals activate astroglia to produce and release S100B that bind to TLR4 and RAGE receptors on microglia. Activated microglia produce IL-1β that bind specific receptors (IL-1R1/IL-1RAcPb) on neurons and induce sickness behavior. There are no specific receptors for IL-6 that can modulate neurons in the same manner as for IL-1β, but higher levels of IL-6 may through stimulation of TNFα synthesis reduce the amount of Hcrt-1 in neurons. Low levels of Hcrt-1 can lead to or modulate fatigue through unknown pathways

elicit an IL-1Ra response independent of IL-1β secretion [28]. Nevertheless, these observations expand the knowledge of how fatigue can be generated and allows a more comprehensive understanding of the biological basis of fatigue in inflammatory and possibly also in non-inflammatory conditions.

In the proposed model for fatigue (Fig. 2), IL-1β can either originate from the periphery after passing the blood-brain barrier or be produced intrathecally by activated microglia. In the latter case, protein S100B secreted by activated astrocytes can signal through RAGE and TLR4 on microglia and lead to IL-1β production [29, 30]. Astrocytes are the most numerous cells in the mammalian brain and have a wide range of functions. They comprise an important component of the blood-brain barrier and contact endothelial cells with their end-feet. The importance of astrocytes in innate and adaptive immune responses in the brain has become more clear in recent years [31]. Activation of astrocytes and microglia may therefore in states of inflammation be the initial step in fatigue generation and could possibly also represent a mechanism by which states with cellular stress or "danger" such as degenerative diseases and cancer induce fatigue through microglia IL-1β production.

Sickness behavior is regarded as an important survival factor in evolution, and it is therefore plausible that pathways other than IL-1-driven mechanisms may have developed and can be operative in non-inflammatory and malignant conditions. In this context, Hcrt1 is an interesting candidate. Lack of Hcrt1 is the cause of narcolepsy type 1 [32, 33], and chronic fatigue is prevalent and strong in this condition [34]. It is known that low levels of Hcrt1 also lead to reduced appetite [35]. Low levels of Hcrt1 in CSF may therefore influence both appetite and fatigue, two prominent components of the sickness behavior response.

Studies on Hrct1 and fatigue are rare. There are several case reports of multiple sclerosis patients with hypothalamic lesions with low Hrct1 in CSF and with accompanying hypersomnia or fatigue, but cohort studies are conflicting; for a review, see Burfeind et al. [36]. In cancer, fatigue is common and worsens during cytotoxic treatment. One study in rodents showed that suppression of activity in the hypothalamic Hcrt-producing neurons and low Hcrt1 concentrations in CSF occurred during cytotoxic treatment and had a causal role in chemically induced fatigue [37]. When administered to humans, hypocretin receptor antagonists typically are associated with the side effects of sleepiness and fatigue, indicating that when hypocretin receptors are blocked, subjects experience fatigue [38].

In our study, high inflammatory activity in CSF (IL-6 high) was accompanied by low Hcrt1 (Fig. 1a, b). IL-6 that can be produced by a variety of cells induces production of TNF-α, which again is able to downregulate mRNA prepro-hypocretin, the precursor of Hrct1 [39]. Unfortunately, we did not measure TNF-α in this study and can therefore only speculate that in states of infection, damage or immunological danger, IL-6 leads to low levels of Hcrt1 and induces fatigue via a TNF-α-dependent mechanism. Although the target receptor or cells are unknown, this pathway may represent an alternative fatigue mechanism besides IL-1 signaling.

Limitations of this study include the small number of patients in the cohort and the low number of variables included in the analysis. A larger cohort would provide higher statistical power for both univariate and multiple regression analyses and the PCA. TNF-α analyses in CSF would have provided a greater impact on the Hcrt1 hypothesis for fatigue signaling. Measures of IL-β would have provided a better understanding of its role in the proposed network. Due to the very low CSF concentrations, robust data is difficult to obtain. However, since IL-1Ra downregulates IL-1β activity, it can be considered as a surrogate marker for IL-1β. Strong arguments in support of this is a study of patients with aseptic meningitis revealing CSF IL-1Ra levels approximately 2,000 times the level of IL-1β. IL-1Ra levels peaked about 12 h after IL-1β levels [40]. Another study in animals found that hypothalamic IL-1β mRNA peaked 1 h after intraperitoneal LPS injections, whereas IL-1Ra mRNA peaked after 3–6 h [41].

