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CHRONIC FATIGUE SYNDROME
with an emphasis on interleukin-1 blockade

Megan Elisabeth Roerink

CHRONIC FATIGUE SYNDROME
with an emphasis on interleukin-1 blockade

Proefschrift
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aan de Radboud Universiteit Nijmegen
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*'We may lose and we may win,
though we will never be here again.'*

Jackson Browne

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The background of the slide features a close-up, high-angle shot of several large, dark green monstera leaves. The leaves are characterized by their iconic fenestrated (holey) pattern and are arranged in a way that creates a sense of depth and texture. The lighting is soft, highlighting the veins and the glossy surface of the leaves.

Chapter 1

General introduction and outline of the thesis

General introduction

Chronic fatigue syndrome

Chronic fatigue syndrome (CFS) is a complex condition that is characterized by severe fatigue present for at least six months leading to disability ⁽¹⁾. In addition to fatigue, a patient has to report at least four out of eight accompanying symptoms in order to fulfill the US Center for Disease Control (CDC) criteria for CFS (table 1) ^(1,2). These symptoms include: memory/concentration problems, post-exertional malaise, non-refreshing sleep, muscle pain, joint pain, headache, sore throat, and tender lymph nodes in the neck or armpit. In both Europe and the USA, the estimated prevalence of CFS ranges between 200 and 1400 per 100000 persons ⁽³⁻⁶⁾, with an estimated prevalence of 1% of the population in the Netherlands ⁽⁷⁾. Approximately 75% of CFS patients is of female gender, and symptoms start at a mean age between 29 and 35 years old ⁽⁸⁾. The majority of patients reports an acute onset of symptoms, for example after an infectious episode or a stressful event ⁽⁸⁾.

A model used to understand this complex illness is that of the four P's ⁽⁸⁾. This model discerns predisposing factors (e.g. childhood trauma), precipitating factors (infection, surgery, etc.) and perpetuating factors (e.g. focus on bodily symptoms, poor sense of control). The fourth P stands for prognostic factors, as it is known that those patients that attribute their symptoms to a physical cause and patients with psychiatric comorbidity show lower recovery rates ⁽⁹⁾.

Table 1 Diagnostic criteria for the chronic fatigue syndrome

Diagnostic criteria CFS (1, 2):
1. The individual has had severe chronic fatigue for 6 or more consecutive months that is not due to ongoing exertion or other medical conditions associated with fatigue (these other conditions need to be ruled out by a doctor after diagnostic tests have been conducted).
2. The fatigue significantly interferes with daily activities and work.
3. The individual concurrently has 4 or more of the following 8 symptoms: <ul style="list-style-type: none"> • post-exertional malaise lasting more than 24 hours • unrefreshing sleep • significant impairment of short-term memory or concentration • muscle pain • pain in the joints without swelling or redness • headaches of a new type, pattern, or severity • tender lymph nodes in the neck or armpit • a sore throat that is frequent or recurring

With respect to the causes of CFS and optimal methods for its diagnosis and treatment, there are a lot of questions that remain unanswered. For example, the diagnosis of CFS can only be made when other fatigue-causing illnesses (e.g. hypothyroidism, anemia) are ruled out. There is no specific (blood) test that can be used to prove the presence of CFS. This is one of the causes of the often long period between the start of symptoms, and the eventual diagnosis which may take up to five years ⁽³⁾. Another difficulty is the optimal treatment for CFS patients. Both cognitive behavioral therapy (CBT) and graded exercise therapy (GET) are effective treatment modalities ⁽¹⁰⁻¹⁴⁾. These behavioral interventions aim at gradual increase of activity levels with CBT also aiming at a change in cognitive processes thought to maintain fatigue and disability, such as a lack of control over fatigue, and the tendency to focus on symptoms ⁽¹⁵⁾. Despite the fact that these therapies can be highly effective, a substantial proportion of patients does not improve after completion of these time-consuming therapies ⁽¹⁴⁾. Therefore, there is an ongoing search for an effective treatment for those patients not responding to CBT and GET.

This thesis focuses on (biological) aspects of CFS that could be used to diagnose and treat CFS more effectively. Although there is an emphasis on the immune system and the proinflammatory cytokine interleukin-1 (IL-1), we also widened our perspective by investigating the role of the hypothalamic-pituitary-adrenal axis (HPA axis) and the cardiovascular system in CFS. These regulating systems will now be introduced.

The immune system & proinflammatory cytokines

The immune system is the host's defense mechanism against external pathogens (bacteria, viruses, fungi, etc.) and potential harmful internal cells (e.g. tumors, necrotic tissue). It can be divided into the 'innate immune system', and the acquired 'adaptive immune system'. Successful functioning and interaction of these systems is necessary to maintain proper functioning of an organism and prevent the occurrence of illness. Where the innate immune system is important for the first line defense, the adaptive immune system (consisting of B- and T-cells) is involved in immune memory and specific targeting of pathogens ⁽¹⁶⁾. Cytokines are proteins that predominantly belong to the innate immune system. Cytokines are produced by a wide range of immunological and non-immunological cells, and have an important role in coordinating the immune response. Most cytokines will be produced when the cells are exposed to an endogenous (damage associated molecular patterns, DAMP's) or exogenous (pathogen associated molecular patterns, PAMP's) stimulus. An example is activation of monocytes by lipopolysaccharide (LPS) from Gram-negative bacteria, one of the most potent activators of the immune system ⁽¹⁷⁾. An important source of cytokine production in the human body are monocytes and macrophages, which predominantly produce interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor (TNF). In addition to regulating mechanisms, pro-inflammatory cytokines such as interleukin-1 β

(IL-1 β) and IL-6, are able to cause symptoms that often accompany an inflammatory response, such as fever⁽¹⁸⁾ and lethargic behavior⁽¹⁹⁾. This specific effect, which resembles the symptoms of CFS, has contributed to the idea that proinflammatory cytokines could play an important role in CFS.

Measurement of circulating cytokines is difficult. First, since the proinflammatory cytokines are potentially deleterious to the host, their concentrations in the circulation are generally low. Second, a number of factors are known to influence cytokine concentrations. For example, processing protocols, storage time and number of freeze-thaw cycles are known to have a substantial influence on cytokine concentrations^(20, 21), which is important knowledge for interpretation of the studies that have been performed. More details on cytokine regulation and action, especially regarding IL-1 will be presented in **chapter 2**.

The hypothalamic-pituitary-adrenal axis and cortisol

The HPA axis is an important component of the endocrine system. It controls the reaction to stress, and plays a role in regulation of different homeostatic processes⁽²²⁾. The end product of the HPA axis is cortisol, which is produced by the adrenal gland (**figure 1**). The adrenal gland is under control of adrenocorticotrophic hormone (ACTH) released by the anterior lobe of the pituitary, which in turn is stimulated by corticotropin-releasing hormone (CRH) production of the hypothalamus. Cortisol acts on mineralocorticoid and glucocorticoid receptors in the body to influence several processes. It also gives a negative feedback signal to the hypothalamus, decreasing the release of CRH. In healthy individuals, cortisol increases upon awakening and declines during the day⁽²²⁾. In addition, cortisol is released in case of physical or emotional stress, and by its regulation of different functions (homeostasis, cardiovascular, etc.), cortisol promotes survival⁽²³⁾. The HPA axis and the immune system interact in a bidirectional way. For example, high concentrations of glucocorticoids released upon a stressor, are able to suppress the immune system by inhibition of pro-inflammatory cytokines and stimulation of anti-inflammatory cytokines (e.g. interleukin 10)⁽²⁴⁾. Deregulation of the HPA axis results in disease with Addison's and Cushing's disease as its most extreme forms, respectively representing hypo- and hyperfunctioning. As will be discussed in more detail below, more subtle derangements of the HPA axis have been described in CFS and in related disorders as fibromyalgia^(25, 26).

The cardiovascular system & orthostatic intolerance

Blood pressure is tightly controlled in order to maintain adequate cerebral perfusion (**figure 2**). Especially the autonomous nervous system (ANS) plays an important role. The ANS influences the heart rate and vascular resistance of peripheral blood vessels, which together with blood volume and cardiac output are important for the maintenance of an

Figure 1 Schematic overview of the HPA axis

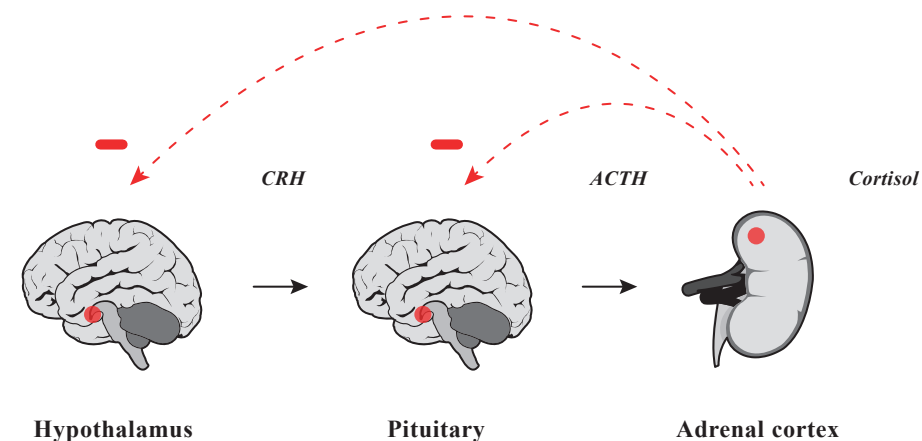
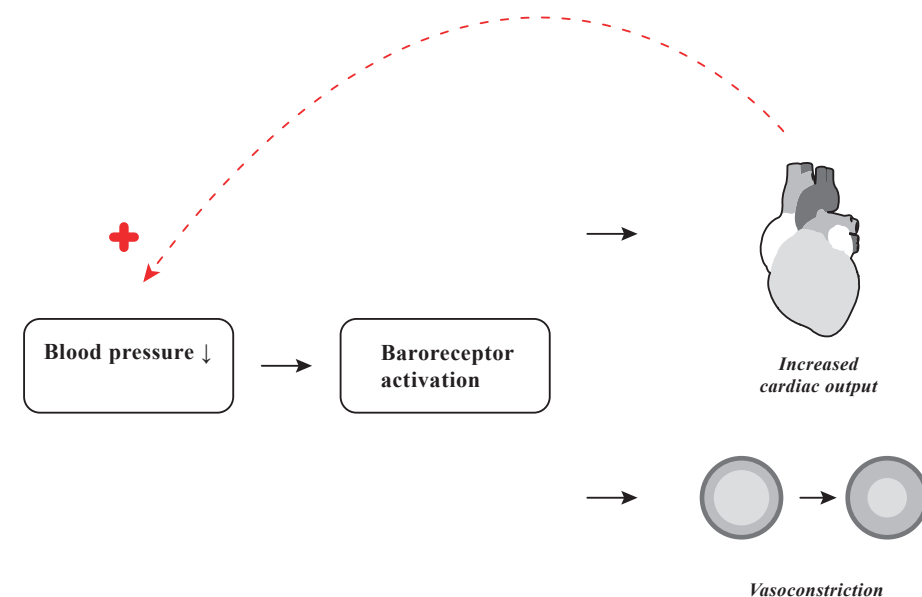


Figure 1 Schematic overview of the hypothalamic-pituitary-adrenal axis (HPA axis). Corticotropin-releasing hormone (CRH) is released by the paraventricular nucleus of the hypothalamus and has a stimulating effect on the anterior lobe of the pituitary, which in turn secretes adrenocorticotrophic hormone (ACTH). ACTH stimulates the adrenal cortex leading to cortisol production. Cortisol has an inhibiting effect on both the pituitary and hypothalamus.

Figure 2 Blood pressure regulation



adequate blood pressure. When a person changes position, from horizontal to vertical, these mechanisms have to respond quickly in order to maintain the blood pressure and warrant cerebral circulation. For example, as a consequence of a drop of blood pressure upon standing (pooling of blood in the lower extremities) the baroreceptors, which are located at the carotid sinus, sense decreased pressure and cause an increased heart rate and peripheral vasoconstriction⁽²⁷⁾. This response and other mechanisms prevent cerebral hypoperfusion, which can result in dizziness, headache, and even syncope. Dysfunction of these regulatory mechanisms can cause orthostatic intolerance, which is a cluster of conditions (orthostatic hypotension, postural orthostatic tachycardia syndrome, etc.) causing the patients to have symptoms in the upright position. As will be discussed in more detail below, orthostatic intolerance has been connected with CFS^(3, 28).

Outline of the thesis

The immune system⁽²⁹⁻³¹⁾, HPA axis^(25, 32), and cardiovascular system^(28, 33, 34) have all been extensively studied in CFS. Despite these previous studies, there is still no proper understanding of pathophysiological mechanisms involved in CFS. In this thesis, I have tried to investigate the role of the aforementioned systems in CFS, with the aim of gaining more insight into mechanisms involved in a complex illness such as CFS.

The role of the immune system in CFS patients has predominantly been investigated by measuring circulating cytokines⁽³¹⁾. This interest in the role of proinflammatory cytokines in CFS comes from studies that show typical CFS-like behavior after the administration of cytokines to healthy individuals⁽³⁵⁻³⁸⁾. Especially IL-1 (α and β) appears to be related to fatigue and has been measured in fatigue-causing illnesses. We reviewed in **chapter 2** the relation between circulating IL-1 α , IL-1 β or the IL-1 receptor antagonist (IL-1Ra) and fatigue in different (inflammatory and non-inflammatory) diseases. In addition, to obtain insight in causality, studies evaluating the effect of lowering IL-1 on fatigue severity were reviewed.

A difficulty with studies inhibiting proinflammatory cytokines, is that most of these drugs are not able to cross the blood-brain barrier because of their large molecular weight. This is no problem when there is a peripheral inflammatory response, but could become a problem when the target is the central nervous system. While evaluating the most effective strategy to inhibit IL-1 in CFS, we came upon studies from Dr Tobinick^(39, 40). In these animal and human studies^(39, 41, 42), it is claimed that rapid central delivery of drugs can be accomplished through injection in the perispinal region. In this region there is a valveless venous plexus, also called the Batons' plexus. Tobinick claims that injection of a drug in the soft tissue surrounding this plexus and placing the patient/animal in an upside-down position, the retrograde flow delivers drugs into the central nervous system (CNS). In **chapter 3** we repeated such animal experiments, and investigated if this indeed is a proper method to bypass more harmful methods of administering drugs into the CNS.

Given the data in the literature pointing to IL-1 as an important mediator of fatigue, the next step was to perform a randomized controlled trial (RCT) in CFS patients, to evaluate the effect of the IL-1Ra anakinra on fatigue severity and disabilities. In **chapter 4** we described the study protocol used to perform this study and in **chapter 5** the outcome of the RCT.

With the RCT, we had the opportunity to study cytokine disturbances in a group of well-defined CFS patients. In addition, all patients were asked to bring a healthy, age-matched, neighborhood control at their first study which served as a proper control group. Methods

to measure a large group of cytokines simultaneously with high specificity and sensitivity have been improved over the past years. In **chapter 6** we investigated if one of these new methods ‘proximity extension assay’ can help us to identify different cytokine profiles in CFS patients compared to controls or identify individually altered cytokines.

In a recent systematic review on circulating cytokines in CFS, TGF- β was the only cytokine that was elevated in CFS in the majority of studies performed ⁽³¹⁾. In **chapter 7** we aimed at replicating this finding in two separate CFS cohorts.

Altered functioning of the HPA axis has been investigated frequently in CFS. In a review on this subject, it was concluded that there is substantial evidence for hypocortisolism in CFS patients ⁽²⁵⁾. In **chapter 8** we asked ourselves the question if we could replicate and extend these findings in a large case-control study. To that end, we not only measured cortisol in saliva, as was done in most previous studies, but also used hair cortisol which reflects long-term cortisol output. In addition, we investigated if lowering IL-1 had an effect on salivary and hair cortisol.

While conducting these studies, the Institute of Medicine (IOM), published a new case-definition for CFS based on an extensive literature search. The IOM proposed a new name for CFS, i.e., Systemic Exertion Intolerance Disease (SEID) ⁽³⁾. The aim of this new definition was to accelerate the diagnostic procedure in CFS, and providing objective measurements to diagnose patients. One of the criteria of the SEID diagnosis is the presence of orthostatic intolerance, with postural orthostatic tachycardia syndrome (POTS) as its most frequent form. In **chapter 9** we investigated the prevalence of POTS in a large cohort of CFS patients, and investigated whether these patients are clinically different from CFS patients without POTS. In addition, to investigate the diagnostic value of POTS, we compared the prevalence of POTS in CFS patients to the prevalence of POTS in fatigued patients who did not fulfill the CFS criteria.

Chapter 10 summarizes the results of the studies and implications of the findings for future research are discussed.

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Chapter 2

Interleukin-1 as a mediator of fatigue in disease: a narrative review

Journal of Neuroinflammation. 2017 Jan 21;14(1):16.

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Abstract

Fatigue is commonly reported in a variety of illnesses, and it has major impact on quality of life. Previously it was thought that fatigue originates in the skeletal muscles, leading to cessation of activity. However, more recently it has become clear that the brain is the central regulator of fatigue perception. It has been suggested that pro-inflammatory cytokines, especially interleukin-1 alpha (IL-1 α) and interleukin-1 beta (IL-1 β), play a prominent role in the development of central fatigue, and several studies have been performed to elucidate the connection between inflammation and these central processes.

In this narrative review, mechanisms of action of IL-1 are described, with special attention to its effect on the central nervous system. In addition, we present a summary of studies that i) investigated the relationship between circulating IL-1 α and IL-1 β and fatigue severity, and/or ii) evaluated the effect of inhibiting IL-1 on fatigue. We aim to improve the understanding of fatigue in both inflammatory and non-inflammatory illnesses, which could help develop strategies to treat fatigue more effectively.

Reviewing the studies that have been performed, it appears that there is a limited value of measuring circulating IL-1. However, inhibiting IL-1 has a positive effect on severe fatigue in most studies that have been conducted.

Background

General introduction and aims

There is growing evidence supporting the theory that the central nervous system plays an important role in the perception of fatigue. The central nervous system processes and values sensory information, as well as guides motivational behavior involving decisions to discontinue activity or to invest effort. Cytokines have been suggested as prominent mediators in the induction of this central fatigue.

In this narrative review, we explored the evidence for the connection between pro-inflammatory cytokines, especially interleukin-1 (IL-1), and the perception of fatigue. Next to investigations that have examined whether there is a relation between circulating IL-1 and severity of fatigue (*table 1*), the effect of blocking IL-1 on fatigue severity has also been reported (*table 2*). For example, trials have been performed in rheumatoid arthritis^(1,2), Sjögren's syndrome⁽³⁾, and diabetes⁽⁴⁾. In this review, the different mechanisms of action of IL-1 will be discussed, especially considering its action in the CNS. We also review studies performed up to this writing that searched for a relation between IL-1 and fatigue in a variety of inflammatory and non-inflammatory illnesses.

Interleukin-1

To elucidate the contribution of IL-1 to the experience of fatigue, it is important to have a view of the pleiotropic action this cytokine. Because of the important role of IL-1 in the innate immune system and other physiological systems, it has become a field of great interest. Of the 11 members of the IL-1 family, two prominent members, IL-1alpha (IL-1 α) and IL-1beta (IL-1 β), have been described most frequently in the literature on fatigue. IL-1 α , IL-1 β and the IL-1 receptor antagonist (IL-1Ra) bind to the type 1 IL-1 receptor (IL-1R1). Whereas IL-1 α and IL-1 β activate an inflammatory signal upon binding to the IL-1R1, IL-1Ra binds to the same receptor but does not activate a signal.

IL-1 α is constitutively present as a bioactive precursor inside a wide range of cells. It is present, for example, in epithelial cells of the lungs, keratinocytes of the skin, and in vascular endothelial cells ⁽⁵⁾. During necrosis resulting in cell death, the bioactive IL-1 α precursor is released. Furthermore, IL-1 α is present on the surface of monocytes and B lymphocytes ⁽⁶⁾. IL-1 β is produced by more specific subsets of cells; it is a product of monocytes, tissue macrophages and dendritic cells ⁽⁵⁾. In order to become biologically active the IL-1 β precursor is first cleaved by caspase-1, an intracellular enzyme that is activated by a complex of intracellular proteins termed “the inflammasome” ⁽⁷⁾. There is also an alternative mechanism by which the inactive IL-1 β precursor is converted into an active cytokine. In presence of a high numbers of neutrophils, enzymes released by these cells, such as elastase and proteinase-3, will cleave the IL-1 β precursor and yield the bioactive moiety ⁽⁸⁾. After binding of IL-1 α or IL-1 β to the IL-1R1, a complex signaling cascade is activated, eventually leading to ‘nuclear factor kappa-light-chain-enhancer of activated B cells’ (NF κ B) production and subsequent gene transcription ⁽⁹⁾. In this manner, IL-1 action leads to a variety of biological events, ranging from activation of the acquired immune system to the induction of fever and slow-wave sleep ⁽¹⁰⁾. For the scope of this review, we will focus on the ability of IL-1 to induce fatigue.

Important when investigating the involvement of IL-1 in disease, is to note that circulating concentrations of IL-1 β often are at best only slightly elevated (picograms/mL) even under conditions of severe pathology ⁽¹¹⁾. A large part of IL-1 β remains inside the cell, and in the circulation it is bound to other proteins, such as the type 2 IL-1 receptor (IL-1R2), which serves as a decoy receptor, leading to a decrease in bioactivity ⁽¹²⁾. Therefore IL-1Ra, which is secreted by various cells in an inflammatory environment, has been proposed as a surrogate marker for IL-1 β activity ^(12, 13).

Effect of Interleukin-1 on the central nervous system

The central nervous system (CNS) plays an important role in cytokine-induced fatigue. As stated earlier, IL-1 α and IL-1 β are produced by a broad range of immunocompetent and non-immunological cells. Elevation of IL-1 in the brain contributes to behavioral alterations described as ‘sickness behavior’, which includes increased feelings of fatigue and depressed mood, loss of interest in social interactions and reduction of physical activity both in animals and in humans treated for different malignancies ⁽¹⁴⁻¹⁹⁾. The observed behavioral alterations in response to the intrathecal administration of pro-inflammatory cytokines indicate that, in addition to its peripheral effect on the immune response, IL-1 also signals to the brain via several immune-to-brain communication pathways.

Before peripherally produced cytokines can have an effect on the brain, they have to find a way to reach the CNS. In most diseases described in this review, there is no disruption of the blood-brain barrier (BBB) to allow proteins to gain access to the CNS. However, there are several mechanisms by which this barrier can be bypassed (**figure 1**). Some parts of the BBB are more permeable, especially those surrounding the circumventricular organs (CVOs), and cytokines like IL-1 can cross the BBB in this area by diffusion through the fenestrated endothelium **(1)** ⁽²⁰⁻²²⁾. For IL-1 α , IL-1 β and IL-1Ra, there is a saturable transport system from blood to the CNS **(2)** ⁽²⁰⁾, and production of cytokines by locally activated perivascular endothelial cell and macrophages has also been described **(3)** ⁽²³⁾. These three routes combined are often described as the humoral pathway. There is also a neuronal pathway, which uses the vagal nerve and sometimes also other peripheral afferent nerve fibers **(4)**, directly transmitting the cytokine signal to relevant brain regions ⁽²⁴⁾. The fifth route, activated by both the humoral and neuronal pathways, is activation of the immunocompetent cells of the brain, being the microglia **(5)**. These cells are able to produce IL-1 β locally once they have become activated ^(25, 26). In chronic fatigue syndrome (CFS), a syndrome characterized by severe fatigue, evidence for microglial activation has already been reported in a small group of patients ⁽²⁷⁾.

The IL-1R1 is distributed throughout the brain, although human studies on this topic are scarce ⁽²⁸⁾. The intracellular pathways after IL-1R1 activation in the brain are similar to those in the periphery, eventually leading to NF κ B activation and subsequent gene transcription ⁽²⁸⁾. In an animal experiment, an increase of IL-1 β mRNA was found in the hypothalamus directly after peripheral injection of IL-1 β , where it is able to induce fever ⁽¹⁵⁾. While the concentration in the hypothalamus decreased within 24 hours, upregulation of IL-1 β mRNA persisted in the cerebral cortex, and this was accompanied by a decrease in spontaneous activity lasting several days. Hypothetically, such persistence of IL-1 β transcription might be due to epigenetic changes in microglial cells, a process that is thought to play a role in several neuroinflammatory disorders ^(29, 30).

Once cytokines have reached the brain, there are changes in behavior through dopamine and serotonin neurotransmitter systems. Cytokines can influence dopamine synthesis via oxidative stress and disruption of the enzyme tetrahydrobiopterin (BH4), which is important for conversion of phenylalanine to the dopamine precursors tyrosine and L-3,4-dihydroxyphenylalanine (L-dopa). In addition, cytokines can enhance dopamine transporter activity and dopamine receptor functioning. Alternatively, cytokines can affect serotonin functioning through the activation of indoleamine 2,3-dioxygenase (IDO) in peripheral immune cells or microglia and kynurenine pathways ⁽³¹⁻³⁶⁾. Immunotherapy models have identified a dissociation between the role of dopamine and serotonin in symptom expression, with mood and cognitive symptoms being more responsive to treatment with

serotonin reuptake inhibitors (SSRIs) and fatigue and psychomotor functioning being more responsive to treatment with dopaminergic medications⁽³⁷⁻³⁹⁾. This suggests that fatigue symptoms may involve alterations in dopamine functioning. Indeed, animal studies show that dopamine depletion alters motivational behaviour in a way similar to cytokine administrations⁽⁴⁰⁻⁴³⁾, and it has been demonstrated that immune-induced reductions in physical activity and effort expenditure can be reversed with dopamine treatment^(14, 44). In addition, fatigue is a common symptom in many psychiatric and neurological conditions that have been associated with alterations of the dopamine system including Parkinson's disease and depression^(37, 45-47). Besides their effects on brain neurotransmitter systems, IL-1 can also influence brain functioning through their effect on hippocampal neuroplasticity, neurogenesis⁽⁴⁸⁾ or via neuro-endocrine mechanisms involving the hypothalamic pituitary-adrenal axis (HPA) functioning⁽⁴⁹⁾. These effects have been associated with the development of mental problems that often concur with fatigue-symptoms, such as impairments in learning and memory and depressive-like behaviour.

To give a clear view of the possible role of IL-1 in the development of fatigue in different diseases, we will discuss the studies that have been performed.

Figure 1 Overview of routes by which peripherally produced IL-1 is able to influence IL-1 levels in the brain

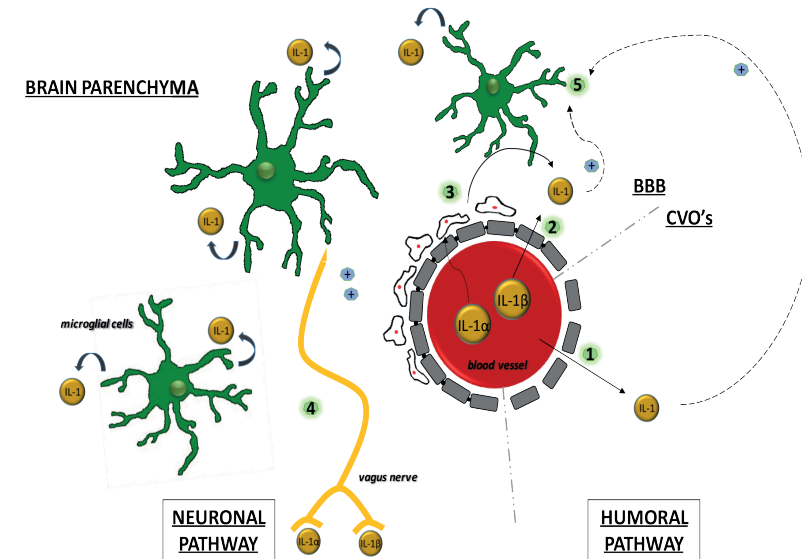


Figure 1 gives an overview of the five different routes that can be used by peripherally produced IL-1 α and IL-1 β to access the CNS. The first route (1) is diffusion of IL-1 through the fenestrated endothelium surrounding blood vessels in the circumventricular organs (CVO's). The rest of the brain microvasculature is surrounded by the blood-brain barrier (BBB), where diffusion is not possible due to tight junctions between cells. In these areas IL-1 can be transported across the BBB by a saturable transport system (2), or it can activate perivascular macrophages at the brain side of blood vessels, stimulating them to produce IL-1 (3). These three routes combined are frequently described as the humoral pathway, which is able to activate microglial cells in the brain parenchyma (5). Another important system is the neuronal pathway, where peripherally produced IL-1 stimulates afferent nerves, especially the vagal nerve, causing local IL-1 production in the CNS by microglial cells (4). Increased concentrations of IL-1 in different areas of the brain are suspected to influence neurotransmitter systems (e.g. dopamine and serotonin), thereby exerting its effect on behavior and the development of fatigue.

Table 1 Overview of studies measuring IL-1 in patients reporting fatigue

Reference	Disease activity	Number of patients	Fatigue questionnaire	IL-1 measurement	Main outcome
Rheumatoid arthritis					
<i>Lampa et al., 2014</i>	No neurological disease or generalized pain, nr of swollen joints 4.9±3.8	Patients (n=14), controls (n=12)	VAS-fatigue	CSF IL-1 and IL-1Ra	Higher IL-1 β and lower IL-1Ra in RA vs controls (p<0.001, p<0.05), positive correlation between IL-1β and fatigue (R=0.55, p<0.05).
Sjögrens syndrome					
<i>Harboe et al., 2009</i>	No acute illness in the week prior to or after sampling, no CRP/ESR elevation	Patients (n=54), controls (n=53)	FSS, VAS-fatigue	CSF IL-1 β , IL-1Ra and IL-1sRII	Higher IL-1Ra in patients (p=0.026), correlation IL-1Ra and VAS-fatigue (R²=0.11, p=0.015).
Sarcoidosis					
<i>Korenromp et al., 2011</i>	No disease activity	Fatigued patients (n=34), non-fatigued patients n=38)	CIS-fatigue (severe fatigue when ≥ 35)	Plasma IL-1 α , IL-1 β , and IL-1Ra	No significant differences.
<i>Baydur et al., 2010</i>	Pulmonary sarcoidosis	Patients (n=22), controls (n=22)	MFI-20	Whole blood production of IL-1 α and IL-1 β after stimulation with LPS (1ng/ml) Plasma IL-1 β before, directly after and 4-6h after exercise	Higher fatigue scores in sarcoidosis patients (p<0.0001), IL-1 β not different between patients and controls or among the three collection times. Correlation between pre-exercise IL-1β and MFI-20 in patients receiving immunomodulatory medication (R² 0.63, p=0.03).
Cancer					
<i>De Raaf et al., 2012</i>	Advanced cancer or 1-5 years post cancer treatment	Advanced cancer (n=45), cancer survivors (n=47)	MFI	Plasma IL-1Ra	Advanced cancer patients had higher IL-1Ra concentrations (p<0.01). In these patients physical fatigue was correlated with IL-1Ra (r=0.32, p=0.03). In cancer survivors, IL-1Ra was related to mental fatigue (r=0.35, p=0.02).
Table 1 Continued					
Prostate cancer					
<i>Greenberg et al., 1993</i>	Men undergoing localized radiotherapy	Patients (n=15)	VAS-fatigue daily during 8 weeks	Serum IL-1 β , at baseline and weekly thereafter	A rise in fatigue was seen between week 1 and 4, fatigue stabilized during week 5 and increased again in weeks 6 and 7. Rise in fatigue during the first four weeks was accompanied by increased IL-1β concentrations (p-value not reported).
<i>Bower et al., 2009</i>	Patients undergoing external beam radiation therapy	Prostate cancer (n=20), breast cancer (n=28)	FSI/fatigue during the past week/baseline, after 5/10/20 days of treatment, final week of treatment, and 2 weeks and 2 months after treatment	Serum IL-1 β , IL-1Ra in a subset of patients, at same time-points as the questionnaires.	Fatigue increased in both groups during treatment. Significant quadratic trend for IL-1 β during treatment (p=0.034). Treatment dose was not associated with IL-1 β and IL-1Ra concentrations. There was no correlation between IL-1 β and fatigue severity. IL-1Ra was associated with fatigue (β=0.63, p=0.016).
<i>Dirksen et al., 2014</i>	Non-metastatic cancer prior to radiation therapy	Patients (n=30)	POMS fatigue (inertia subscale)/pre-treatment en post-treatment	Serum IL-1 β , pre-treatment and post-treatment (<2 weeks after radiotherapy, <10 weeks after brachytherapy)	Fatigue was increased post-treatment (p=0.027). No differences in IL-1 β concentrations, no correlation with fatigue severity.
<i>Jim et al., 2012</i>	Non-metastatic or asymptomatic metastatic prostate cancer	Patients (n=53)	FSI (fatigue over the past week)/at baseline and after 6 months	SNP in IL1B gene (rs16944)	IL1B had no significant effect on fatigue-related outcomes.
Breast cancer					
<i>Greinitz et al., 2000</i>	Women undergoing postoperative radiotherapy (no chemotherapy), without metastatic disease	Patients (n=41)	FAQ, and VAS-fatigue/ during previous week/ at baseline, end of week 1-5, and 2 months after treatment	Serum IL-1 β , same time points as questionnaires	VAS-fatigue increased until week 4 (p<0.001). During week 4 and 5 FAQ physical (p=0.035 and 0.015) and cognitive (p=0.008 and 0.007) subscales were significantly elevated. IL-1 β did not increase during treatment.
<i>Ion Ah et al., 2008</i>	Stage 0-IIIa breast cancer before adjuvant therapy	Patients (n=44)	Piper-fatigue scale/ at baseline and at 3 months (during adjuvant therapy) and 6 months after baseline (initial recovery)	Whole blood production of IL-1 β after stimulation with PHA (10ug/ml)	IL-1β predicted fatigue before adjuvant therapy (β=0.30, p<0.05).

<i>Liu et al., 2012</i>	Stage I-III breast cancer prior to ≥ 4 3-week cycles of chemotherapy	Patients (n=53)	MFSI-SF/fatigue during past week/at baseline and during cycle 1 and 4 of chemotherapy (last 2 weeks)	Plasma IL-1Ra, at the same time points as questionnaires	Fatigue significantly increased over time ($p < 0.05$). IL-1Ra dropped at cycle 1 week 3 ($p < 0.0001$). There was no association between IL-1Ra and fatigue.
<i>Schmidt et al., 2015</i>	Stage 0-III breast cancer prior to adjuvant radiation therapy	Patients (n=92)	FAQ/ at baseline, after completion of radiotherapy (week 7), and the end of the intervention (week 13, resistant exercise/relaxation)	Serum IL-1Ra, at the same time points as questionnaires	Moderate correlation between IL-6/IL-1Ra at the end of radiotherapy with physical fatigue at the same time ($r=0.25$, $p=0.022$) and at 6 weeks after chemotherapy ($r=0.23$, $p=0.046$).
<i>Bower et al., 20002</i>	Stage 0-II breast cancer 1-5 years after diagnosis, after completion of treatment	Fatigued (n=20), non-fatigued (n=20)	Energy/fatigue subscale RAND-36 (score 0-50=high fatigue, score 70-100=low fatigue)/fatigue during past 4 weeks FSI/fatigue during past week	Serum IL-1 β and IL-1Ra	Fatigued women had significantly higher IL-1Ra concentrations ($p=0.006$).
<i>Bower et al., 2011</i>	Stage 0-IIIa breast cancer, after completion of primary cancer therapy (within past 3 months) i.e. surgery, radiation, and/or chemotherapy	Patients (n=103)	FSI (cut-off 3)/fatigue during the past week	Plasma IL-1Ra	64% scored above 3 on the FSI, these patients did not have a higher IL-1Ra concentration. There was no significant association between IL-1Ra and fatigue or chemotherapy exposure.
<i>Bower et al., 2007</i>	Stage 0-II breast cancer survivors (6.5-10 years after diagnosis)	Fatigued (n=10), non-fatigued (n=15)	Vitality scale SF-36 (<50=significant fatigue, >70=absence of significant fatigue)	Whole blood production of IL-1 β after stimulation with LPS (100pg/ml) or cortisol (0 , 10^{-8} , 10^{-7} , 10^{-6} M), at baseline, directly after TSST, and after 30min recovery	No differences at baseline. IL-1β increased significantly in fatigued patients after completion of the TSST ($p=0.02$).

Table 1 Continued

<i>Collado-Hidalgo et al., 2006</i>	Stage 0-III breast cancer survivors, 1-5 years post-diagnosis	Fatigued (n=32), non-fatigued (n=18)	Vitality scale SF-36 (<50=significant fatigue, >70=absence of significant fatigue)	Plasma IL-1Ra	IL-1Ra was significantly higher in fatigued breast-cancer survivors ($p=0.05$).
<i>Orre et al., 2011</i>	Stage II-III breast cancer patients, 2.7-7.2 years after postoperative locoregional radiotherapy	Patients (n=299)	Fatigue questionnaire	Serum IL-1Ra	There was no significant association between IL-1Ra and fatigue.
<i>Collado-Hidalgo et al., 2009</i>	Stage 0-III breast cancer survivors, 1-5 years post-diagnosis	Fatigued (n=33), non-fatigued (n=14)	Vitality scale SF-36 (≤ 55 =significant fatigue, >70=absence of significant fatigue), MFSI	IL-1B-511 (CT) polymorphism	Fatigued survivors had a substantial overrepresentation of CC alleles, and underrepresentation of TT alleles. The prevalence of at least one cytosine was more frequent among fatigued patients ($p=0.007$) and associated with fatigue in multiple regression ($p=0.021$), which was no longer significant after controlling for depressive symptoms ($p=0.052$).
<i>Reinertsen et al., 2011</i>	Stage II-III breast cancer survivors	Fatigued (n=101), non-fatigued (n=201)	Fatigue questionnaire (cut-off 4, clinical significant fatigue), chronic fatigue was defined as fatigue being present for at least 6 months	IL-1B rs16944 (A/G) SNP, and IL-1 β mRNA expression	There was no association between chronic fatigue and the IL-1B SNP or IL-1 β mRNA expression.
Testicular cancer					
<i>Orre et al., 2009</i>	Patients 5-20 years after unilateral orchiectomy	Fatigued (n=92), non-fatigued (n=191)	Fatigue questionnaire (cut-off 4, clinical significant fatigue), chronic fatigue was defined as fatigue being present for at least 6 months	Plasma IL-1Ra	Fatigued patients had significant higher IL-1Ra ($p=0.002$). In multiple regression analysis, IL-1Ra corrected for age had an OR of 1.93 (95%CI 1.21-3.08). Although age an IL-1Ra explained only 4% of the variance. IL-1Ra was not included in the final model.

Table 1 Continued

Uterine cancer <i>Ahlgberg et al., 2004</i>	Patients receiving external radiation therapy after hysterectomy	Patients (n=15)	MFL20/at baseline, after 30Gy (+3 weeks) and after 46Gy (+5-6 weeks)	Plasma IL-1 (α or β unknown), same time-points as questionnaires	Fatigue increased during treatment, IL-1 remained below the detection limit during the entire study period (4pg/mL).
AML/MDS <i>Meyers et al., 2005</i>	Newly diagnosed AML/MDS before undergoing chemotherapy.	Patients (n=54)	Brief fatigue inventory (cut-off score ≥ 4 , moderate-severe fatigue)/fatigue in the past 24 hours/baseline and after 1 month of treatment	Plasma IL-1 (α or β unknown) and IL-1Ra, at baseline.	The was a positive correlation of IL-1Ra and fatigue ($r=0.52$, p-value not reported).
Post-stroke fatigue <i>Ormstad et al., 2011</i>	Acute stroke patients	Patients (n=45)	FSS (dichotomized as a score ≥ 4 or <4)/ at 6, 12, and 18 months after stroke	Serum IL-1 β and IL-1Ra, <24 h (n=35), 24-48h (n=7), and 48-72h (n=3) after stroke onset	Significant correlation between IL-1β and fatigue at 6 months ($r=0.37$, $p=0.015$). Negative correlation between IL-1Ra and fatigue at 12 months ($r=-0.38$, $p=0.013$). Fatigued patients had significant lower IL-1Ra concentrations.
<i>Becker et al., 2015</i>	Acute stroke patients	Patients (n=39)	FAS 30/90/180/365 days after stroke	IL1RN SNP rs4251961	Carriers of a C allele reported more fatigue ($p=0.03$). At 30 and 90 days, patients with at least one C allele had higher scores on fatigue ($p<0.05$).
CFS <i>Hornig et al., 2015</i>	CFS	Patients (illness duration ≤ 3 years n=52, illness duration >3 years n=246), controls (n=348)	MFI	Plasma IL-1 α , IL-1 β and IL-1Ra	There were no differences when comparing all patients combined to controls. However, patients with a short illness duration had significantly higher IL-1α ($p<0.05$) and IL-1Ra ($p<0.05$) compared to controls. In patients with a long illness duration IL-1β was significantly lower compared to controls ($p<0.05$). IL-1α, IL-1β and IL-1Ra were higher in short illness patients compared to long illness patients ($p<0.01$).

Table 1 Continued

<i>Russell et al., 2016</i>	CFS (female)	Patients age:1. ≤ 18 / illness duration ≤ 2 years (n=18), 2. age 18-50/average illness duration 7 years (n=22), 3. age ≥ 50 and average illness duration 11 years (n=28), controls (n=81)	Chalder fatigue in adolescents, and MFI in other patients	Plasma IL-1 α and IL-1 β	Looking at individual expression, there were no differences between patients and controls. IL-1α appeared in a linear classification model in the adolescent group, but not in the other 2 groups.
<i>Hardcastle et al., 2015</i>	Moderate (mobile) or severe (housebound) CFS	Moderate CFS (n=22), severe CFS (n=19), controls (n=22)	FSS	Serum IL-1 β and IL-1Ra	Significant IL-1β increase in moderate compared with severe CFS patients ($p=0.002$). For other subgroups and IL-1Ra there were no differences.
<i>Landi et al., 2016</i>	CFS	Patients (n=100), controls (n=79)	MFI	Plasma IL-1 α and IL-1 β	No significant differences.
<i>Chao et al., 1991</i>	CFS	Patients (n=9), controls (n=7)	VAS-fatigue	Serum IL-1 β	No differences in serum IL-1 β . IL-1β production after LPS stimulation was significantly higher in CFS patients ($p<0.05$)
<i>Swanink et al., 1996</i>	CFS	Patients (n=76), controls (n=69)	CIS	PBMC production of IL-1 β after stimulation with LPS (1ng/mL) or PHA (4ug/mL) Plasma IL-1 α , IL-1 β , and IL-1Ra	No differences in circulating cytokine concentrations. Significant lower IL-1β production after LPS stimulation ($p<0.05$), no correlation between production and fatigue severity.
<i>Mawle et al., 1997</i>	CFS	Patients (n=26), controls (n=50)	-	Whole blood production of IL-1 α , IL-1 β , and IL-1Ra after stimulation with LPS PBL production of IL-1 α and IL-1 β after stimulation with PHA	IL-1α production was lower in severely ill patients (n=13) and those with a gradual disease onset (n=17) compared to controls ($p=0.038$, $p=0.011$). IL-1β was also lower in patients with a gradual disease onset ($p=0.039$).