The study's strengths include a well-characterized patient cohort not under drug treatment that potentially could affect the analysis and the use of CSF for analysis instead of peripheral blood.

These results need to be validated in future studies, and especially it would be interesting to further explore the role for Hrct1 signaling of fatigue and also to apply more sensitive and accurate assays for IL-1β measures in CSF.

## Conclusion

The main findings of this study indicate a functional network in which several IL-1β-related molecules in CSF influence fatigue in addition to the clinical factors of depression and pain. The neuropeptide Hcrt1 seems to participate in fatigue signaling, but probably not through the IL-1 pathway.

## Additional files

**Additional file 1: Figure S1.** The visual analog scale (fVAS) used for scoring of fatigue. (DOC 25 kb)

**Additional file 2: Figure S2.** a) Scree-plot of the eigenvalues of components 1–5 from PCA model with biochemical variables only. Components 1 and 2 had eigenvalues > 1 and were retained in the analysis. b) Scree-plot of the eigenvalues of components 1–6 from the PCA model with biochemical variables and fatigue (fVAS). The first three components had eigenvalues > 1 and were retained in the analysis. (TIFF 533 kb)

**Abbreviations**
Hcrt1: Hypocretin-1; IL1-Ra: Interleukin-1 receptor antagonist; IL1RII: Interleukin-1 receptor type II; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; S100B: S100 calcium-binding protein B

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**Availability of data and materials**
Can be obtained upon request.

**Authors' contributions**
KB, CB, and RO designed the study. Patient data was collected and analyzed by ABT and RO. Laboratory experiments were performed by KB and IK. Data was analyzed by KB, CB, IK, PR, and RO. Statistical analysis was performed by KB, CB, JTK, and RO. KJ performed the pathway analysis. KB and RO wrote the first draft, and all authors contributed to the final version of the manuscript and revised it critically for intellectual content and final sign-off. RO provided overall guidance and support in all responsibilities. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**
The study was performed according to the Declaration of Helsinki and approved by the Regional Ethics Committee West (2010/1455). All participants gave written consent to participate in the study.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

## Publisher's Note


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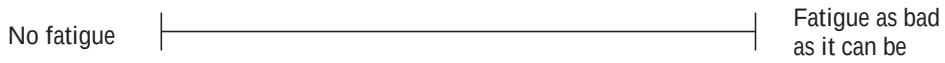
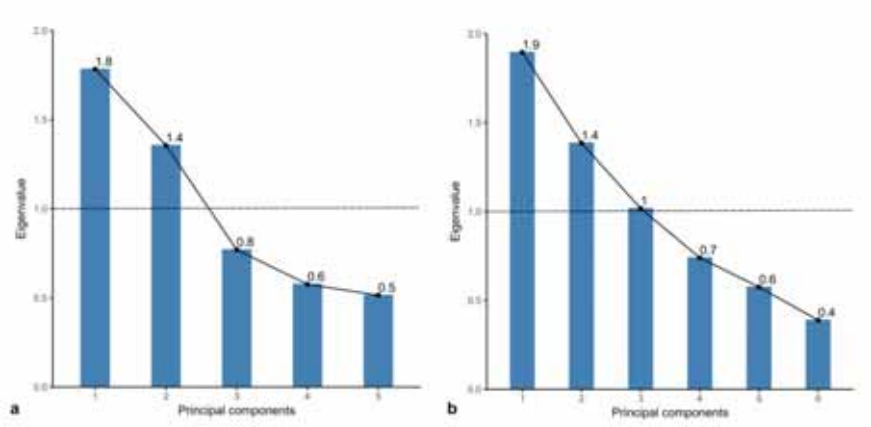
Figure S1

# Fatigue Visual Analogue Scale

This questionnaire asks about how much of a problem fatigue has been for you in the last week.

To the left is “no fatigue” and to right “fatigue as bad as it can be”. Rate your fatigue by placing a marker at the point along the line that best represents your perception of fatigue over the last week.




Eigenvalue
2.0
1.5
1.0
0.5
0.0
1.8
1.4
0.8
0.6
0.5
1
2
3
4
5
Principal components
a
Eigenvalue
2.0
1.5
1.0
0.5
0.0
1.9
1.4
1
0.7
0.6
0.4
1
2
3
4
5
6
Principal components
b