Table 1 Continued

<i>Cannon et al., 1997</i>	Sudden onset CFS	Patients (n=16), controls (n=15)	-	PBMC production of IL-1 β , IL-1Ra, and IL-1sRII after stimulation with LPS (1ng/ml), indomethacin, or a combination, before and daily after a 15min exercise on day 2	At baseline, controls had a significant increase in IL-1 β production during the luteal phase (unstimulated, p=0.021). This increase was absent in CFS patients. In the follicular phase control group had an increase IL-1 β production 48h after exercise. In CFS patients there was no alteration over time. In the follicular phase IL-1Ra secretion was higher in CFS patients (unstimulated, p=0.023). IL-1sRII was higher in patients (unstimulated, p=0.0002). No significant differences.
<i>Tomoda et al., 2004</i>	CFS	Patients (n=15), controls (n=23)	-	IL-1 β production of PBMCs after stimulation with PHA (5 u/ml) or LPS (50ng/ml)	No significant differences.
<i>Lloyd et al., 1991</i>	CFS	Patients (n=25), controls (n=28)	-	Serum and CSF IL-1 β	No significant differences.
<i>Peterson et al., 2015</i>	CFS	Patients (n=18), controls (n=5)	-	CSF IL-1 β and IL-1Ra	No significant differences.
<i>Natelson et al., 2005</i>	CFS	Patients (n=44), controls (n=13)	MFI	CSF IL-1 α and IL-1 β	No significant differences.
<i>Hornig et al., 2016</i>	CFS	Patients (n=32), MS controls (n=40), and controls (n=19)	-	CSF IL-1 α , IL-1 β and IL-1Ra	CFS patients had significant lower IL-1 β and IL-1Ra concentrations compared to normal controls (p=0.003 and p=0.014). And compared to MS patients IL-1 α (p=0.0007), IL-1 β (p=0.0018) and IL-1Ra (p=0.0003) were decreased in CFS.

Table 1 Continued

(Post) infectious fatigue					
<i>Vollmer-Conna et al., 2004</i>	Patients with acute Q-fever, EBV, or RRV	Q-fever (n=18), EBV (n=24), RRV (n=24)	Physical symptom checklist/fatigue in the past two weeks	Serum IL-1 β PBMC production of IL-1 β after stimulation with LPS (10ng/ml)	Fatigue was reported in 100% of Q-fever patients, >75% of EBV patients, and >50% of RRV patients. In Q-fever IL-1β correlated significantly with fatigue (r=0.47, p=0.04), which was also found in the EBV/RRV combination group (r=0.39, p=0.01). All significant results were obtained from the unstimulated samples. No significant differences.
<i>Vollmer-Conna et al., 2007</i>	Patients with post-infectious fatigue and post-infectious patients without fatigue	EBV (n=11), RRV (n=6), Q-fever (n=5), and controls after EBV (n=17), RRV (n=14) or Q-fever (n=11)	Somatic and psychological health report (fatigue was defined as a score ≥ 3 on the SOMA subscale) at 1, 2, 3, 6, and 12 months after onset of the infection	Serum IL-1 β PBMC production of IL-1 β after stimulation with LPS (10ng/ml), mouse anti-human or anti-CD3	No significant differences.

Table 1 gives an overview of all studies that investigated the relationship between IL-1 and fatigue severity.

Abbreviations; AML=acute myeloid leukemia; CFS=chronic fatigue syndrome; CSF=cerebrospinal fluid; CIS=checklist individual strength; CRP=C-reactive protein; EBV=Epstein-Barr virus; ESR=erythrocyte sedimentation rate; FAS=fatigue assessment scale; FAQ=functional activity questionnaire; FSI=fatigue symptom inventory; FSS=fatigue severity scale; LPS=lipopolysaccharide; MDS=myelodysplastic syndrome; MFI=multidimensional fatigue inventory; MFSI=multidimensional fatigue symptom inventory; MS=multiple sclerosis; PBL=peripheral blood leukocytes; PBMC=peripheral blood mononuclear cell; PHA=phytohaemagglutinin; POMS=profile of mood states; RRV=Rosser river virus; SF=short form; TSST=Trier social stress test; VAS=visual analog scale.

Table 2 Overview of studies evaluating the effect of inhibiting IL-1 on fatigue severity

Reference	Disease activity	Design	Number of patients	Fatigue questionnaire	IL-1 intervention	Main outcome
Rheumatoid arthritis <i>Alten et al., 2011</i>	≥6 of 28 tender and swollen joints, elevated hsCRP and/or ESR	Randomized, double-blind, placebo-controlled, dose-parallel-group, dose-finding trial	274	FACIT-F at 12 weeks	MTX combined with canakinumab: 1. 150mg s.c. every 4 weeks (n=69), 2. 300mg s.c. every 2 weeks (n=64), 3. 600mg i.v. followed by 300mg s.c. every 2 weeks (n=71) or placebo s.c. every 2 weeks (n=70)	Decrease in fatigue canakinumab group 1 (p=0.006) and 3 (p=0.028) compared to placebo.
<i>Omdal et al., 2005</i>	Mean DAS28 6.2±1.1	Pilot, non-blinded, no control group	8	FSS and VAS-fatigue at baseline, week 4, and week 8	100mg s.c. anakinra daily	Decrease in FSS (p=0.002) and VAS-fatigue (p=0.0001) during the 8 weeks, accompanied by a decrease of the DAS28 score (p<0.0001).
Sjögrens syndrome <i>Norheim et al., 2012</i>	No elevation CRP/ESR	Randomized, double-blind, placebo-controlled, parallel-group trial	26, 1 not included in analysis	FSS and VAS-fatigue at baseline, week 0, week 2, week 4, and week 5	100mg s.c. anakinra (n=12) or placebo (n=13) daily during 4 weeks	No difference FSS scores after 4 weeks, more frequent reduction of VAS-fatigue of >50% in anakinra group (50% vs 8%, p=0.03).
Table 2 Continued						
CAPS <i>Kone-Paut et al., 2011</i>	Moderate or severe disease activity	Part 1. open-label, followed by part 2. which was a double-blind withdrawal phase in responders, ending with open-label part 3	35	5-point likert scale, daily first 15 days of part 1, weekly thereafter (physician and patient), FACIT-F	Single canakinumab (150mg) dose in part 1 (n=35), followed by canakinumab (n=15) or placebo (n=16) every 8 weeks for 24 weeks in part 2. At relapse or at end of part 2, patients were treated with canakinumab for 16 more weeks (n=31)	Fatigue absent or minimal at the end of part 1 in >85% of patients paralleled by decreased disease activity. Increase FACIT-F at the end of part 1 (p<0.05). Fatigue relapse in patients randomized to placebo in part 2.
<i>Huemmerle-Deschner, 2011</i>	Disease activity requiring medical intervention	Open label, phase II trial	7 (pediatric)	5-point likert scale at post-treatment day 1 and 2, and week 1 and 5 (physician)	Canakinumab 150mg or 2mg/kg, repeated after seven days in absence of complete response	Fatigue was absent or minimal 1 day after canakinumab in all patients. This was accompanied by a decrease in disease activity.
<i>Hoffman et al., 2008</i>	NLRP3 mutation combined with classic FCAS/MWS symptoms	Part 1. 6-week randomized controlled trial, part 2A. open-label, 2B. randomized controlled trial	47	DHAF rating fatigue over previous 24 hours	Part 1 loading dose of 320mg rilonacept/placebo s.c. (n=47), followed by weekly s.c. injections of 160mg rilonacept/placebo. Part 2 (n=46) weekly s.c. rilonacept 160mg during 9 weeks followed by 9 weeks rilonacept/placebo	Decrease in fatigue in part 1 (p<0.001), relapse in those patients treated with placebo in part 2 (p<0.001).

Table 2 Continued

Diabetes <i>Cavelti-Weder et al., 2011</i>	Type 2 diabetes	Randomized, double-blind, placebo-controlled trial	30	Fatigue scale for motor and cognitive functions	XOMA052/placebo (0.01 mg/kg – 1mg/kg)	At baseline 53% of patients experienced mild-severe fatigue. One month after treatment fatigue was increased in the placebo and lowest dosing group, in the two medium dosing groups fatigue was slightly decreased, and in the two highest dosing groups fatigue was remarkably decreased. Effect size dose-dependent effect $d=0.3$. The highest dose of 1.0 mg/kg had a favorable effect on motor fatigue ($d=1.05$).
Cancer <i>Hong et al., 2015</i>	Advanced non-small cell lung cancer	Open label dose escalation trial	16	EORTC-QLQ, at baseline and after 8 weeks	Intravenous MABp1 every 3 weeks trough 4 dosing levels (0.25/0.75/1.25/3.75 mg/kg, and 3.75mg/kg every 2 weeks (until disease progression)	Non significant improvement in fatigue severity. Median disease free progression was 57 days.
<i>Hickish et al., 2016</i>	Metastatic colorectal cancer refractory to standard chemotherapy	Randomized controlled trial	309	EORTC-QLQ	MABp1 plus best supportive care or placebo (2:1)	Significant improvement of fatigue, increase in appetite, and decrease in pain severity

Table 2 gives an overview of all studies that investigated the relationship between IL-1 and fatigue severity.

Abbreviations: CAPS=cryopyrin-associated periodic syndrome; CRP=C-reactive protein; DAS=disease activity score; DHAF=daily health assessment form; EORTC-QLQ=European organization for research and treatment of cancer quality of life questionnaire; ESR=erythrocyte sedimentation rate; FACIT-F=functional assessment of chronic illness therapy subscale fatigue; FCAS=familial cold autoinflammatory syndrome; FSS=fatigue severity scale; hsCRP=high sensitive C-reactive protein; MTX=methotrexate; MWS=Muckle-Wells syndrome; s.c.=subcutaneous; VAS=visual analog scale.

Overview of studies investigating the role of Interleukin-1 in disease

Inflammatory illnesses

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic disease characterized by recurrent, often symmetrical destructive arthritis. In addition to local joint inflammation, RA is known for systemic symptoms such as fatigue. The prevalence of fatigue in the RA population varies between 40% and 88%, depending on criteria and questionnaires used⁽⁵⁰⁻⁵²⁾. Although the exact causal mechanism of fatigue is unknown⁽⁵³⁾, it can be predicted by pain, sleep disturbances and depression, rather than by disease activity⁽⁵⁴⁾. The contribution of cytokine disturbances to the development of fatigue remains to be elucidated, but could be prominent as treatment with tumor necrosis factor alpha (TNF- α) inhibitors has a positive effect on fatigue compared to treatment with methotrexate alone⁽⁵⁵⁾.

Cytokine disturbances in RA are well known, and are predominantly driven by increased TNF- α and IL-1, although TNF- α is measured more frequently. Both concentrations of IL-1 β and IL-1Ra are slightly elevated in RA, and both correlate with disease severity, reflected by elevated pain scores and an increased erythrocyte sedimentation rate (ESR)^(56, 57). Several findings suggest a central activation of the immune system in RA patients. A study evaluating IL-1 concentrations in cerebrospinal fluid (CSF) in 14 female RA patients with moderate disease activity and 12 healthy subjects found IL-1 β concentrations in CSF are increased in patients, and positively correlated to fatigue severity ($R=0.55$, $p<0.05$)⁽²⁶⁾. Such a correlation was not present for pain or tender joint count. IL-1Ra in CSF was lower in RA patients compared to healthy subjects. Furthermore, IL-1 β concentrations in CSF were significantly higher than in plasma, which suggests a central proinflammatory state in RA patients.

The next step is to assess the effect of IL-1 blockade on fatigue severity in RA, which has been investigated by using monoclonal antibodies against IL-1 β (canakinumab, Ilaris) and recombinant IL-1Ra (anakinra, Kineret) in patients with current disease activity^(1, 2). In both studies there was a significant decrease of fatigue severity. The double blind study performed by Alten et al.⁽¹⁾ measured fatigue using the ‘Functional Assessment of Chronic Illness Fatigue’ (FACIT-F) questionnaire in patients on different canakinumab dosing regimens next to methotrexate, compared to patients who used placebo. At 12 weeks, two out of three canakinumab groups reported a small but significant decrease in fatigue compared to placebo. With respect to disease response rate, measured by joint inflammation and other disease specific characteristics, there was only a significant response in one of the groups (150mg canakinumab s.c. once every four weeks). An inherent problem with canakinimab, being a monoclonal antibody, is its failure to reach the CNS, and hence only fatigue driven

by peripherally produced IL-1 that may gain access to the brain is being countered. In case of apparent peripheral inflammation, which is the case in RA, this appears to be effective as can also be concluded from a study lowering TNF- α using a monoclonal antibody; here a rapid effect on central nociceptive brain activity was found ⁽⁵⁸⁾.

In the study using anakinra in RA, eight patients were treated daily for eight weeks, although there was no placebo-treated control group ⁽²⁾. The decrease of fatigue severity was most profound in the first four weeks with visual analog scale (VAS) scores being almost reduced by 50 percent. Decrease of fatigue was paralleled by a decrease in disease activity.

Sjögren's syndrome

Another disease that is often accompanied by joint pain is Sjögren's syndrome, although diminished salivary and lacrimal gland function are the hallmarks. Sjögren's syndrome is characterized by autoantibody production against ribonucleoproteins and mononuclear cell accumulations in exocrine glands. Besides sicca complaints, fatigue is one of the most frequently noted symptoms in this disease reported by up to 85% of patients ⁽⁵⁹⁾. Fatigue for some part can be explained by an altered sleeping pattern ⁽⁶⁰⁾, but IL-1 might also be a contributor.

Harboe et al. assessed IL-1 alterations in CSF in 54 adult patients with primary Sjögren syndrome (pSS) compared to 53 controls ⁽⁶¹⁾. IL-1 β concentrations were below the detection limit of 1 pg/ml for both patients and controls. IL-1Ra concentrations were significantly elevated in patients and correlated to fatigue severity using a visual analog scale (VAS) independent of age and depression, although this correlation was very weak ($r=0.11$, $p=0.015$).

The effect of IL-1 inhibition on fatigue severity was assessed by the same study group in 26 pSS patients ⁽³⁾. Patients were treated with either daily anakinra or placebo for a period of four weeks, and were randomized on a 1:1 basis. Fatigue scores measured with the fatigue severity scale (FSS) after four weeks compared to baseline did not differ between groups. However, significantly more patients in the anakinra group had a fatigue reduction of more than 50% when using the VAS fatigue scale ($p=0.03$). This study suggests anakinra could be effective for treating fatigue in pSS, although the study was probably underpowered to detect significant changes.

Cryopyrin-associated periodic syndrome

In cryopyrin-associated periodic syndrome (CAPS), a group of rare diseases with an estimated prevalence of 1 in 360 000 persons ⁽⁶²⁾, increased IL-1 β activity plays a crucial role. CAPS consists of three auto-inflammatory disorders: familial cold autoinflammatory

syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurologic cutaneous and articular syndrome (CINCA). These syndromes are all caused by a mutation in the NLRP3 gene encoding cryopyrin, a protein which is responsible for inflammasome activation ⁽⁶³⁾. Different stimuli, for example, cold temperature in FCAS, can lead to cryopyrin production in these patients, causing a systemic inflammatory response mainly caused by IL-1 β . FCAS, MWS and CINCA are all characterized by intermittent episodes of fever, headache, urticarial rash, and arthralgia ⁽⁶⁴⁾. Although these symptoms are typically present during exacerbations, overall quality of life is also significantly affected and fatigue is reported by more than 75% of FCAS patients ^(65, 66).

The influence of blocking IL-1 on disease severity and fatigue was assessed in several studies. It should be noted that the MWS and CINCA patients tend to have sterile chronic meningitis, which probably results in inhibitors having greater entry into the brain ⁽⁶⁷⁾. Koné-Paut et al. assessed the influence of treatment with canakinumab in 35 CAPS patients ⁽⁶⁸⁾. At baseline mean FACIT-F scores for the whole group were 27.4; after 8 weeks of treatment the score increased to 40.6, which is a significant decrease in fatigue ($p<0.05$). Symptoms of fatigue, as rated by the physicians, were already absent in more than 85 percent of patients after eight days of treatment. In the second part of the study, patients were randomized to either canakinumab or placebo. In those patients randomized to placebo, fatigue recurred. In another study, the influence of canakinumab on fatigue was assessed in seven pediatric CAPS patients ⁽⁶⁹⁾. At several time points physicians scored fatigue severity using a 5-point scale. At baseline fatigue was reported to be severe in two patients, moderate in three patients and mild in one patient. After one day of treatment, fatigue was absent in five patients and minimal in two patients and this effect was maintained until the next relapse of fever.

The effect of rilonacept (Regeneron), a soluble IL-1 decoy-receptor construct, was assessed in 47 CAPS patients in two sequential phase III studies ⁽⁷⁰⁾. In the first double-blind part of the trial, patients were randomized between weekly rilonacept or placebo for a duration of six weeks. In the subsequent second study, patients were treated with active drug for nine weeks, followed by another placebo-controlled period of nine weeks. Fatigue severity was measured using a 10-point rating scale by both patients and investigators. In both groups, fatigue decreased significantly during the first phase of the trial, with a larger decrease in the rilonacept group. In the third phase of the trial patients on placebo had a relapse of symptoms, while patients receiving rilonacept remained without fatigue.

The influence of anakinra on the development of symptoms in FCAS, was assessed in patients who were exposed to a cold challenge ⁽⁷¹⁾. In three patients, anakinra was given twenty-four hours and one hour prior to the challenge. None of the patients developed

acute symptoms which they developed without prior anakinra treatment. Although not measured objectively, patients reported less fatigue and increased well being, a feeling that lasted 48-72 hours after the second anakinra dose. In all of the described studies, the decrease in fatigue was accompanied by less inflammatory activity both clinically and biologically. These studies demonstrate the effect of IL-1 on clinical symptoms, and the fast improvement of these symptoms when IL-1, especially IL-1 β , is inhibited.

Sarcoidosis

In sarcoidosis, an inflammatory disease of unknown etiology, patients develop granulomas in involved organs. The lungs are affected most often, but extra-pulmonary manifestations are present in up to 30% of patients ⁽⁷²⁾. Young patients are most often affected, and symptoms usually resolve within 2-4 years. Even when in clinical remission of the disease, prevalence of fatigue is rather high. In a Dutch post-sarcoidosis cohort of 75 patients, 49% of patients reported severe fatigue, which was associated with psychological distress and reduced health status ⁽⁷³⁾.

To explore the involvement of pro-inflammatory cytokines in post-sarcoidosis patients with fatigue, 72 patients were included in a study by Korenromp et al. ⁽⁷⁴⁾. Patients were categorized as being fatigued based on a Checklist Individual Strength subscale fatigue (CIS-f) score ≥ 35 (n=34), or non-fatigued when the score was below 35 (n=38). Whole blood IL-1 α and IL-1 β production was measured after lipopolysaccharide (LPS) stimulation. In plasma, these cytokines were also determined in addition to IL-1Ra. No differences for these proteins could be found between groups. The contribution of IL-1 β was also assessed in 22 patients with active sarcoidosis compared to 22 controls ⁽⁷⁵⁾. Fatigue was measured using the Multidimensional Fatigue Inventory (MFI-20), and IL-1 β concentrations were determined before and after 11-15 minutes of cardiopulmonary exercise testing. Between patients and controls, there were no differences measured in IL-1 β concentrations. However, pre-exercise circulating IL-1 β concentrations in patients significantly correlated with fatigue severity in those patients who used immunomodulatory drugs (n=13). Thus, fatigue in sarcoidosis patients seems to be a consequence of treatment rather than of the disease itself. However the study population is too small to draw firm conclusions. The effect of IL-1 inhibition on fatigue severity in sarcoidosis patients has not been assessed.

Non-inflammatory illnesses

Diabetes Mellitus

During the past 3 decades, a large number of studies have documented a role of IL-1 β in Type 1 and Type 2 diabetes. IL-1 β causes selective pancreatic beta-cell toxicity, resulting in decreased insulin production ⁽⁷⁶⁾. Anakinra might be able to reduce this, disease-characterizing, islet inflammation in newly diagnosed type 1 diabetes patients ⁽⁷⁷⁾, but

probably has to be combined with T-cell targeting therapy to reach a maximal effect. The effect of anakinra on diabetes regulation was also assessed in type 2 diabetes ⁽⁷⁸⁾. After 13 weeks of treatment, patients needed less diabetes lowering drugs to obtain the same glycemic control. A similar positive response on glycemic control was established using an anti-IL-1 β antibody in type 2 diabetes ⁽⁷⁹⁾.

The interaction between peripheral inflammation and deregulation of central mechanisms was demonstrated in type 2 diabetic mice ⁽⁸⁰⁾. After administration of LPS or IL-1 β , diabetic mice had prolonged sickness behavior compared to controls. The mechanism for this diabetes-induced brain immune alteration is unclear, but it appears that diabetes has an effect on the IL-1 β counter regulation, as IL-1Ra did not increase after LPS administration in diabetic mice.

Both patients with type 1 and type 2 diabetes experience fatigue, although literature on this subject is scarce. In a recent study in 214 patients with type 1 diabetes, severe and persistent fatigue was present in 40% of patients ⁽⁸¹⁾. Diabetes appeared to be correlated with behavioral variables rather than with blood glucose concentrations. These results lead to the development of a behavior-based therapy to treat fatigue in type 1 diabetes ⁽⁸²⁾. Cavelti-Weder et al. assessed the efficacy of XOMA052, a monoclonal anti-IL1 β antibody, compared to placebo in 30 type 2 diabetes patients ⁽⁴⁾. Fatigue was reported by 53% of patients and significantly correlated to diabetes duration, but not to age, HbA1c, weight, body temperature, and C-reactive protein. After treatment for one month, fatigue decreased in the groups treated with moderate and high dosed XOMA052, whereas an increase of fatigue was seen in the low-dose and placebo groups.

Cancer

In cancer, fatigue is one of the most prominent symptoms during all stages of disease, leading to substantial impairment and disability. A recent study evaluated the prevalence of fatigue in patients with breast, prostate, colorectal, and lung cancer undergoing active treatment (n=2177) or who had survived cancer (n=515) ⁽⁸³⁾. Moderate-to-severe fatigue was reported by 45% and 29% of patients, respectively. The impact of fatigue on daily functioning in these patients is even greater than that of nausea or cancer-related pain ⁽⁸⁴⁾. The exact mechanism causing fatigue during and after cancer treatment is not clear, but it is suspected that proinflammatory cytokines, especially TNF- α and IL-1 β play an important role ⁽⁸⁵⁾. One of the major reasons for this suspected relationship is that chemotherapeutic agents are known to trigger IL-1 β release, as mentioned previously ⁽⁸⁶⁾. In the acute situation such cytokine release promotes survival, but during the course of anti-cancer treatment it is associated with a variety of manifestations of illness, including fatigue ⁽⁸⁷⁾. A systematic review evaluating the relationship between IL-1 and fatigue in different types

of cancer during and after treatment, could not prove IL-1 β concentrations to be significantly correlated to fatigue severity⁽⁸⁸⁾. Patients in different stages of disease were analyzed as one group, which could have influenced the results. It is known that different biological processes take place during treatment and in the post-treatment situation. However, fatigue could be associated with an increase in circulating IL-1Ra ($r=0.24$, $p<0.001$) in this review, thus probably pointing to IL-1 activity.

In addition to a possible effect of IL-1 during cancer treatment, IL-1 may also influence the persistence of symptoms after treatment. This was evaluated in a group of advanced cancer patients ($n=45$) and cancer survivors ($n=47$)⁽⁸⁹⁾. In both patient groups, IL-1Ra correlated with physical fatigue ($r=0.32$, $p=0.03$ and $r=0.24$, $p=0.10$, respectively). In cancer survivors, IL-1Ra not only correlated with physical fatigue, but also with mental fatigue ($r=0.35$, $p=0.02$). When comparing both groups, inflammatory markers were higher in patients with advanced cancer than in cancer survivors. Concentrations of circulating IL-1 β and/or IL-1 α were not determined.

Prostate cancer

A possible relationship between IL-1 and fatigue in patients treated for prostate cancer has already been addressed more than two decades ago⁽⁹⁰⁾. In this study, 15 men undergoing external beam radiation therapy for prostate cancer were evaluated for a period of eight weeks. Radiation therapy initiates an immunological response to stimulate tissue repair, which is accompanied by an increase in pro-inflammatory cytokines⁽⁹¹⁾. Patients reported on fatigue daily using a VAS. IL-1 β was determined in serum before start of therapy, and weekly thereafter. Both concentrations of IL-1 β and fatigue increased during treatment, with a maximum after four weeks of treatment. A correlation between these measurements was not determined. Although performed in a small number of patients, this study was the first study on this subject. More recently, other investigators evaluated inflammatory markers during radiation therapy in patients with breast ($n=28$) and prostate ($n=20$) cancer⁽⁹²⁾. Circulating IL-1 β increased significantly during treatment, although there was a large variation between patients, and there was no correlation between IL-1 β and fatigue severity. However, in a subset of 22 patients, IL-1Ra was determined, which did correlate with reported fatigue. In another study, a correlation between IL-1 β and fatigue was not found⁽⁹³⁾.

A study conducted in patients with prostate cancer evaluated the influence of single nucleotide polymorphisms (SNPs), which are associated with the production of pro-inflammatory cytokines. The study assessed the development of fatigue during androgen-deprivation therapy⁽⁹⁴⁾. Testosterone is suspected to modulate cytokine concentrations, especially IL-1 β , IL-6 and TNF- α . Variation in IL-1 β genotypes did not predict changes

in fatigue scores in the 53 patients evaluated. Interventions directed towards inhibition of IL-1 have not been performed in prostate cancer.

Breast cancer

Several studies have been performed in breast cancer patients undergoing radio- or chemotherapy. Geinitz and colleagues investigated the association between fatigue and cytokine concentrations during adjuvant radiotherapy in breast cancer patients⁽⁹⁵⁾. In accordance with prostate cancer patients undergoing radiotherapy, fatigue severity reached a maximum after four weeks of treatment, IL-1 β concentrations in serum did not change and did not correlate with fatigue severity. Another study examined potential predictors of fatigue before, during and after adjuvant therapy in 44 women after breast cancer surgery⁽⁹⁶⁾. Blood samples were collected before adjuvant therapy had started. Questionnaires were repeated during and after therapy. Before adjuvant therapy, higher IL-1 β concentrations predicted fatigue severity. During and after adjuvant therapy this association was no longer present, but cytokine concentrations were not determined during this period. Liu et al. measured fatigue and IL-1Ra in a group of 53 women diagnosed with breast cancer before and during chemotherapy⁽⁹⁷⁾. At baseline, IL-1Ra did not correlate with higher fatigue levels, and had no influence on changes of fatigue severity during treatment. The most recent study, performed by Schmidt et al., did find a small but significant influence of increased IL-6/IL-1Ra ratio after treatment, which could not be found for IL-1Ra levels ($r=0.25$)⁽⁹⁸⁾.

Besides experiencing fatigue during cancer treatment, breast cancer survivors up to two years after completing treatment also report more fatigue than healthy controls⁽⁹⁹⁾. This symptom may be due to the cytokine response initiated by tissue damage during the acute treatment phase and persists after several years. To investigate the contribution of proinflammatory cytokines to fatigue after treatment, Bower et al. compared 20 fatigued women with 20 women without fatigue between one and five years after breast cancer diagnosis⁽¹⁰⁰⁾. Fatigued women had significantly higher concentrations of IL-1Ra in serum ($p=0.006$), there were no differences for IL-1 β concentrations, which were below the detection limit in almost half of the patients. These observations were not confirmed in a study in 103 patients 1-3 months after treatment for breast cancer⁽¹⁰¹⁾. Bower et al. also evaluated *ex vivo* whole blood IL-1 β production after LPS stimulation in 10 fatigued and 15 non-fatigued breast cancer survivors at baseline, and after completion of the Trier Social Stress Test (TSST)⁽¹⁰²⁾. At baseline there were no differences with regard to IL-1 β production. However, after completing the TSST, fatigued patients had significant higher IL-1 β concentrations. These findings suggest a higher pro-inflammatory response to psychological stress in fatigued patients. Circulating IL-1Ra concentrations were determined by the same study group in 50 fatigued and non-fatigue breast cancer survivors and were found to

be significantly higher in fatigued patients ⁽¹⁰³⁾. Again, this finding was contradicted by a cross-sectional study evaluating IL-1Ra levels in 299 disease-free breast cancer survivors, who did not find any positive correlations between this marker and fatigue severity ⁽¹⁰⁴⁾.

The presence of SNPs in promoters of cytokine genes was also studied in breast cancer survivors (fatigued n=33, non-fatigued n=14). The presence of at least one cytosine nucleotide at the IL-1 β gene (rs16944), a common SNP in many diseases, was reported to be associated with fatigue ⁽¹⁰⁵⁾. However, in a larger cohort (n=302) this association could not be confirmed ⁽¹⁰⁶⁾.

Other types of cancer

In two other types of solid tumors, the involvement of IL-1 in the development of fatigue has been assessed. Orre et al. evaluated 92 fatigued testicular cancer survivors, compared to 191 non-fatigued survivors at a median of 11 years after diagnosis ⁽¹⁰⁷⁾. Cases had significant higher IL-1Ra concentrations than controls. Increased IL-1Ra concentrations significantly correlated with physical fatigue, although they explained only 4% of variance in logistic regression analysis. A study investigating IL-1 in 15 patients with uterine cancer before, during and after undergoing curative radiation therapy, failed to prove a correlation, as IL-1 concentrations remained below the detection limit during the whole study ⁽¹⁰⁸⁾. No distinction was made between IL- α and IL-1 β in this small pilot study.

In hematologic malignancies, a single study has been performed that assessed the correlation between fatigue and IL-1 and IL-1Ra in 54 patients with acute myeloid leukemia or myelodysplastic syndrome undergoing pretreatment evaluation ⁽¹⁰⁹⁾. IL-1Ra concentrations correlated with fatigue severity ($r=0.52$). Concentrations of circulating cytokines were higher in patients than in healthy controls.

The effect of IL-1 α inhibition, using a neutralizing antibody, on fatigue was determined in 16 patients with metastatic, treatment-resistant non-small cell lung cancer ⁽¹¹⁰⁾. Quality of life was assessed at baseline and after eight weeks of treatment using the European Organization for Research and Treatment of Cancer, Quality of Life Questionnaire (EORTC QLQ C-30). After eight weeks fatigue was reported to be less severe, although this difference was not significant probably due to the small patient numbers. A significant improvement of fatigue after blocking IL-1 α was seen in a large group of patients treated for metastatic colorectal cancer, in addition to improvement of appetite and a decrease in pain severity (personal communications) ⁽¹¹¹⁾.

Post-stroke fatigue

In patients who experienced a stroke, fatigue is reported by 29-77% of the population. The prevalence of fatigue is equally distributed over patients after ischemic stroke and those who had an intracerebral hemorrhage ⁽¹¹²⁾. With respect to inflammation, high levels of circulating IL-6 during the acute phase of stroke have been associated with poor outcome (odds ratio 3.1, 95% CI: 1.9-5.0); these data are derived from a large prospective study consisting of 844 patients ⁽¹¹³⁾. In subarachnoid hemorrhage patients, IL-6 concentrations can be lowered using intravenous anakinra infusion ⁽¹¹⁴⁾ and might prove to increase survival in future studies.

The relationship between post-stroke fatigue and inflammation was described by Ormstad et al., who included 45 patients after a first stroke in a longitudinal study ⁽¹¹⁵⁾. Serum samples were collected <24, 24-48, and 48-72 hours after stroke onset in 35, 7, and 3 of the 45 patients. IL-1 β and IL-1Ra were measured in available samples. Fatigue was measured using the Fatigue Severity Scale (FSS) up to 18 months after stroke. Directly after stroke, IL-1 β concentrations correlated with fatigue severity after six months ($r=0.37$, $p=0.015$); this correlation could no longer be found after 12 and 18 months. At 12 months however, a negative correlation between IL-1Ra in the acute phase and fatigue was found ($r=-0.38$, $p=0.013$), a correlation that was not present at 6 and 18 months. Age, gender, comorbidity and use of medication were not confounders for these associations. These results imply that the acute inflammatory response during stroke has an impact on the occurrence of fatigue in the chronic phase.

In a study of 39 stroke patients, the presence of a C allele at a SNP located in the promoter region of *IL1RN*, was related to the severity of post-stroke fatigue ⁽¹¹⁶⁾. The presence of a C allele in this region has been associated with lower IL-1Ra concentrations and higher concentrations of circulating IL-1 β ⁽¹¹⁷⁾. In this study patients were included within 72 hours of stroke onset, fatigue was assessed using the Fatigue Assessment Scale (FAS) at one or more time points (30-365 days after stroke). In patients with severe fatigue, a C/T or C/C genotype was significantly more present (88%) than in patients with moderate (57%) and low fatigue (24%, $p=0.03$). This small study is the only study performed in this field, and circulating cytokine concentrations were not determined.

Chronic fatigue syndrome

Chronic fatigue syndrome (CFS) is a condition of unknown origin that is characterized by the presence of severe fatigue for a duration of at least six months, next to several accompanying symptoms such as headaches, sore throat, muscle and joint pain ⁽¹¹⁸⁾. Over the past decades, CFS has been attributed to a range of different causes, but a unifying cause has not been found. Even if a distinct abnormality is found repeatedly, for example relative

hypocortisolism⁽¹¹⁹⁾, it is difficult to determine whether this is a causative factor or rather an epiphenomenon as a consequence of inactivity, depressive symptoms, sleep problems, etc. Perhaps more than any other chronic disease associated with fatigue, cytokines have been measured by several investigators. A relationship between IL-1 and fatigue severity has often been assessed. The studies reveal a large heterogeneity, not only with respect to patient characteristics, but also with respect to selection of controls and sample handling. In addition, there is a large variation in questionnaires used to measure fatigue dimensions and fatigue related symptoms. These issues make it difficult to draw reliable conclusions.

A systematic review focusing on circulating cytokines in CFS was published recently by Blundell et al.⁽¹²⁰⁾, who reviewed all studies published on this subject between the publication of the first CFS case definition in 1988⁽¹²¹⁾ to March 2015. All 38 studies measuring circulating cytokines in diagnosed CFS patients compared to controls were included. As mentioned earlier, there were large differences with respect to recruitment of controls, sample handling, and exclusion of concomitant diagnoses. IL-1 α was measured in 11 of the described studies, 27% of studies found increased concentrations, and 73% found no significant differences. IL-1 β was determined in 28 studies, with only 25% reporting increased concentrations, the other studies did not find any significant differences. One of the more recent studies included in the review also discriminated patients with a short duration of illness (≤ 3 years, n=52) from patients with a long illness duration (n=246) and controls (n=348)⁽¹²²⁾. It appeared that patients with a short duration of illness had significantly higher IL-1 α and IL-1Ra concentrations than controls. This was also found when comparing IL-1 β levels in patients with short versus long illness duration. IL-1 β appeared to be elevated in patients with a short duration and decreased in patients with a long illness duration (when compared to controls). After this extensive review of the literature by Blundell, three more studies on circulating cytokines were published⁽¹²³⁻¹²⁵⁾. A study by Rusell et al. also tried to discriminate between patients with different illness durations⁽¹²⁵⁾. Comparing IL-1 concentrations, no differences could be found, although it has to be noted that patients with a 'short' illness duration had been fatigued for a mean of seven years, which is longer than the study mentioned earlier. In the linear classification model however, IL-1 α appeared to have predictive value in recently ill adolescent patients. Hardcastle et al. compared severely ill, house-bound patients (n=19), to moderately ill patients (n=22)⁽¹²³⁾. Although groups are rather small, IL-1 β was significantly elevated in the moderately ill patients ($p=0.002$). There were no differences for IL-1Ra. The third study could not find any differences between patients and controls for either IL-1 β or IL-1 α in a group of 100 patients and 79 controls⁽¹²⁴⁾. We conclude from the literature that there is limited evidence for increased circulating IL-1 in CFS patients, although there might be a more pro-inflammatory pattern in those with a short illness duration⁽¹²²⁾.

The effect of physical exercise on circulating cytokines was discussed in a separate systematic review⁽¹²⁶⁾, although some of the studies discussed were also included in the review by Blundell⁽¹²⁷⁻¹³¹⁾. The conclusion of this review is that also after exercise of varying intensity, there are no consistent differences with respect to IL-1 β .

Another approach is to compare cytokine production capacity of PBMCs after stimulation between CFS patients and controls. An early study reported increased IL-1 β production after LPS stimulation in a small group of CFS patients (n=9) compared to controls (laboratory personal, n=7)⁽¹³²⁾. Swanink et al. recruited neighborhood controls and found the opposite: lower LPS-induced IL-1 β concentrations in patients (n=76) than in controls (n=69), with a large overlap between concentrations of cytokines⁽¹³³⁾. Lower IL-1 β and IL-1 α production after PHA stimulation was also reported by Mawle et al. in patients with a gradual onset of symptoms, no differences were observed when those with a gradual and acute onset analyzed together⁽¹³⁴⁾. A fourth study by Cannon et al., published in the same period, investigated IL-1 β production in women during different phases of the menstrual cycle⁽¹³⁵⁾. In controls, spontaneous IL-1 β production by PBMCs increased during the luteal phase, which already has been observed in healthy subjects many years ago⁽¹³⁶⁾. However this could not be found in CFS patients. One recent study reported no differences between CFS patients and controls⁽¹³⁷⁾.

IL-1 β production by PBMCs in relation to fatigue has also been studied during the acute phase of an infection⁽¹³⁸⁾ and in the phase of persisting symptoms⁽¹³⁹⁾. During the acute phase the IL-1 β concentration correlated significantly with fatigue-symptoms, however this relationship disappeared in the persistent phase. The perpetuation of fatigue symptoms in the absence of peripherally increased cytokine concentrations suggest that other, most likely central mechanisms, may be involved in persistent fatigue after an acute infection.

With the brain as the suspected target organ for immunological dysregulation in CFS, a limited number of studies measured cytokine concentrations in cerebrospinal (CSF) fluid of patients. The first study, performed in 1991 by Lloyd et al., found no differences in IL-1 β concentrations between patients and controls⁽¹⁴⁰⁾. Others had similar findings, and both IL-1 α and IL-1 β tended to be below the detection limit^(141, 142). A more recent study compared 32 CFS patients to 40 patients with multiple sclerosis (MS) and 19 controls⁽¹⁴³⁾. CFS patients had lower CSF concentrations of both IL-1 β and IL-1Ra compared to the MS and control group. When CFS patients were compared with MS patients only, IL-1 α levels were also significantly lower.

Instead of creating more insight into pathological mechanisms in CFS, the described studies tend to raise more questions with respect to the role of IL-1 in CFS. It could be that disturbances of IL-1 signaling are only present in certain groups, for example only in those patients with short illness duration or those who experience fatigue after an infection, instead of when all patients are considered together. One possible way to elucidate the role of IL-1 in CFS, is to investigate the effect of blocking IL-1 on fatigue severity in CFS patients ⁽¹⁴⁴⁾.

Conclusions

In this review, we first described the mechanism by which IL-1 is able to influence certain brain regions, thereby leading to the development of fatigue. Next, we reviewed the literature describing studies where i) fatigue was correlated to IL- α , IL-1 β , or IL-1Ra activity, or ii) the effect of lowering IL-1 concentrations on fatigue severity was measured. In addition to inflammatory diseases such as CAPS, we also focused on non-inflammatory diseases characterized by profound fatigue, such as several malignancies and CFS. There might be a distinctive underlying mechanism causing fatigue in inflammatory disorders, compared to the other groups of fatigue causing illnesses. In inflammatory diseases fatigue often has an acute pattern, however in subgroups of patients fatigue persists even when the inflammation phase has subsided.

It can be concluded that there is no solid evidence that increased concentrations of circulating IL- α and IL-1 β are associated with fatigue in any of the diseases described. This is not surprising, given the fact that circulating concentrations of these cytokines usually are very low, as discussed previously ⁽¹²⁾. However, IL-1Ra seems to be correlated with fatigue in some diseases, for example in cancer. However, in each of the studies described in this review, but especially in CFS, studies are rather contradicting. For a large part this can be caused by the fact that there is a large heterogeneity between studies. Selection of controls and sample handling, which is known to be very important when measuring cytokines, differed significantly between studies or was not described ⁽¹⁴⁵⁾. Furthermore, studies differed with respect to questionnaires used to measure fatigue, the presence of comorbid diseases, use of medication in the patients studied, sample size, time since onset of the disease, duration of the fatigue (acute versus chronic) and the presence or absence of inflammatory processes.

For blocking IL-1 activity, most of the currently available inhibitors do not reach effective concentrations in the brain when the blood brain barrier is intact. This particularly is the case for the large molecular inhibitors (like canakinumab and rilonacept). For anakinra, which has a smaller molecular weight of 17kDa, the available pharmacological data show that the drug is able to reach the CNS after peripheral administration, although it is not clear if the local concentration in the CNS is high enough to have a substantial influence on neural processes ^(146, 147).

In diseases such as rheumatoid arthritis ⁽²⁾ and Sjögren's ⁽³⁾ blocking IL-1 using anakinra reveals promising effects on fatigue. In addition specific inhibition of either IL-1 α ⁽¹¹¹⁾ or IL-1 β ⁽⁴⁾ also has a positive influence on fatigue severity. Unfortunately, the majority of the studies were not randomized controlled trials ^(2, 4), or were most likely underpowered

to detect significant effects⁽³⁾. If IL-1 blockade effectively diminishes fatigue, the question of course remains whether this is a direct effect on central fatigue, whether the effect on fatigue is due to inhibition of inflammation, or whether IL-1 blockade directly affects central neurotransmitter systems. Also, it is important to determine if the positive effects of IL-1 blockade are limited to acute fatigue or are also present in patients who report persistent fatigue without evidence of being ill. Especially in this last group, persistent fatigue may involve maintenance of alterations in central brain systems, potentially triggered by acute inflammation.

With regard to future studies it is our hope that these will be performed in more controlled settings, which will make it easier to draw conclusions and to establish whether fatigue should or should not be added to the growing list of diseases in which blocking IL-1 is effective⁽¹⁴⁸⁾.

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Chapter 3

Perispinal injection as a method to access the central nervous system

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Abstract

Introduction: Alzheimer's disease is a debilitating condition, and the search for an effective treatment is ongoing. Inflammation, in reaction to amyloid deposition, is thought to accelerate cognitive decline. With tumor necrosis factor alpha being an important pro-inflammatory cytokine, a recent trial investigated the effect of the tumor necrosis factor alpha inhibitor etanercept after peripheral administration in Alzheimer's patients. Although there was no significant effect, others have claimed spectacular effects of etanercept after perispinal injection. In the present study, the central delivery of drugs with a large molecular weight was evaluated after injection in the cervical perispinal region. When successful, this might increase therapeutical options for Alzheimer's disease.

Methods: Nine male Sprague-Dawley rats were injected with Iodine-125 labeled cetuximab (146 kDa), etanercept (51 kDa) and anakinra (17 kDa). Each radioiodinated drug was injected in two rats into the perispinal region, and in one rat into the dorsal tail vein. Directly after injection, the rat was placed in a head down position for 3 minutes, to direct blood flow into the valveless vertebral venous system. A single-positron emission computed tomography scan was acquired starting 5 minutes after injection, subsequently the rats were euthanized and bio-distribution was determined.

Results: Intracranial delivery of the radiolabeled drugs could not be visualized in all but one of the rats. Injected drugs accumulated locally in the perispinal region.

Conclusions: In this study, no evidence could be found for the delivery of drugs to the central nervous system after perispinal injection. Additional research is needed before this treatment can be used in patients with Alzheimer's disease.

Introduction

During the past few years, there has been an increase in the use of targeted therapies for different kinds of inflammatory disorders. Most of these drugs, for example, the tumor necrosis factor alpha (TNF- α) inhibitor etanercept, have a high molecular weight, which prohibits them from passing the blood-brain barrier (BBB). This is no problem when treating diseases such as rheumatoid arthritis, but it becomes an obstacle when the brain is the primary focus of inflammation. The latter is the case in brain injury in Alzheimer's disease (AD). It is suspected that inflammation, as a consequence of amyloid deposition, plays an important role in the cognitive decline in AD ^(1,2), and that the intensity of this inflammatory reaction influences the speed of cognitive decline in individual patients ⁽³⁾.

TNF- α is a proinflammatory cytokine known for its activity in several disease conditions ⁽⁴⁾. It plays an important role in immune-to-brain communication ⁽⁵⁾, and increased TNF- α is associated with rapid neurocognitive decline ⁽⁶⁾ and neuropsychiatric symptoms ⁽⁷⁾ in AD. The effect of peripheral inhibition of TNF- α in AD has been assessed recently in a trial evaluating the effect of etanercept and placebo in 41 AD patients ⁽⁸⁾. Although the drug was well tolerated, there were no significant effects on cognitive functioning and behavior. However, earlier uncontrolled studies by Tobinick et al., claim benefit from perispinal administration of etanercept in AD ^(9,10) and post-stroke patients ⁽¹¹⁾. If true, these results, which were heavily criticized by Whitlock ⁽¹²⁾, would open up potential new treatment avenues.

The first question to be asked is whether there is a causal relationship between increased TNF- α activity and cognitive decline in AD. As mentioned above, the role of inflammation in AD has not been fully elucidated at this moment. Therefore, TNF- α inhibition as a strategy in this category of patients is questionable. As said, a recent phase II study using peripheral etanercept administration in patients with AD was ineffective ⁽⁸⁾. Still, this lack of an effect could have been caused by insufficient inhibition of TNF- α in the central nervous system (CNS) after peripheral administration. This leads to the second question, which is: Does perispinal administration of drugs with a high molecular weight lead to adequate concentrations in the brain? Etanercept does not cross the blood BBB after peripheral administration because of its high molecular weight (51 kDa). Tobinick searched for a method to bypass the BBB, without having to use more harmful methods of administering drugs, and relies on the vertebral venous system (VVS) to accomplish this ⁽⁹⁾.

In humans, the musculature and the (sub)cutaneous tissues of the back and neck are drained by the external vertebral venous plexus (EVVP) ^(13,14). The EVVP connects with the internal vertebral venous plexus (IVVP), and both plexuses connect with the basivertebral veins. These

three venous entities represent the vertebral venous system (VVS), also known as Batson's plexus, which is thought to be valveless and forms a separate and discrete venous network, paralleling, joining and at the same time bypassing, the longitudinal veins of the thoraco-abdominal cavity. The VVS also connects with the intracranial basilar venous plexus and the intracranial dural sinuses ⁽¹⁵⁾. In nonhuman primates, Batson injected radiopaque material into the deep dorsal vein of the penis, and demonstrated the existence of a connection between the pelvic venous plexus with the VVS; he also showed that the contrast medium can be redirected into the intracranial venous sinuses after compression (and subsequent temporary obstruction of the inferior vena cava) of the abdomen ⁽¹³⁾. With this study, he found an explanation for the observation that metastases of retroperitoneal cancers in humans preferably tend to distribute to the vertebral skeleton and spinal epidural space, and via the cranial sinuses into the brain. Based on these findings and assumptions, Tobinick designed his concept for drug delivery into the brain ⁽⁹⁾. He assumed that, after injection into the perispinal soft tissues, and subsequently placing the patient in the Trendelenburg position, the retrograde flow within the VVS would result in the delivery of the medication, through the cranial veins, into the brain. Tobinick et al. have performed and published only one single experiment in rats, visualizing the intracranial distribution of etanercept labeled with the positron emitter Cu-64 ⁽¹⁶⁾, and speculate about the mechanism without providing further proof ⁽¹⁷⁾. Positron emission tomography (PET) studies in healthy controls or patients have not been performed.

In this brief article, the methods published by Tobinick were explored by injecting drugs with a large molecular weight to the cervical perispinal region of Sprague-Dawley rats. As this technique is claimed to be safe and an effective way to administer drugs to the CNS, it could provide a new treatment option for patients with Alzheimer's disease.

Methods

Preparation of radio-labeled drugs

Cetuximab 5 mg/mL (Erbix®[®], Merck, Darmstadt, Germany), etanercept (Enbrel®[®], Pfizer, New York, United States) and anakinra 149 mg/ml (Kineret®[®], Swedish Orphan Biovitrum AB, Stockholm, Sweden) were dialysed against 50 mM phosphate buffer, pH 7.4 and radiolabeled with I-125 (PerkinElmer). For radioiodination the iodogen method was applied. Briefly, 100 µg cetuximab, 300 µg etanercept and 300 µg anakinra were incubated in an iodogen-coated tube (100 µg) with 50-72 MBq I-125 in phosphate buffer, pH 7.4 during 10-60 minutes. Labeling efficiency was 68-90% and the radioiodinated products were purified by gel filtration on a PD-10 column (GE) that was eluted with PBS, 0.5% BSA. The radiochemical purity (RCP) was determined by instant thin layer chromatography (ITLC) and exceeded 95% for all preparations.

Animals and procedures

All animal procedures were conducted in accordance with the Radboud Animal Welfare guidelines. Nine male Sprague-Dawley rats with an average weight of 250 g were anesthetized with 2.5-3% isoflurane inhalation anesthesia, and injected with 150 µL I-125-labeled cetuximab (146 kDa), etanercept (51 kDa) and anakinra (17 kDa). Drugs with different molecular weights were selected to determine the effect of molecular weight on central delivery. For each drug, two rats were injected in the area overlying the cervical spine at the C6-7 level using a 30 gauge needle at a depth of 6 mm, as described by Tobinick et al. ⁽¹⁶⁾. One rat was injected in the dorsal tail vein followed by flushing with 1ml of saline. Directly after injection, the rats were placed in head down position for 3 minutes. Five minutes after the injection, a single-positron emission computed tomography scan (SPECT) was performed using a microSPECT/CT scanner (U-SPECT II scanner, MIlabs, Utrecht, the Netherlands). After completing the scan, which took 20 minutes, all rats were euthanized and bio-distribution was determined.

Results

SPECT imaging could not reveal intracranial accumulation of the radioiodinated drugs in all but one of the animals (*figure 1a*). This animal, the first rat that was injected, died during acquisition of the SPECT-scan and distribution of the tracer was compatible with the anatomical margins of the ventricular system and the intraspinal CSF-space. In the other animals, radiolabeled drugs could only be detected in the injection region (*figure 1b*), without any sign of penetration into the central nervous system.

The biodistribution of the radiolabeled drugs is summarized in (*table 1*), in which the uptake in the heart, blood, brain, and perispinal injection region is specified. Except for the first rat, deposition of drugs in the brain was ≤ 0.10 %ID/g. After injection in the dorsal tail vein, the average distribution was higher in blood and in the heart. After perispinal injection, the drugs accumulated locally, and did not reach the brain.

Figure 1 In vivo distribution of I-125 labeled Cetuximab after perispinal injection using SPECT

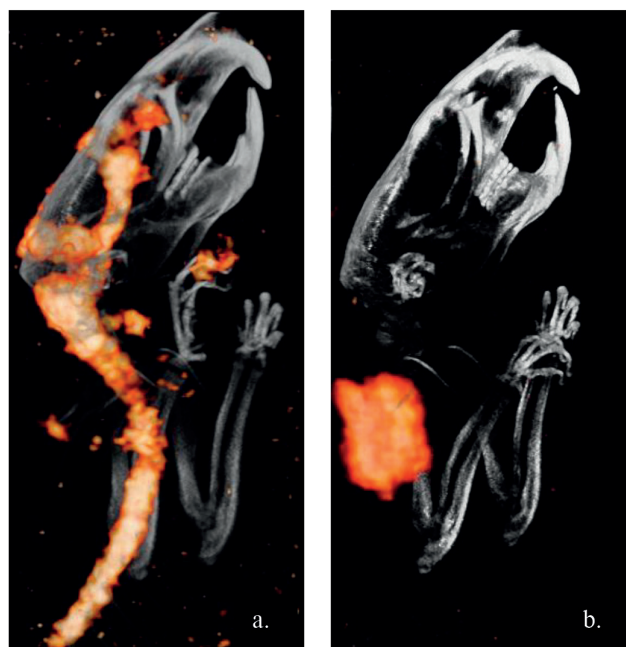


Figure 1 Biodistribution of I-125 Cetuximab in the animal in figure 1a suggested injection to be intrathecal, instead of perispinal. The animal died during acquisition of the SPECT scan. The biodistribution as displayed in figure 1b is in line with images of the other four perispinal injected animals. The radiolabeled drug in these animals was only found at the injection site.

Table 1 In vivo distribution of I-125 labeled drugs measured in blood, heart, brain, and the injection regions.

	AC* (%ID/g) Blood	AC (%ID/g) Heart	AC (%ID/g) Brain	AC (%ID/g) Perispinal region	AC (%ID/g) Dorsal tail vein
Cetuximab					
<i>perispinal</i>					
Rat 1	2.06	0.39	4.05	0.06	-
Rat 2	0.19	0.05	0.01	17.15	-
<i>dorsal tail vein</i>					
Rat 3	5.24	1.61	0.10	-	0.46
Etanercept					
<i>perispinal</i>					
Rat 4	0.14	0.04	0.01	1.10	-
Rat 5	0.10	0.03	0.01	0.93	-
<i>dorsal tail vein</i>					
Rat 6	2.98	0.91	0.08	-	0.20
Anakinra					
<i>perispinal</i>					
Rat 7	0.30	0.11	0.06	10.86	-
Rat 8	0.16	0.07	0.02	7.31	-
<i>dorsal tail vein</i>					
Rat 9	0.37	0.19	0.04	-	0.15

Table 1 *AC: average concentration

Discussion

In this study, the previous claim that radiolabeled drugs accumulate in the brain after perispinal injection^(11, 16, 18), could not be replicated. In only one of the rats, deposition of I-125 labeled cetuximab in the brain regions compatible with the intraventricular system could be detected. In the other animals, drugs accumulated locally at the injection site.

The first rat that was injected died in the SPECT-scanner (< 20 min after administration). In the same rat, accumulation of the tracer in the perispinal region was very low compared to the other rats (0.06%ID/g), which supports the suspicion of a “false route” during injection. When, according to the protocol, the drug is blindly injected in the perispinal region at a depth of 6 mm, there is a high change of injecting intrathecally. In this study, drugs had to be administered carefully to prevent this misrouting. In the experiment by Tobinick et al., injection might have been directly into the intrathecal space, and not into the perispinal soft tissues. As such, the recommendations by Tobinick et al. to use the VVS as a delivery-route for drugs into the central nervous system lacks any scientific basis.

Conclusion

In conclusion, we believe that there is a lack of proof that perispinal injection of drugs like etanercept would lead to effective concentrations in the brain. Before this technique can be recommended for treatment in patients with AD and other neurological diseases, proof of concept is needed. However, our present experiments falsified the claim by Tobinick et al. about the vertebral venous system being an anatomical route to bypass the blood-brain-barrier and to deliver high molecular drugs to the central nervous system. Based on the literature, and the now available new data, we feel that there is even insufficient basis to propose an RCT at this point.

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Chapter 4

Cytokine inhibition in Chronic Fatigue Syndrome patients: Study Protocol for a Randomized Controlled Trial

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Abstract

Background: Chronic fatigue syndrome (CFS) is a medically unexplained syndrome for which no somatic or pharmacological treatment has been proven effective. Dysfunction of the cytokine network has been suspected to play a role in the pathophysiology of CFS. The disturbances of the cytokine network detected in CFS patients are highly variable, in part due to the lack of adequate controls in many studies. Furthermore, all studies have been performed on peripheral venous blood of patients. As cytokines mainly act in the tissues, for example, the brain, the information that can be derived from peripheral blood cells is limited. The information regarding the possible role of cytokines in the pathophysiology, could come from intervention studies in which the activities of relevant cytokines are reduced, for example, reducing interleukin-1, interleukin-6 or tumor necrosis factor. In this study the clinical usefulness of anakinra, an IL-1 antagonist, will be assessed in patients with CFS.

Methods/design: A randomized placebo-controlled, double-blind trial will be conducted. Fifty adult female patients meeting Centers for Disease Control (CDC) criteria for CFS, and without psychiatric co-morbidity will be included. After inclusion, patients will be randomized between treatment with anakinra (recombinant human interleukin-1 receptor antagonist) or placebo. Each group will be treated four weeks. Outcome measures will be assessed at baseline, after four weeks of intervention, and six months after baseline assessment. The primary outcome measure will be fatigue severity measured with the validated Checklist Individual Strength (CIS). Secondary outcome measures are functional impairment, physical and social functioning, psychological distress, pain severity, presence of accompanying symptoms, and cytokine and cortisol concentrations.

Discussion: This is the first randomized placebo-controlled trial that will evaluate the effect of interference with IL-1 on the experience of fatigue in patients with CFS. The results of this study may expand treatment options for patients with CFS, for whom at this moment graded exercise therapy and cognitive behavioral therapy are the only evidence based interventions.

Background

Chronic fatigue syndrome (CFS) is a medically unexplained syndrome characterized by severe disabling fatigue for a period of at least 6 months, which leads to considerable impairment in daily functioning ⁽¹⁾. Various accompanying symptoms may be present, such as headache, joint and muscle pain, sore throat, impaired memory and concentration, and exercise intolerance. In the Netherlands the prevalence of CFS is at least 27.000 persons ⁽²⁾. So far, the cause for CFS is unclear ⁽³⁾. Cognitive behavioral therapy (CBT) and graded exercise therapy (GET) are the only interventions that have shown positive results in randomized controlled clinical trials for treating fatigue associated CFS-symptoms and disability ⁽⁴⁻⁸⁾.

Cytokines are hormone-like proteins that convey messages between cells. Originally they were thought to act only within the host defence system, but soon it became clear that they mediate an array of diverse effects in normal physiology and disease. Since pro-inflammatory cytokines play a key role in inflammation (e.g. by causing fever, inducing muscle pain, fatigue, sleep, and other flu like symptoms), they have been hypothesized to be responsible for the symptoms in CFS ^(9,10).

Several studies have been performed to investigate whether there is an excess of cytokines in CFS, but so far the findings are inconsistent ^(11, 12). A recent systematic review on circulating cytokines in CFS reported that the majority of studies performed during the past years did not find significant increased concentrations of proinflammatory cytokines ⁽¹³⁾. A major problem is that many studies did not use adequate controls, and use different methods to handle blood samples. Cytokine responses are under genetic control, but they are extremely vulnerable to other influences, such as hormonal status, food, exercise, stress, behavior, drugs, and vaccines ⁽¹⁴⁾. Therefore, it is not easy to compose a good control group. An additional problem is that almost all studies have been performed on peripheral venous blood. As cytokines mainly act in tissues, with the brain being the most important target organ in CFS, information that can be derived from studying circulating cytokine concentrations (which are generally in the picogram/ml range) is limited. The only information regarding a role of cytokines that is pathophysiologically relevant could come from intervention studies in which crucial cytokines in tissue are inhibited. A potentially relevant cytokine, which can be blocked in humans without severe side effects is interleukin-1 (IL-1) ⁽¹⁵⁾.

Although it is plausible that cytokines play a role in the pathophysiology of CFS, there is only indirect evidence for this theory:

1. The complaints of patients with CFS are often described as that of a persistent flu. During infections like influenza, symptoms are generally ascribed to the action of cytokines (like IL-1, IL-6, tumor necrosis factor alpha (TNF) and interferons) ⁽⁹⁾.
2. Many disease states are accompanied by anorexia, loss of interest, somnolence and fatigue, a symptom complex coined as sickness behavior. The cytokines IL-1beta, TNF and IL-6 are thought to be responsible for it. Administration of either IL-1, IL-6, TNF or each of the interferons to humans and animals is accompanied by flu-like symptoms ⁽¹⁶⁻¹⁸⁾.
3. Previously, it was reported that IL-8 and IL-10 were significantly elevated in cerebrospinal fluid in patients with CFS, compatible with induction of IL-1 ⁽¹⁹⁾.
4. Beta amyloid precursor protein has also been found to be elevated in cerebrospinal fluid of CFS patients ⁽²⁰⁾. Production of this protein is under control of IL-1 and TNF ⁽²¹⁻²³⁾.
5. Our group has previously established that patients with CFS have a significant loss of grey matter in the brain ^(24, 25). This loss of grey matter might be caused by enhanced cytokine activity.

Drugs that interfere with the proinflammatory cytokine IL-1 are commonly used nowadays for a variety of inflammatory disorders ⁽²⁶⁾. The recombinant IL-1 receptor antagonist (anakinra) reduces the activity of both IL-1 α and IL-1 β by binding to the IL-1 receptor. This intervention is highly targeted and hence would allow investigators to draw conclusions regarding the pathophysiology of CFS, and the effect of reducing cytokine concentrations in CFS patients. Moreover, compared to blocking TNF- α or IL-6, blocking IL-1 with anakinra has a long safety record with respect to side-effects, and is not associated with increased susceptibility to opportunistic infections such as *Mycobacterium tuberculosis*.

The primary aim of this study is to assess the effect of anakinra on fatigue severity in patients with CFS. Fatigue is the most central and characterizing symptom of CFS and, in contrast to the accompanying symptoms, it is reported by all patients. It is also strongly related to the functional impairments reported by patients. As a secondary study aim, we will assess the effect of anakinra on level of functional impairment, physical and social functioning, pain severity, presence of accompanying symptoms and psychological distress. In this paper we describe the protocol to evaluate the effects of anakinra. Other studies with anakinra or anti-IL-1 β revealed a decrease in fatigue ⁽²⁷⁻³⁰⁾.

Methods/design

Study design

A randomized placebo-controlled trial (RCT) will be performed to determine whether interference with IL-1 is able to reduce fatigue and disabilities in CFS patients. Within each study arm, treatment will be double blind. The study will be performed in the Radboud University Medical Center, in the Department of Internal Medicine and in the Expert Center for Chronic Fatigue (ECCF), located in Nijmegen, the Netherlands. All female CFS patients visiting the outpatient clinic of the department of Internal Medicine or visiting the ECCF, will be considered for participation. Furthermore, patients connected to the 'ME/CVS-stichting', a Dutch foundation for CFS patients, will be asked to participate in the study. To increase homogeneity in our study population, we decided to only include female patients, as CFS is a disease that mostly affects women. After inclusion, each patient will receive an individual study code. Patients will be asked to bring a healthy, age-matched, neighborhood control at their first study visit. If patients decide not to participate in this study, an attempt will be made to elucidate the reason for this, but patients are not obligated to explain their refusal.

Study population

Fifty subsequent patients will be included and equally randomized between treatment arms. Inclusion criteria for participation comprise the CDC diagnosis of CFS ⁽³¹⁾, fatigue duration ≤ 10 years or recent progression of fatigue severity, female gender, age between 18 and 59 years old, a score ≥ 40 on the subscale fatigue severity of the Checklist Individual Strength (CIS), and a score ≥ 700 on the Sickness Impact Profile (SIP). The exclusion criteria include females who are pregnant or nursing, intend to get pregnant during the study, use or have used psychotropic medication in the past month, received an alive vaccine during the last four weeks, had substance abuse in the past 3 months, have symptoms more than 10 years, are taking any medication except oral contraceptives and/or paracetamol, have evident somatic co-morbidity, have current engagement in CFS research, do not have the ability to understand the nature and the extent of the trial and the procedure required, have psychiatric co-morbidity (major depression, psychosis, eating disorders, anxiety disorders, bipolar disease and post traumatic stress disorder) assessed with 'The Mini-International Neuropsychiatric Interview' (M.I.N.I.) ⁽³²⁾, and are currently engaged in a legal procedure with respect to disability claims.

Ethical approval

The study is approved by the Medical Ethical Review Committee of the RadboudUMC Nijmegen (registration number 2014/025). The study is registered at the European Union Drug Regulating Authorities (EudraCT: 2013-005466-19) and will be conducted according

to the Declaration of Helsinki, Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP) guidelines. The inclusion of patients started in July 2014. Written informed consent will be obtained from all patients before participating in the trial.

Study medication, randomization and follow-up

Eligibility for participation of patients is determined at the pre-study visit. After giving informed consent patients will be screened for in- and exclusion criteria by means of the examinations listed in *figure 1*. Laboratory investigations performed earlier will be evaluated for all patients and will be repeated if not performed recently, or when essential measurements, as recommended by others ⁽³¹⁾, are missing. Patients who qualify to be included will be randomized 1:1 to receive either anakinra (100mg/day) or placebo. Randomization will be performed by the study pharmacist (department of Clinical Pharmacy, Radboud University Medical Center), the randomization assignment is known only by the pharmacist and will only be exposed in case of emergency. If the code is broken, it will render the participant not eligible. When the study is completed, the randomization list will be made available by the study pharmacist.

Study medication will be provided by the Swedish Orphan Biovitrium (Sobi) and stored at the Department of Pharmacy of the RadboudUMC. Preparation and labelling of anakinra and placebo will be done according to the current guidelines. This will be performed by the Clinical Trials Unit department of Clinical Pharmacy of the RadboudUMC. Anakinra and placebo syringes will be identical in appearance, the placebo syringes contain a mixture of sodium citrate, sodium chloride, and polysorbate. Medication is used once daily, during a period of four weeks. Anakinra and placebo will be provided by the main investigator. On the first study day, patients will be instructed how to self-inject the study medication, as described by others ⁽²⁷⁾. Administration takes place in the subcutaneous tissue, most often the abdomen or the thighs. During the study, patients will be advised to set their alarm clock daily at the same time to remind them of using the medication correctly. Drug adherence will be evaluated after one week and after completion of treatment. Patients will return all used and unused syringes after four weeks.

During the intervention period, use of co-medication is only allowed when used for ≤ 14 consecutive days, on the condition that there are no known interactions with anakinra. Oral contraceptives and/or paracetamol can be used without a limitation. During the follow-up period, there are no limitations regarding the use of medication. All co-medication will be registered and reported afterwards.

Since the anakinra arm is difficult to blind because of local reactions at the injection site, the research physician and the principal investigator will not be informed of the side effects.

After the injection instruction by the physician assistant (PA), the patient will be instructed to report adverse effects to the PA and not to the research physician. The PA will report all side effects to an independent physician. The independent physician will examine patients if needed and is mandated to stop treatment. To evaluate blinding of treatment, patients will be asked which medication they thought they were using during the trial, after they have completed the study.

Study visits are carried out at week zero, week four and, if needed, after six months. After one week patients will be contacted by telephone to evaluate the occurrence of any problem regarding the use of medication and drug utilization will be recorded. If there are serious side effects an additional study visit can be performed by the independent physician at any time. Between study visits, subjects will be asked to fill out web-based questionnaires up to six months after their first study visit (*figure 1*).

Outcome measures

The primary outcome measure is the fatigue severity measured with the subscale fatigue severity of Checklist Individual Strength (CIS) at four weeks, the primary endpoint of the study. Scores on the CIS subscale range from 8-56 (8 items, 7-point Likert Scale).

This questionnaire has been validated extensively in patients with chronic fatigue syndrome ^(33, 34). Patients will fill out this web-based questionnaire weekly during the course of the trial, and monthly during the follow-up period (*figure 1*).

Secondary outcome measures

Level of *functional impairment* measured with the Sickness Impact Profile (SIP8) total score. The SIP8 is a validated instrument to evaluate sickness-related dysfunction. The total score gives an indication of the experienced disabilities in all domains of functioning ⁽³⁵⁾.

Physical and social functioning assessed with the subscale physical functioning and subscale social functioning of the Short Form (SF)-36 ⁽³⁶⁾.

Level of *psychological distress* assessed with the total score on the Symptom Checklist-90 (SCL-90). A high total score reflects psychological distress ⁽³⁷⁾.

Pain severity assessed with a visual analogue scale (VAS). This score can vary between 0 (no pain) and 10 (worst pain ever experienced).

Presence of *accompanying symptoms* will be evaluated using the CDC-criteria. The number of symptoms can vary between 0 and 8.

Cytokine concentrations in blood (plasma and blood in Pax-gene tubes). Our study can provide additional information regarding cytokine levels, because we have the opportunity to compare cytokine concentrations with healthy neighborhood controls. Also we will compare pre-treatment concentrations with post-treatment concentrations.

Cortisol concentrations in hair and saliva, because of the possible role of the hypothalamus-pituitary-adrenal axis in CFS. For the baseline assessment, comparison will be made with cortisol concentrations in matched neighborhood controls. All patients will collect saliva during two consecutive days at four different time points (directly after awakening, 30 minutes after awakening, 11 a.m., 8 p.m.), using the passive drool method. Saliva will be stored at -80°C until further analysis.

Microbiome determination in stools. This will give more insight into the microbial flora of the host, which is a new field of interest in a wide range of diseases. The availability of well-defined patients with CFS and matched controls is a great opportunity in an unexplored area of CFS research, to assess whether the microbiome of CFS patients is peculiar. Patients will collect feces at home, all samples will be stored at -80°C until further analysis.

All secondary outcome measures will be assessed at baseline and directly following the intervention (*figure 1*). Only the VAS pain scale will be filled in weekly (together with the CIS) during the trial.

At 6 months, all questionnaires evaluating primary and secondary outcomes will be repeated again to evaluate if the expected effects of the medication are maintained during the follow-up period of five months.

Other study parameters collected at baseline will be: demographic data, medical history, psychiatric history, serology results collected before inclusion in the study, use of medication, smoking and the use of alcohol and drugs.

Withdrawal of individual participants

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator or independent physician can decide to withdraw a subject from the study for urgent medical reasons. In general, non-completers are not to be replaced. Subjects withdrawn from the study for a medical reason or adverse events will receive adequate follow up. All analysis will be done according to the Intention to Treat principle (ITT). In case of discontinuation, efforts will be made to continue all study measurements. Withdrawn patients, or patients who are still severely fatigued following the intervention, will be offered regular care, which is cognitive behavioral therapy at the ECFE.

Adverse events

Adverse events (AE) are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded. Anakinra is known as a very safe drug. Side effects are mainly those related to irritation at the injection site, these reactions usually wane with prolonged treatment. We have reported from our own experience with long-term use of anakinra in patients with Schnitzler syndrome that the risk for infection is not enhanced, even in this elderly group⁽³⁸⁾. Nevertheless, the package insert warns against infectious complications in patients with underlying illness. Rarely, neutropenia develops during treatment, which is a reason to discontinue therapy.

Statistical analysis

The primary analysis will be the comparison between the two different treatment groups (anakinra or placebo) at 4 weeks. Data will be analysed on an ITT basis. Missing values will be replaced using multiple imputation with fully conditional specification with at least five imputations. The imputation method that will be used is predictive mean matching for missing data in primary and secondary outcome measures. Aside from condition we will use the following variables assessed at baseline to generate the imputations: duration of symptoms, age, BMI and baseline values of the outcome measures.

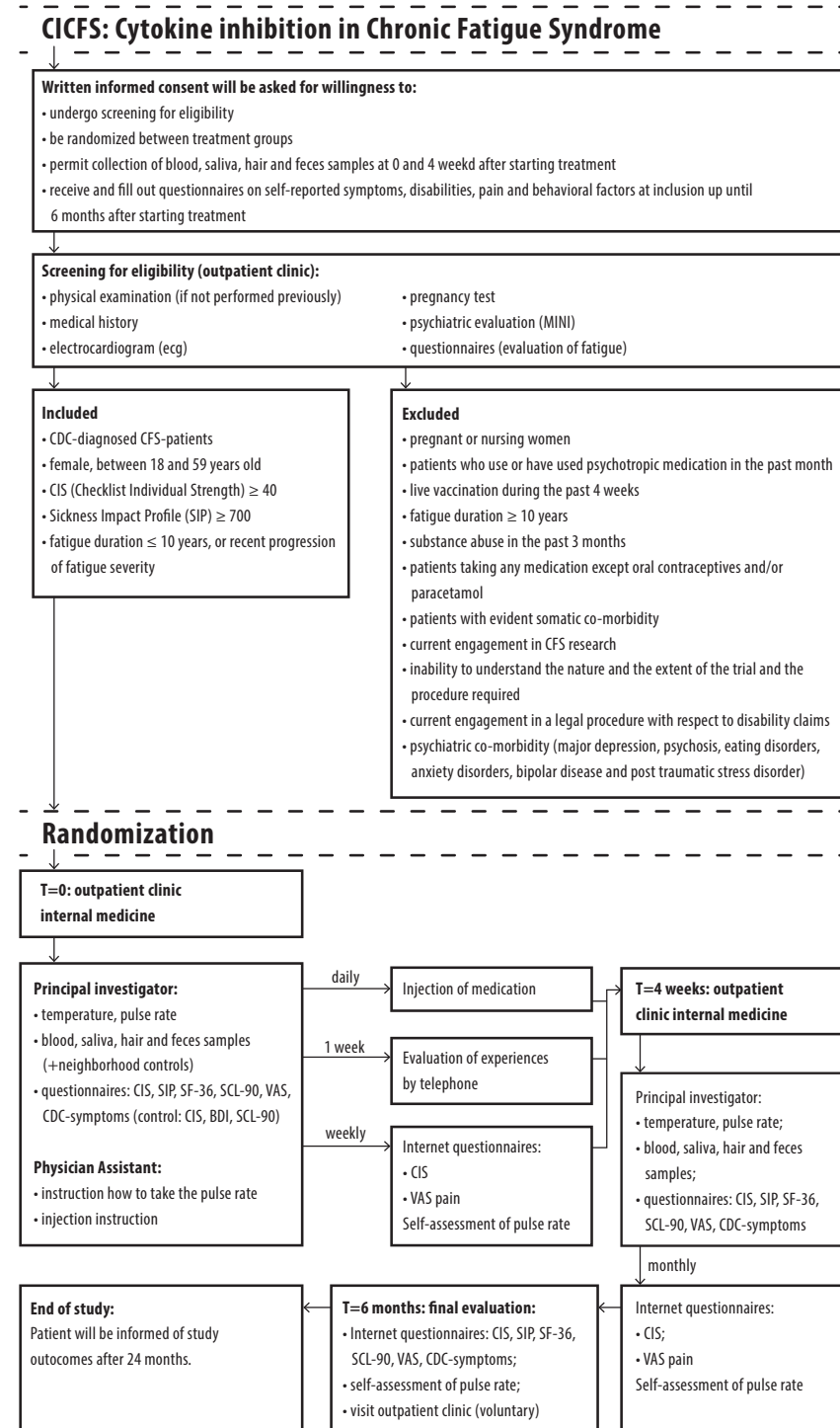
The results will be analysed with SPSS for Windows. To determine if there is a significant difference between the intervention arm and placebo condition, ANCOVA will be used with the outcome measure on the second assessment as dependent measure, the baseline score as covariate, and condition as fixed factor⁽³⁹⁾. We will test if significant differences exist between both groups in mean age and BMI at baseline, both known to influence circulating cytokine levels. If so, these variables will be entered as covariates in the ANCOVA. When a statistical significant difference is found in the primary analysis, a sensitivity analysis will be performed on the basis of different assumptions about the values of missing data. For the secondary outcome measures, the same analysis will be repeated, but now with the secondary outcome measures at second assessment as dependent variable, and the scores at baseline as covariate.

Power calculation

The sample size is based on the fatigue subscale score of the CIS at 4 weeks. In the present study the power calculation is based on results from comparable studies with CFS patients where fatigue severity was determined with the CIS-fatigue scale⁽⁴⁰⁾. If interleukin-1 plays a central role in CFS symptomatology, we expect a considerable reduction of fatigue following treatment. Assuming a large controlled effect size of 0.85, an alpha of 0.05 and a

power of 0.80, 23 patients are needed in each arm of the study. The number of patients can be further reduced by using ANCOVA, with the outcome measure on the second assessment as dependent measure, the baseline score as covariate, and condition as fixed factor. In this kind of trials ANCOVA yields greater power than other statistical methods⁽³⁹⁾. Based on the correlation between the pre- and post CIS fatigue scale, the sample size of 23 can be multiplied with 0.883 (1-0.342²). Including an assumed dropout rate of 20 percent, we will have to include 25 patients in each group to demonstrate a significant difference between the medication group and the placebo.

Figure 1



Discussion

This study will be the first randomized placebo-controlled trial to evaluate the effect of blocking IL-1 on symptoms in patients with CFS. Earlier studies investigated cytokine production to be of relevance in CFS patients, but conflicting results have been published^(11, 13). A possible explanation is that good controls have not been used in these studies, and cytokines were measured in peripheral blood instead of in tissues.

Blinding the study for anakinra is a difficult procedure, because of the occurrence of local skin reactions in a significant amount of patients. To maintain the double-blind design of this trial, side-effects will be evaluated by an independent Physician Assistant (P.A.). In earlier ‘double blind’ trials medication was injected in the presence of the main investigator⁽²⁷⁾. The effect of anakinra will be measured up to six months after the start of treatment. In this manner we can evaluate the long-term effect of blocking IL-1 for a short period. It will provide more insight into the best treatment for CFS patients, when blocking IL-1 appears to be effective.

In conclusion, this study will provide more insight into the pathophysiology and treatment of patients with CFS. If an effective treatment can be found, this will drastically improve quality of life in patients with this disabling disease.

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Chapter 5

Cytokine inhibition in patients with chronic fatigue syndrome: a randomized trial

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Abstract

Background: Interleukin-1 (IL-1), an important proinflammatory cytokine, has been suspected to play a role in the chronic fatigue syndrome (CFS).

Objective: To evaluate in female CFS patients the effect of subcutaneous anakinra versus placebo on fatigue severity.

Design: Randomized, placebo-controlled trial from July 2014 until May 2016. Patients, providers, and researchers were blinded to treatment assignment.

Setting: University hospital in the Netherlands.

Patients: 50 women, between 18 and 59 years old, with CFS and severe fatigue leading to functional impairment.

Interventions: Participants were randomized to daily subcutaneous anakinra (100mg/day, n=25) or placebo (n=25) for four weeks and followed for an additional 20 weeks off treatment (n=50).

Measurements: Primary outcome: fatigue severity measured by the Checklist Individual Strength (CIS-fatigue) subscale at four weeks; Secondary outcomes: level of impairment, physical and social functioning, psychological distress and pain severity at four and 24 weeks.

Results: At 4 weeks, 8% (2/25) of anakinra participants and 20% (5/25) of placebo participants reached a fatigue level within the range reported by healthy individuals. There were no clinically important or statistically significant differences between groups in fatigue severity at 4 weeks (mean difference CIS-fatigue score: 1.5 points; 95% CI -4.1 to 7.2) or at the end of follow-up. There was no statistically significant difference between groups for any secondary outcome at four weeks or the end of follow-up. One patient in the anakinra group discontinued treatment due to an adverse event. Patients in the anakinra group had more injection site reactions (68% (17/25) vs. 4% (1/25)).

Limitations: Small sample size, wide variability in duration of symptoms; inclusion not limited to patients with symptoms after an infection.

Conclusions: Peripheral IL-1 inhibition using anakinra for four weeks does not result in a clinically significant reduction of fatigue severity in women with CFS and severe fatigue.

Introduction

Chronic fatigue syndrome (CFS) is characterized by severe, persistent and disabling fatigue. Although different case definitions for CFS/myalgic encephalomyelitis exist, the Center for Disease Control (CDC) criteria are most often used in research. According to the CDC definition, patients with fatigue have to report at least four out of the following eight accompanying symptoms: post-exertional malaise, unrefreshing sleep, impairment of short-term memory or concentration, headache, muscle pain, tender lymph nodes, sore throat or arthralgia^(1,2). The pathogenesis of CFS is unknown. The only effective treatments are cognitive behavioral therapy and graded exercise therapy⁽³⁻⁵⁾ though a substantial proportion of patients do not improve after these therapies⁽⁶⁾.

The immune system has been a focus in the search for the pathogenesis of CFS because of the resemblance of CFS symptoms with ‘sickness behavior’, in which proinflammatory cytokines such as interleukin-1 α and β (IL-1 α and IL-1 β) and tumor necrosis factor play a role⁽⁷⁾. These findings are derived from studies in mice^(8,9) and humans⁽¹⁰⁻¹³⁾. After peripheral administration or production, the cytokines can reach the central nervous system and influence neurotransmitter systems⁽¹⁴⁾.

Several studies searched for elevated circulating concentrations of cytokines, particularly IL-1 and tumor necrosis factor in CFS⁽¹⁵⁻¹⁸⁾. Some studies found CFS patients had increased concentrations of IL-1 α (19, 20), IL-1 β (19, 21), and the IL-1 receptor antagonist (IL-1Ra)⁽¹⁵⁾, while others found no such increases⁽¹⁸⁾. Of note, measuring circulating cytokines is complex and sample handling is critical⁽²²⁾. Circulating concentrations, especially of IL-1 β , are often below detection. Most cytokines remain in the intercellular environment^(23,24). In CFS increased cytokine activity might be confined to the brain. This forces us to explore other methods to investigate the role of proinflammatory cytokines in CFS, for example by intervening with cytokine effects.

IL-1 is one of the proinflammatory cytokines associated with fatigue most frequently⁽²⁵⁾. There is extensive experience with blocking IL-1 in different diseases⁽²⁶⁾. Both IL-1 α and IL-1 β can be inhibited by the IL-1 receptor antagonist anakinra. Anakinra has mild side-effects, mainly limited to the injection site^(27,28). Anakinra reaches the CNS, albeit in low concentrations^(29,30). Central effects of anakinra are most impressive in patients treated for cryopyrin-associated periodic syndromes, a rare autoinflammatory syndrome^(31,32). However, these patients have overt inflammation with increased permeability of the blood-brain barrier. Several studies in inflammatory⁽³³⁻³⁵⁾ and non-inflammatory⁽³⁶⁾ illnesses, assessed the effect of IL-1 inhibition on fatigue severity. Most of these studies found positive effects of IL-1 blockade on fatigue. Studies using daily

anakinra injections (100mg/day), found a decrease of fatigue within four weeks of treatment^(33, 34).

To investigate the role of proinflammatory activity in CFS, we conducted a randomized placebo-controlled trial in female CFS patients using the IL-1 receptor antagonist anakinra. All patients had severe fatigue leading to functional impairment.

Methods

Design overview

The study protocol including a detailed description of the intervention has been published (<http://www.ncbi.nlm.nih.gov/pubmed/26438161>)⁽³⁷⁾. This randomized parallel-group placebo-controlled trial was performed at the Department of Internal Medicine and Expert Center for Chronic Fatigue (ECCF) of the RadboudUMC. The local ethics committee approved the protocol (2014/025). Patients were randomized 1:1 between daily subcutaneous anakinra or placebo for four weeks, followed by a follow-up period of 20 weeks. Participants, all study personal (main investigator, physician assistant), and the sponsor were blinded to treatment allocation throughout the study.

Recruitment started in July 2014 and lasted until November 2015. Follow-up was completed in May 2016. After approval of the original protocol but before the start of the trial, the number of patients was increased to 50 and a decision was made to exclude patients with an illness duration of >10 years. During the trial, an amendment was made to allow medication use for ≤14 consecutive days for treatment of adverse events if the medication had no known interactions with anakinra (use of paracetamol and oral contraceptives already was allowed at baseline and during the trial). The published protocol mistakenly mentioned the number of accompanying CDC symptoms as a secondary outcome measure whereas the original protocol did not specify those symptoms as a secondary outcome.

Setting and participants

All female CFS patients visiting the outpatient clinic of the Department of Internal Medicine or the ECCF were considered for participation. Regional hospitals and a CFS-treating center could refer patients. Patients connected to a Dutch patient advocacy foundation for CFS were also invited to participate. Patients willing to participate were screened for eligibility after giving written informed consent. Patients were asked to fill out web-based questionnaires and were evaluated by the research physician by means of medical history, physical examination, electrocardiogram, pregnancy test, psychiatric evaluation using the Becks Depression Inventory-Primary Care version and the Mini International Neuropsychiatric Interview⁽³⁸⁾.

Patients were included if they met the CDC criteria^(1, 2), were aged between 18 and 59 years old, had a maximal fatigue duration of ten years or recent progression of symptoms, had a score of ≥40 on the fatigue severity subscale of the Checklist Individual Strength (CIS-fatigue), and had a score of ≥700 on the Sickness Impact Profile. These questionnaire scores reflect severe fatigue leading to substantial impairment. The exclusion criteria were described previously⁽³⁷⁾ and are listed in *supplementary table 1*.

Randomization and blinding

The randomization list was computer-generated by the Department of Pharmacy⁽³⁹⁾. During the study, the list was only known by the pharmacist, and could only be exposed in case of emergency. The pharmacy provided sequentially numbered boxes, which were given to the patient by the research physician on the patients' starting date. After completion of the trial and closing of the data file, the list was made available to the research physician. Both anakinra and placebo were provided by the Swedish Orphan Biovitrium (Sobi, Stockholm, Sweden). Syringes of both arms were visually inspected by the pharmacy to secure identical appearance. Each consecutive patient received a study number corresponding with a number on the randomisation list and one of the medication boxes [1-50].

Patients were instructed to report side-effects to an independent physician assistant, to keep the research physician blinded. After one week of treatment, patients were contacted by telephone to evaluate the presence of adverse events. Patients were instructed to call the physician assistant or internist on call in case of adverse events. After completion of the intervention at four weeks, adverse events were evaluated once more. All adverse events were graded by the physician assistant based on their severity (mild, moderate, severe), treated if necessary, and followed until they had abated⁽³⁷⁾. Anakinra frequently causes a reaction at the injection site, which could influence blinding if noticed by the research physician. The physician assistant was supervised by an independent senior internist. After completion of the intervention period, the patients were asked which medication they thought they had been using. Data analysis was performed by a researcher blind for allocation of patients.

Interventions

At the first study day, patients received medication boxes providing study drugs for 4 weeks. Medication was provided by the research physician. The boxes contained either anakinra (100mg/day) or placebo, which was administered subcutaneously on a daily basis. The placebo contained a mixture of sodiumcitrate, sodiumchloride, and polysorbate. Each box contained 28+4 syringes. The physician assistant instructed all participants on the subcutaneous injections during the first study visit and handed out written instructions to them. After the instruction, the first injection was self-administered in the presence of the assistant. Patients were advised to set a daily alarm to remind themselves of using the medication. After 1 and 4 weeks, drug adherence was evaluated, and after completion of the trial remaining syringes were returned and counted. In case of problems with adherence or drug administration during the study, monitoring frequency was intensified. After 4 weeks patients were seen at the outpatient clinic by the research physician. After six months, at the end of the follow-up period, the research physician phoned the patients⁽³⁷⁾.

Outcomes

Primary and secondary outcome measures were assessed using web-based questionnaires, which were completed at baseline, after four weeks and 24 weeks post-baseline. Questionnaires on fatigue and pain severity were filled out weekly during the intervention, and monthly during follow-up.

The primary study outcome was fatigue severity after completion of the intervention (4 weeks), measured with the CIS-fatigue subscale^(40,41). The CIS is a validated questionnaire often used in CFS patients. One of the subscales assesses fatigue severity. Scores range between 8 and 56 with higher scores reflecting more severe fatigue. A previous non-inferiority trial suggested that a clinical meaningful change was unlikely if the mean difference between treatment groups was less than 5.2 points⁽⁴²⁾. Secondary outcome measures were level of functional impairment assessed with the Sickness Impact Profile (range 0-5799)⁽⁴³⁾, physical and social functioning assessed with the respective subscales of the Short Form (SF)-36 (range 0-100)⁽⁴⁴⁾, level of psychological distress assessed with the Symptom Checklist-90 (SCL-90, range 90-450)⁽⁴⁵⁾, and pain severity assessed with a visual analogue scale (VAS, range 0-10) after completion of the intervention period. For all outcome measures, with the exception of the SF-36, a higher score means more symptoms. Additional outcome measures on body temperature, pulse rate, cytokine and cortisol concentrations, and microbiome were collected and will be reported elsewhere. All safety issues were discussed with an independent Drug Safety Monitoring Board during the trial.

Statistical analysis

The sample size estimate was based on the effect size reported in previous studies that had evaluated CBT in CFS patients^(46,47). In each arm, 23 patients were needed assuming an effect size of 0.85, a power of 0.80, and an alpha of 0.05 (two-sided). This would reflect a mean between group difference in the CIS-fatigue at post-treatment assessment of 8.5 to 9 points assuming a within group SD of 12.1 in the anakinra condition and 8.7 in the placebo condition⁽⁴⁶⁾. The sample size was set assuming analysis of covariance (ANCOVA) and a correlation between baseline and post-baseline measurement of 0.342. With an estimated dropout of 20 percent, 25 patients had to be included in each study arm.

All analyses were performed using IBM SPSS Statistics software for Windows (version 22.0). Primary and secondary outcome measures were analyzed based upon the randomized treatment assignment. Data were complete for all baseline and post-baseline measures of primary and secondary outcomes, except for a missing maximum VAS-pain score at 8 weeks in an anakinra patient.

For the analysis of all outcome measures ANCOVA was used in order to determine if there were any differences between the study arms. The score on the outcome measure at four weeks was entered as dependent variable, with treatment as fixed factor, and score at baseline as covariate. For evaluation of the effect of illness duration on outcome, illness duration was added as a covariate in a separate analysis. Outcomes were considered as significantly different in case of a p-value <0.05.

Role of the funding source

This study was partially supported by the Interleukin Foundation and an independent donor that wishes to stay anonymous. Both anakinra and placebo were provided by the Swedish Orphan Biovitrium (Sobi, Stockholm, Sweden). Neither the funders nor the manufacturer of the medication had a role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

To reach the required 50 participants, 82 severely fatigued female patients were screened for eligibility. After inclusion, 50 patients were randomly assigned to one of the treatment arms (*figure 1*). Reasons for exclusion are displayed in *figure 1*. During the trial, one patient discontinued treatment due to a local skin infection which recovered after antimicrobial treatment. No patients were lost to follow-up. Concomitant medication that was used during the intervention period is shown in *supplementary table 2*. None of the patients received a co-intervention during the intervention period of the trial.

Figure 1 Inclusion and randomization procedures.

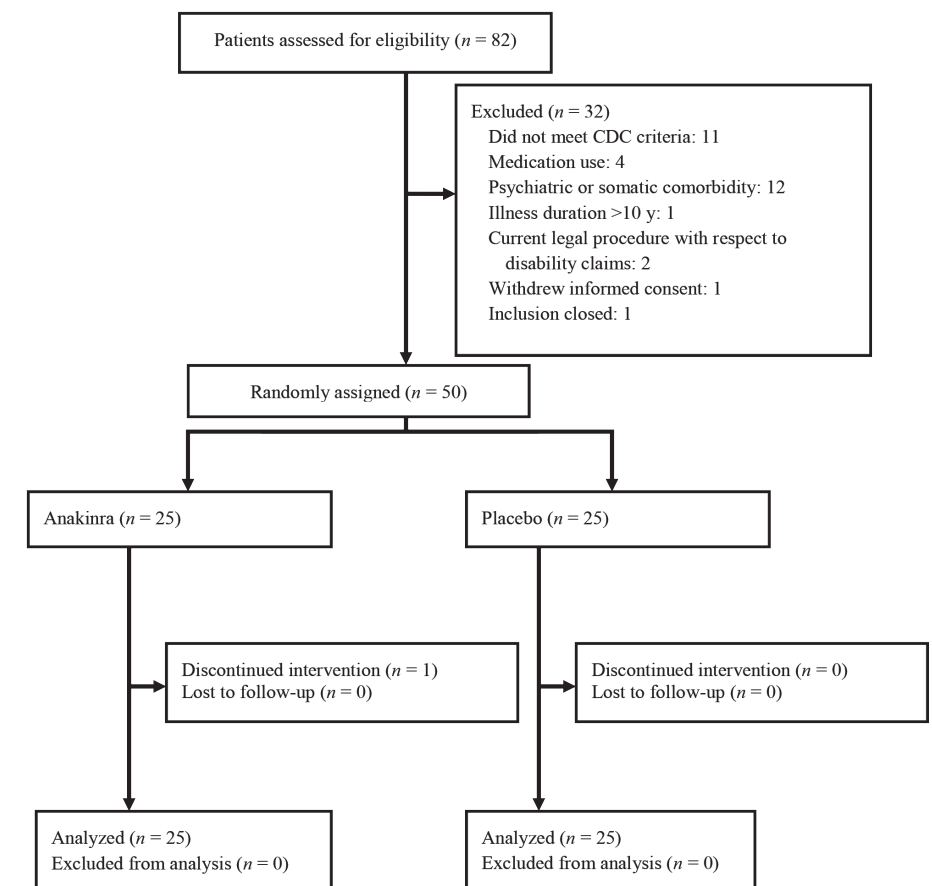


Figure 1 CDC = Centers for Disease Control and Prevention.

Baseline characteristics of groups were similar (**table 1**). Symptoms started after an infection in 24 patients, 14 of these patients were randomized to anakinra. One patient randomized to placebo was post-menopausal. 23 patients within the anakinra group and 25 patients within the placebo group adhered to $\geq 90\%$ of the study medication. Only one patient in the anakinra group missed $>50\%$ of the injections due to medication discontinuation because of an adverse event.

Table 1 Baseline Characteristics of the Study Population

Characteristic	Anakinra (n = 25)	Placebo (n = 25)
Mean age (SD), y	30 (9)	32 (11)
Median illness duration (range), mo	44 (7–109)	39 (9–108)
Mean body mass index (SD), kg/m ²	25 (5)	25 (4)
Mean fatigue severity (CIS-fatigue score) (SD)*	52 (4)	51 (4)
Mean functional impairment (SIP score) (SD)†	1647 (584)	1706 (721)
Mean social functioning (SF-36 score) (SD)‡	33 (24)	39 (23)
Mean physical functioning (SF-36 score) (SD)‡	48 (23)	56 (19)
Mean psychological distress (SCL-90 score) (SD)§	152 (31)	148 (30)
Mean maximum pain score (VAS) (SD)	7 (2)	7 (2)
Mean CDC symptoms (SD), n	7 (1)	6 (2)

Table 1 CDC = Centers for Disease Control and Prevention; CIS-fatigue = fatigue severity subscale of the Checklist Individual Strength; SCL-90 = Symptom Checklist-90; SF-36 = Short Form 36; SIP = Sickness Impact Profile; VAS = visual analogue scale.

* Ranges from 8 to 56; higher scores indicate worse fatigue.

† Ranges from 0 to 5799; higher scores indicate greater impairment.

‡ Ranges from 0 to 100; higher scores indicate better functioning.

§ Ranges from 90 to 450; higher scores indicate greater distress.

|| Ranges from 0 to 10; higher scores indicate worse pain.

Primary outcome

The primary outcome measure was assessed for all patients. After four weeks of treatment there was a mean difference of 1.5 on the CIS-f between patients treated with anakinra and controls (95% CI -4.1 to 7.2, $p=0.59$, **table 2**). Both groups showed an overall decrease in fatigue severity over the complete follow-up period, but most participants remained severely fatigued with a mean CIS-f score >35 (**figure 2**). In the anakinra group, 2 patients (8%) were no longer severely fatigued after the intervention period, reflected by a CIS-f <35 (47), compared to 5 patients (20%) in the placebo group (difference -12.0%, 95% CI: -31.8% to 7.8%, $p=0.22$). In those patients who reported symptoms after an infection, there was also no difference in fatigue severity after four weeks between both study arms

Table 2 Treatment Effect Directly After Treatment (4 Weeks) and at the End of Follow-up (24 Weeks) in the Intention-to-Treat Population*

Outcome Measures	Anakinra (n = 25)	Placebo (n = 25)	P Value†	Difference (95% CI)
Primary				
CIS-fatigue score (after treatment)‡	46.7 (42.7 to 50.7)	45.1 (41.2 to 49.1)	0.59	1.5 (-4.1 to 7.2)
Secondary				
CIS-fatigue score (follow-up)‡	45.3 (40.6 to 50.0)	44.0 (39.2 to 48.7)	0.69	1.3 (-5.3 to 8.0)
SIP score§				
After treatment	1472.2 (1243.0 to 1701.5)	1353.7 (1124.4 to 1582.9)	0.47	118.6 (-205.8 to 443.0)
Follow-up	1351.5 (1092.2 to 1610.9)	1260.4 (1001.0 to 1519.7)	0.62	91.2 (-275.8 to 458.1)
SF-36 score				
Physical functioning				
After treatment	58.2 (51.5 to 64.8)	61.2 (54.6 to 67.9)	0.53	-3.0 (-12.5 to 6.5)
Follow-up	60.8 (53.0 to 68.6)	64.8 (57.0 to 72.6)	0.47	-4.0 (-15.1 to 7.1)
Social functioning				
After treatment	41.2 (32.1 to 50.3)	49.3 (40.2 to 58.4)	0.22	-8.0 (-20.9 to 4.9)
Follow-up	46.0 (35.5 to 56.6)	52.5 (41.9 to 63.0)	0.39	-6.5 (-21.4 to 8.5)
SCL-90 score¶				
After treatment	144.4 (136.6 to 152.2)	139.9 (132.1 to 147.7)	0.42	4.5 (-6.6 to 15.6)
Follow-up	143.5 (135.3 to 151.7)	140.5 (132.3 to 148.7)	0.61	3.0 (-8.6 to 14.6)
Maximum VAS pain score**				
After treatment	7.4 (6.5 to 8.3)	6.3 (5.4 to 7.2)	0.104	1.0 (-0.2 to 2.3)
Follow-up	6.9 (5.9 to 7.9)	6.6 (5.6 to 7.6)	0.63	0.34 (-1.1 to 1.7)

Table 2 CIS-fatigue = fatigue severity subscale of the Checklist Individual Strength; SCL-90 = Symptom Checklist-90; SF-36 = Short Form 36; SIP = Sickness Impact Profile; VAS = visual analogue scale.

* Values are mean questionnaire scores (95% CIs) corrected for baseline scores. † Derived by analysis of covariance. ‡ Ranges from 8 to 56; higher scores indicate worse fatigue. § Ranges from 0 to 5799; higher scores indicate greater impairment. || Ranges from 0 to 100; higher scores indicate better functioning.

¶ Ranges from 90 to 450; higher scores indicate greater distress. ** Ranges from 0 to 10; higher scores indicate worse pain.

Figure 2 Fatigue, impairment, and pain scores throughout the study.

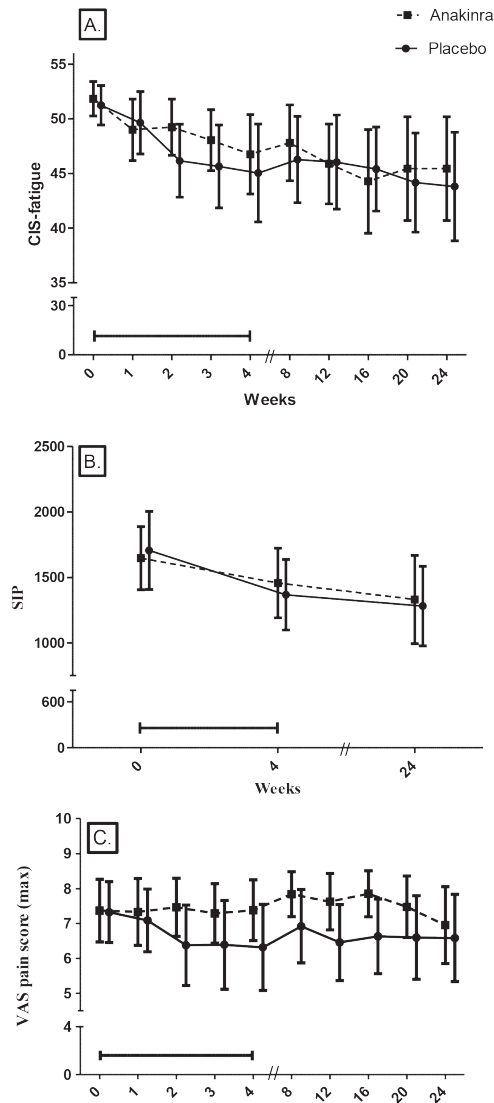


Figure 2 The mean (95% CI) CIS-fatigue (top), SIP (middle), and maximum VAS pain (bottom) scores for both treatment groups during the complete intervention and follow-up periods are shown. Summaries are based on questionnaire scores for all 25 anakinra recipients and all 25 placebo recipients, with the exception of the maximum VAS pain score at 8 wk, for which 1 anakinra recipient was missing. CIS = Checklist Individual Strength; SIP = Sickness Impact Profile; VAS = visual analogue scale.

($p=0.45$). In addition, there was no effect of duration of symptoms on fatigue severity after treatment (data not shown).

Secondary outcomes

Secondary outcome measures were assessed in all patients. After four weeks and at follow-up, there were no statistically significant differences in impairment, physical and social functioning, psychological distress and pain severity between study arms (table 2).

Adverse events

The adverse events during the intervention period are listed in table 3. Adverse events were more frequent in the anakinra group compared to controls [24/25 (96%) vs. 14/25 (56%)] due to injection site reactions in the anakinra group [17/25 (68%) vs. 1/25 (4%)]. All injection site lesions resolved completely. There were no serious adverse events (SAE).

Within the anakinra group, 17 patients (68%) correctly guessed which treatment they had been using compared to 15 patients (60%) in the placebo group.

Table 3 Adverse Events and Serious Adverse Events Reported During the 4-Week Intervention*

Adverse Event	Anakinra (n = 25)	Placebo (n = 25)
Death	–	–
Withdrawal due to adverse event	1 (4)	–
≥1 adverse event	24 (96)	14 (56)
Serious adverse event	–	–
Injection site reaction	17 (68)	1 (4)
Infection	6 (24)	4 (16)
Gastrointestinal symptoms	1 (4)	3 (12)
Central nervous system symptoms	3 (12)	3 (12)
Psychological symptoms	1 (4)	1 (4)
Other	9 (36)	4 (16)

Table 3 * Adverse events were evaluated at 1 and 4 wk. Patients were instructed to contact the hospital by telephone if an adverse event occurred. Values are numbers (percentages).

Discussion

Though 2 anakinra participants and 5 placebo participants reached a fatigue level within the range reported by healthy individuals, our study did not find that anakinra led to a clinically meaningful decrease of fatigue severity. The CIS-f at four weeks, the primary outcome of this study, was 1.5 points higher in the anakinra group and the 95% CI for the anakinra-placebo difference was -4.1 to 7.2. The lower bound of the confidence interval of this difference is consistent with a maximal potential benefit with anakinra of 4.1 points on the CIS-f. A prior non-inferiority study suggested that a clinical meaningful change between treatment groups was unlikely if the mean difference was less than 5.2 points on the CIS-f⁽⁴²⁾. We also found no statistically significant differences between groups for secondary outcome measures, such as functional impairment and physical impairment.

Despite many studies reporting circulating cytokines in CFS⁽¹⁸⁾, intervention studies on this subject are scarce. We searched for intervention studies (English language search of Pubmed to December 2016 and clinicaltrials.gov) and found no published or registered study evaluating the effect of directly lowering cytokines on fatigue severity in CFS patients. However, a positive effect of IL-1 inhibition on fatigue severity has been found in several other illnesses such as rheumatoid arthritis⁽³³⁾, Sjögren's syndrome⁽³⁴⁾, and diabetes⁽³⁶⁾.

We considered several methodological and study population explanations for our findings. Compliance did not differ between treatment groups, although one patient in the anakinra group discontinued treatment after two weeks as a consequence of a local skin infection. As at least 90% of the injections were administered by 96% of patients, it is unlikely that poor adherence explains the findings. As CFS typically affects women, we only included female patients to investigate a homogeneous group. Since recent studies suggest an inflammatory pattern early in the course of CFS⁽¹⁵⁾, we excluded patients with an illness duration of >10 years. Psychiatric illnesses, mainly depression, are known to have an influence on inflammation⁽⁴⁸⁾. To solely investigate the effect of blocking IL-1 on fatigue, patients with active psychiatric comorbidity were excluded. We believe it is unlikely that any of the study population selection issues influenced the outcome.

A possible explanation for the results of this study is that peripherally administered anakinra does not reach the brain in sufficient concentrations to have a biological effect. Previous studies have demonstrated that IL-1Ra is able to reach the cerebrospinal fluid (CSF) in low concentrations after intravenous^(30,49) and subcutaneous⁽⁵⁰⁾ administration. No human data on CSF concentrations after s.c. administration exist, but a study in rats found CSF concentration of 170⁽⁵⁰⁾ ng/mL after a single IL-1Ra injection both in naïve rats and after brain ischaemia⁽⁵⁰⁾. However, penetration of the brain parenchyma only occurred after brain ischaemia. Thus

therapeutically active concentrations in the brain might be only reached in case of disruption of the blood-brain barrier, which is probably not present in CFS.

Finally, the lack of an effect in this study could be caused by a limited role of IL-1 in CFS. After peripherally produced IL-1 reaches the CNS, an upstream signaling cascade is activated, eventually leading to alterations in the production of neurotransmitters, especially dopamine⁽¹⁴⁾. Treating fatigue through IL-1 inhibition might only be effective in diseases with a more acute inflammatory pattern, whereas in the chronic situation these upstream effects should also be addressed. This is supported by the fact that IL-1 inhibition especially seems to be effective in diseases with an acute inflammatory pattern⁽²⁵⁾. It could also be that proinflammatory cytokines other than IL-1, for example tumor necrosis factor, are responsible for symptoms in CFS.

Regarding adverse events, there were no large differences in the incidence of infections or CNS symptoms. However, skin reactions at the injection sites were more frequent in the anakinra group. This is a known side-effect of anakinra, mostly present during the first weeks of treatment. It is characterized by macrophage infiltration⁽⁵¹⁾ and causes pain and discomfort, which might have impacted pain scores in the anakinra group during treatment. The degree of skin reaction has been found to correlate with diminished therapeutic effects⁽⁵¹⁾, it is unclear to what extent this contributed to the primary outcome of our study, especially because the severity of the injection site reaction varied among patients and was not scored in an objective manner. As a consequence of the small sample size, uncommon harms of anakinra could have been missed.

Overall, fatigue severity decreased over the total duration of the trial in both study arms, comparable to previous studies performed by our group⁽⁵²⁾. In the placebo group, 20% of patients responded positively to the treatment, similar to a pooled placebo response of 19.6% in previous studies in CFS patients⁽⁵³⁾.

Increased concentrations of IL-1 β correlate with sickness behavior and fatigue during acute infections such as Q-fever and infectious mononucleosis⁽⁵⁴⁾. Therefore, interfering with IL-1 β might be more effective in those patients with persisting fatigue after an infection. A limitation of this study is that the inclusion of patients was not limited to these patients. However, 48% of patients in the current study reported an infection as the initial trigger for their symptoms, and these patients did not respond differently to the treatment. Additional limitations of the current study are the small sample size and wide variability in the duration of symptoms, although the latter did not influence the main outcome.

In conclusion, we found no clinically meaningful effect of anakinra on fatigue severity in CFS patients. It may be concluded that if IL-1 plays a role in CFS, blockade of the IL-1R1 in the peripheral tissues such as the neuromuscular compartment has no effect. Future studies should focus on intracerebral processes in CFS patient and on reducing inflammation with agents that reduce IL-1 activity in the brain ^(55, 56).

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Appendix Table 1 Exclusion Criteria

Use of medication (except for oral contraceptives and/or paracetamol)
Use of psychotropic medication in the past month
Psychiatric comorbidity (major depression, psychosis, eating disorders, anxiety disorders, bipolar disease, and posttraumatic stress disorder) assessed with the Mini-International Neuropsychiatric Interview
Evident somatic comorbidity (as an explanation for fatigue)
Fatigue lasting >10 y (without recent progression)
Substance abuse in the past 3 mo
Pregnancy, nursing, or intended pregnancy during the study
Live vaccine during the past 4 wk
Current engagement in chronic fatigue syndrome research
Inability to understand the nature and extent of the trial and the procedure required
Current engagement in a legal procedure with respect to disability claims

Appendix Table 2 Concomitant Medication Use During the Intervention*

Medication	Anakinra (n = 25)	Placebo (n = 25)
Oral contraceptives	9 (36)	13 (52)
Paracetamol	16 (64)	16 (64)
Nonsteroidal anti-inflammatory drugs	1 (4)	2 (8)
Codeine	1 (4)	–
Antibiotics	3 (12)	1 (4)
Antihistamines	2 (8)	–
Other		
Alutard (ALK-Abelló)	1 (4)	–
Metamucil (Procter & Gamble)	1 (4)	–
Melatonin	1 (4)	–

Appendix table 2 * Values are numbers (percentages) of patients who used the specific medication group.

Chapter 6

Cytokine Signatures in Chronic Fatigue Syndrome Patients: a Case Control Study

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Abstract

Background: Cytokine disturbances have been suggested to be associated with the Chronic Fatigue Syndrome (CFS) for decades.

Methods: Fifty female CFS patients were included in a study on the effect of the interleukin-1-receptor antagonist anakinra or placebo during four weeks. EDTA plasma was collected from patients before and directly after treatment. At baseline, plasma samples were collected at the same time from 48 healthy, age-matched female neighborhood controls. A panel of 92 inflammatory markers was determined in parallel in 1 μ L samples using a 'proximity extension assay' (PEA) based immunoassay. Since transforming growth factor beta (TGFbeta) and interleukin-1receptor antagonist (IL-1Ra) were not included in this platform, we measured these cytokines with ELISA.

Results: In CFS patients, the 'normalized protein expression' value of IL-12p40 and CSF-1 was significantly higher (p-value 0.0042 and 0.049, respectively). Furthermore, using LASSO regression, a combination of 47 markers yielded a prediction model with a corrected AUC of 0.73. After correction for multiple testing, anakinra had no effect on circulating cytokines. TGFbeta did not differ between patients and controls.

Conclusions: In conclusion, this study demonstrated increased IL-12p40 and CSF-1 concentrations in CFS patients in addition to a set of predictive biomarkers. There was no effect of anakinra on circulating cytokines other than IL-1Ra.

Background

Chronic fatigue syndrome (CFS) is a condition of unknown origin that is characterized by severe fatigue for more than six months leading to significant disability. To fulfill the CFS criteria as recommended by the Centers for Disease control (CDC), patients also have to report at least four out of eight of the accompanying symptoms (e.g. muscle pain, post-exertional fatigue, headache, etc.)^(1,2). With CFS being an exclusionary diagnosis, patients often report to have symptoms for several years before being diagnosed⁽³⁾. Most of the current case definitions suggest a collection of mandatory diagnostics to exclude common causes for fatigue such as anemia and thyroid illnesses^(1,4), but there is a need for more specific tests to diagnose patients. Another advantage of such a test is that it might be easier to define CFS subgroups⁽⁵⁾, for example those patients that would or would not respond to an immune intervention. Last but not least, a distinctive marker or set of markers may point to relevant pathogenetic mechanisms that may be further explored.

In the past years, numerous studies have been performed searching for potential biomarkers⁽⁶⁾. Because of the resemblance of CFS with symptoms that characterize immune activation, there has been a particular interest in the immune system with studies measuring lymphocyte subsets^(7,8), cytokine production⁽⁹⁻¹¹⁾, and single nucleotide polymorphisms in immune related genes^(12,13). However, despite a large number of studies conducted, this has not led to a unified conclusion useful for clinical practice. Studies are largely contradicting, and a recent systematic review on circulating cytokines did not find evidence for altered cytokine concentrations in CFS, with the exception of transforming growth factor-beta (TGF- β)⁽¹⁴⁾. TGF- β appeared to be elevated in 63% of the selected studies. This was also found in a recent study on cytokine signatures in CFS⁽¹⁵⁾. Other cytokines were only elevated in a minority of studies, for example interleukin-1 α (IL-1 α) in 27% of the studies, interleukin 12 (IL-12) in 18%, interleukin 23 (IL-23) 25%, and interleukin 8 (IL-8) in 29% of studies. Some studies only found differences when differentiating between patients with long and short illness duration^(9,16).

In order to make progress on the role of the immune system in CFS, we have to critically review the studies that have been performed, and try to clarify the reasons for these discrepancies. When measuring circulating cytokines, several issues have to be taken into account. First, patient selection is important. Studies often combine different cohorts of patients, recruit employees as controls, or controls who participated in previous studies^(9,17), and this may lead to different pre-analytical procedures. The latter is especially an important issue in this context. Cytokines may be released *ex vivo* by different circulating cells, and collection tubes, storage time, number of freeze-thaw cycles, and processing protocols have been found to be of influence^(18,19). To make a reliable comparison between

patients and controls, especially in CFS where circulating cytokines are expected to be low, it is essential that the pre-analytical process in these groups is identical.

Another important issue is the type of analysis used to determine cytokine concentrations. Most studies measuring cytokines use antibody based Enzyme-Linked Immuno Sorbent Assays (ELISA) ⁽¹⁴⁾. However, limitations of this technique are that multiplex forms of the assays often use only one antigen-binding antibody to detect the protein, which limits detection specificity as well as sensitivity ⁽²⁰⁾. Sandwich ELISA achieves better performance by using pairs of antibodies for each targeted protein, but the assays typically need relatively large sample volumes for analyses of single protein species, limiting throughput and spending precious samples. By contrast, the proximity extension assay (PEA) uses dual antibody recognition with oligonucleotide-conjugated antibodies in multiplex assays with modest requirements for sample volumes ^(20, 21). Upon simultaneous binding of the correct pair of antibodies, their attached oligonucleotides anneal to each other and can be enzymatically extended, forming specific DNA sequences that can be quantified using quantitative real-time polymerase chain reactions (qPCR).

In this study, cytokine profiles of female CFS patients participating in a randomized controlled trial on the effect of IL-1 inhibition on fatigue severity ⁽²²⁾ were compared with age- and gender-matched healthy neighborhood controls. In addition, TGF- β and the IL-1 receptor antagonist (IL1-Ra), which were not included in the PEA, were measured separately using an ELISA. Pre-analytical procedures were identical for patients and controls. Furthermore, the effect of IL-1 inhibition using the IL-1-receptor antagonist anakinra for one month on circulating cytokine concentrations was assessed. As reported in detail elsewhere, the study did not demonstrate a beneficial therapeutic effect in these patients ⁽²³⁾.

Methods

Patients & design

All patients participated in a double-blind randomized controlled trial (RCT) on the effect of IL-1 inhibition on CFS-related symptoms, of which the results were reported elsewhere ⁽²³⁾. The study was conducted at the Department of Internal Medicine and Expert Center for Chronic Fatigue (ECCF) of the RadboudUMC, Nijmegen, the Netherlands. Details of the study were described previously ⁽²²⁾. In short, fifty female patients between 18 and 59, years old were included when they fulfilled the CDC criteria for CFS ^(1, 2). As recommended by the CDC criteria, patients can only be included when the body mass index (BMI) is ≤ 40 kg/m². Main exclusion criteria were the presence of a somatic disease that could explain severe fatigue (sleep apnea, anemia, etc.), psychiatric comorbidity (e.g. depression, anxiety disorders) or the use of medication (with the exception of oral contraceptives and paracetamol). Patients were asked to bring a healthy, female, neighborhood control, without complaints of fatigue and within the same age range (± 5 years), to their first study visit.

After inclusion, patients were randomized 1:1 to either daily subcutaneous (s.c.) injections with anakinra (100 mg/day) or placebo (mixture of sodium citrate, sodium chloride, and polysorbate) for a duration of four weeks. Controls did not receive an intervention. Anakinra and placebo were provided by the Swedish Orphan Biovitrum (Sobi, Stockholm, Sweden). The randomization list was computer-generated by the Department of Pharmacy ⁽²⁴⁾. Patients administered the study medication at home on a daily basis. Both the placebo and anakinra syringes had an identical appearance, and drug adherence was evaluated as described previously ^(22, 23).

All participants provided written and oral informed consent before inclusion. The hospitals' ethics committee approved the study protocol (2014/025). The study was performed in accordance with the declaration of Helsinki.

Questionnaires

Fatigue was measured in both patients and controls using the fatigue severity subscale of the checklist individual strength (CIS), which has been used frequently in CFS patients ^(25, 26). Scores on the CIS-f can vary between 8 and 56, and a score ≥ 35 reflects severe fatigue ⁽²⁷⁾. Psychological distress was measured with the total score on the Symptom Checklist-90 (SCL-90) ⁽²⁸⁾.

Peripheral blood collection

Morning blood samples were collected from all patients prior to the first s.c. injection, and after four weeks of treatment. Samples of controls were collected and processed simultaneously with those of patients before treatment. There were no specific instructions with respect to food intake prior to blood sampling. Venous blood was collected in EDTA tubes, and kept on ice until centrifugation, which was performed within 2-3 hours. Next, samples were centrifuged at 2960xg for 10 minutes at 4°C. Plasma aliquots were then frozen at -80°C for a maximal duration of 2 years. Analyses for all patients and controls were run at the same time.

PEA assay

Inflammation biomarker profiles were analyzed by the analysis service of Olink Proteomics AB (Uppsala, Sweden), using their PEA based Proseek[®] Multiplex Inflammation panel^{196*96} (21). This analysis simultaneously measures 92 selected inflammatory proteins, listed in S1, using only 1 µL of plasma. For each protein, there are two separate antibodies connected to one oligonucleotide each. After binding by the antibody pair to its target, the 3' ends of the oligonucleotides hybridize, priming a DNA polymerization reaction that forms a protein-specific reporter DNA-sequence for each detected protein molecule. The reporter DNA strands are then quantified using qPCR. Four internal controls and two external controls were included in each assay. The raw Cq values were normalized for variation between and within runs and converted into Normalized Protein Expression Units (NPX). The NPX values are expressed on a Log2 scale where one unit higher NPX values represent a doubling of the measured protein concentrations. This arbitrary unit can be used for relative quantification of proteins and comparing the fold changes between groups.

Based on the CFS literature, 20 cytokines were selected to be of special interest; CD40L (CD40 ligand), CXCL-9 (chemokine ligand 9), CXCL-10 (chemokine ligand 10), CCL-2 (MCP-1), CCL-11 (eotaxin), IFN-γ (interferon gamma), IL-1α (interleukin-1 alpha), IL-2 (interleukin-2), IL-4 (interleukin-4), IL-6 (interleukin-6), IL-7 (interleukin-7), IL-8 (interleukin-8), IL-10 (interleukin-10), IL-12p40 (interleukin-12 subunit p40), IL-17A (interleukin-17A), CSF-1 (macrophage colony-stimulating factor 1), TNF-β (tumor necrosis factor-beta), TRAIL (TNF-related apoptosis-inducing ligand), TGF-α (transforming growth factor alpha), and TNF (tumor necrosis factor) (9, 14, 29).

ELISA

Total TGF-β1 levels were measured by sandwich ELISA as described in detail previously (R&D systems) (30). All samples were acid activated to activate latent TGF-β (1 M hydrochloric acid, 30 min, room temperature). Analysis was performed at the Leiden University Medical Center. IL-1Ra ELISA (R&D systems) was performed at the Radboud University Medical Center.

Statistical analysis

Study data were analyzed using IBM SPSS statistic package version 22 and R (31). All continuous variables are presented as means and standard deviations (SD) or medians and ranges, and categorical variables as percentages.

Inflammatory markers were excluded if >25% of the measurements were below the detection limit. Remaining missing values were imputed with a random value between 0 and the LOD for the protein at hand, a method that avoids the artificial reduction of the standard deviation that is a consequence of imputing the values LOD/2 or LOD/√2. For the baseline comparison of twenty pre-selected cytokines, analysis of covariance (ANCOVA) was performed with age and BMI added as covariates. Based on the result of a previous study, the same analysis was repeated dividing the patient group into patients with a long illness duration (>3 years) and patients with a short illness duration (9).

In order to establish a predictive model, a logistic regression model was selected using the LASSO regression strategy that aims at eliminating predictors with only marginal predictive performance. As potential predictors for CFS, the cytokine concentrations supplemented with age and BMI were used. To determine the performance of this model, the area under the ROC-curve (AUC) was calculated (32). As the model is evaluated in the same population that is used for construction, the predictive performance will be overestimated. To correct for this optimism, new populations were generated using bootstrap sampling. In each population the same modeling strategy was used. Each prediction model was then evaluated in both the bootstrap population and the original population. After 500 repetitions of this process, the differences between performance in bootstrapped samples and the original population were used to estimate the optimism due to internal validation (33).

To determine the influence of IL-1Ra on cytokine concentrations, analysis ANCOVA was used with the cytokine concentration after four weeks as dependent variable, treatment as fixed factor, and concentration at baseline, age, and BMI as covariates.

Results

Patient characteristics

A total of 50 CFS patients and 48 age-matched, neighborhood controls were included in the study. Two patients were not able to bring a healthy control at baseline. **Table 1** displays demographic and fatigue-related characteristics. Within the CFS group, there were 21 patients with a short illness duration (≤ 3 years, 58%) and 29 patients with a long illness duration (> 3 years, 42%). As expected, CFS patients had a higher CIS-fatigue score than controls (52 ± 4 vs. 20 ± 11 , $p < 0.001$). Total score on psychological distress was also significantly higher in patients (150 ± 30 vs. 119 ± 32 , $p < 0.001$). BMI, ethnicity, and percentage of patients using oral contraceptives did not significantly differ between groups.

Table 1 Baseline characteristics of chronic fatigue syndrome patients (CFS) and healthy controls (HC).

	CFS (n=50)	HC (n=48)	p-value
Age, years	31 (10)	31 (10)	0.98
Ethnicity			0.37
Caucasian (%)	49 (98)	47 (98)	
Other (%)	1 (2)	1 (2)	
Body mass index, kg/m ²	25.1 (4.5)	24.9 (4.4)	0.88
Oral contraceptives (%)	22 (44)	16 (33)	0.28
Paracetamol (%)	22 (44)	8 (16)	0.002
Illness duration, months	43 [7-109]	N/A	
≤ 3 years (%)	29 (58)		
> 3 years (%)	21 (42)		
Fatigue severity (CIS-fatigue)	52 (4)	20 (11)	< 0.001
Psychological distress (SCL-90)	150 (30)	119 (32)	< 0.001

Table 1 Data are number (%), mean (SD), or median [range]. CIS= checklist individual strength. SCL-90= symptom checklist 90. N/A= not applicable.

CFS patients versus controls

Twenty pre-selected cytokines were compared between patients and controls, based on the recent CFS literature^(9, 14, 29). For IFN- γ , IL-1 α , IL-2, IL-4, IL17A, and TNF, more than 25% of samples was under the LOD in both patients and controls. Results for the remaining 14 cytokines are displayed in **figure 1**. In this exploratory analysis, both IL-12p40 and CSF-1 appeared to be elevated in CFS patients (p-value 0.004 and 0.049 respectively). Other cytokines did not differ between patients and controls. Dividing the patient group into those with short illness duration, and those with longer illness duration did not change these results (data not shown).

Figure 1 Normalized Protein Expression Units (NPX) values for patients with chronic fatigue syndrome (CFS, n=50) compared to healthy controls (HC, n=48)

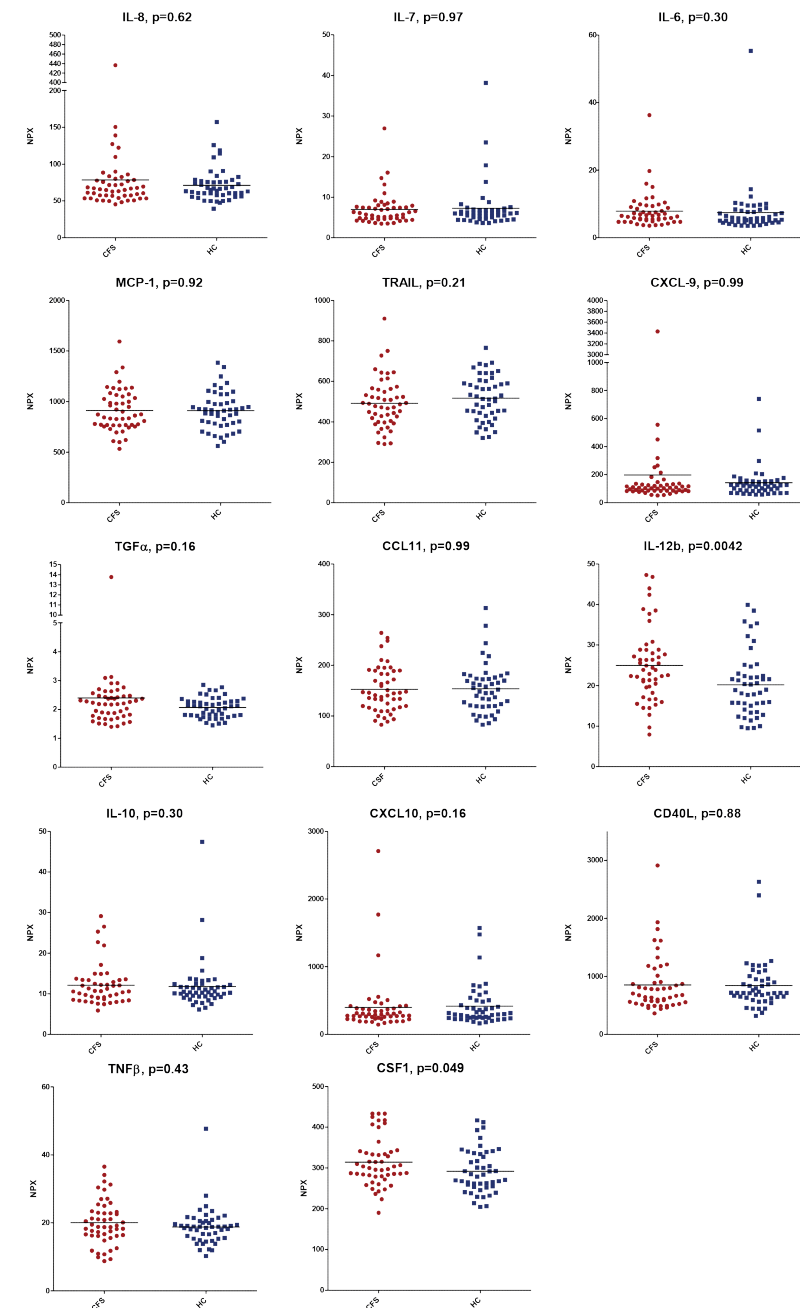


Figure 1 displays linear NPX values for patients and controls. P-values were derived by analysis of covariance.

There were no differences in TGF- β 1 and IL-1Ra between patients and controls, as determined by ELISA.

Prediction model

Of the 92 proteins measured, 22 appeared to be below the detection limit for >25% of samples for both patients and controls (IFN- γ , IL-1 α , IL-2, IL-4, IL17A, TNF, MCP-3, IL-17c, IL-20Ra, IL-2Rb, TSLP, IL-10Ra, IL-22Ra1, PD-L1, IL-24, IL-13, ARTN, IL-20, IL-33, LIF, NRTN, and IL-5). Although theoretically there is still information in the recorded concentrations for these cytokines, these proteins were excluded from the analysis as they were considered not to be candidates with a substantial predictive potential. The remaining 70 proteins were entered into the LASSO regression analysis in addition to age and BMI. Out of this total of 72 variables entered, 47 appeared in the final regression model (*table 2*). 22 variables had a positive association with the risk of being a CFS patient, and a negative association was present for 23 variables. To determine the performance of this model, an AUC was calculated with correction for optimism. Optimism in the current model was 0.265, which resulted in a corrected AUC value of 0.734.

Influence of IL-1Ra on circulating cytokines

In accordance with the analysis of patients versus controls, in 22 cytokines the NPX value was below the detection limit in more than 25% of cases. These cytokines were excluded from the analysis. One patient in the anakinra group discontinued treatment after two weeks as a consequence of an adverse event and was excluded from the analysis. IL-1 α , a cytokine of special interest, was not detectable in more than 75% of samples both before and after treatment. In *figure 2* the influence of anakinra vs placebo is displayed for all detectable cytokines with corresponding 95% confidence intervals (95%CI). In the anakinra group there appeared to be an inhibiting effect of anakinra on CSF-1, IL-18R1, and ENRAGE. In addition there was a stimulating effect on CXCL-9; for the remaining variables there was no influence of anakinra. As expected, IL-1Ra was significantly higher in those patients treated with anakinra ($p < 0.001$, *figure 3*). Of the 25 patients treated with anakinra, 23 patients had a concentration above the detection limit of 2000 pg/ml.

Table 2 LASSO regression analysis: factors associated with the risk of being a CFS patient versus healthy controls.

Proteins that are positively associated with the risk of being a CFS patient:		Proteins that are negatively associated with the risk of being a CFS patient:	
Protein	Weight	Protein	Weight
TWEAK	5,077	BetaNGF	-6,683
CCL4	4,182	LIFR	-4,053
IL12B	3,898	HGF	-2,153
CDCP1	3,611	CXCL6	-1,849
VEGFA	3,138	4EBP1	-1,317
CSF1	2,810	SCF	-1,307
IL10RB	1,737	MMP1	-1,256
CCL11	1,354	ADA	-1,237
CD5	1,087	CXCL10	-1,223
MCP1	1,047	IL18R1	-1,076
CASP8	0,865	CXCL9	-0,697
FGF5	0,758	CCL28	-0,611
IL6	0,737	CCL25	-0,557
CCL23	0,591	OSM	-0,544
CX3CL1	0,568	CCL20	-0,514
ST1A1	0,307	CCL19	-0,507
TNFSF14	0,303	TRANCE	-0,498
CD244	0,302	NT3	-0,489
IL10	0,287	MCP4	-0,458
CXCL5	0,245	TRAIL	-0,406
LAPTGFbeta1	0,166	ENRAGE	-0,356
OPG	0,107	CD6	-0,109
		TNFB	-0,062
		MMP10	-0,033
		FGF23	-0,017

Optimism: 0.2652
AUC: 0.9996
Corrected AUC: 0.7344

Table 2 Given associations are all conditional on the other predictors.

Figure 2 Treatment effect of the four week intervention period on circulating cytokines.

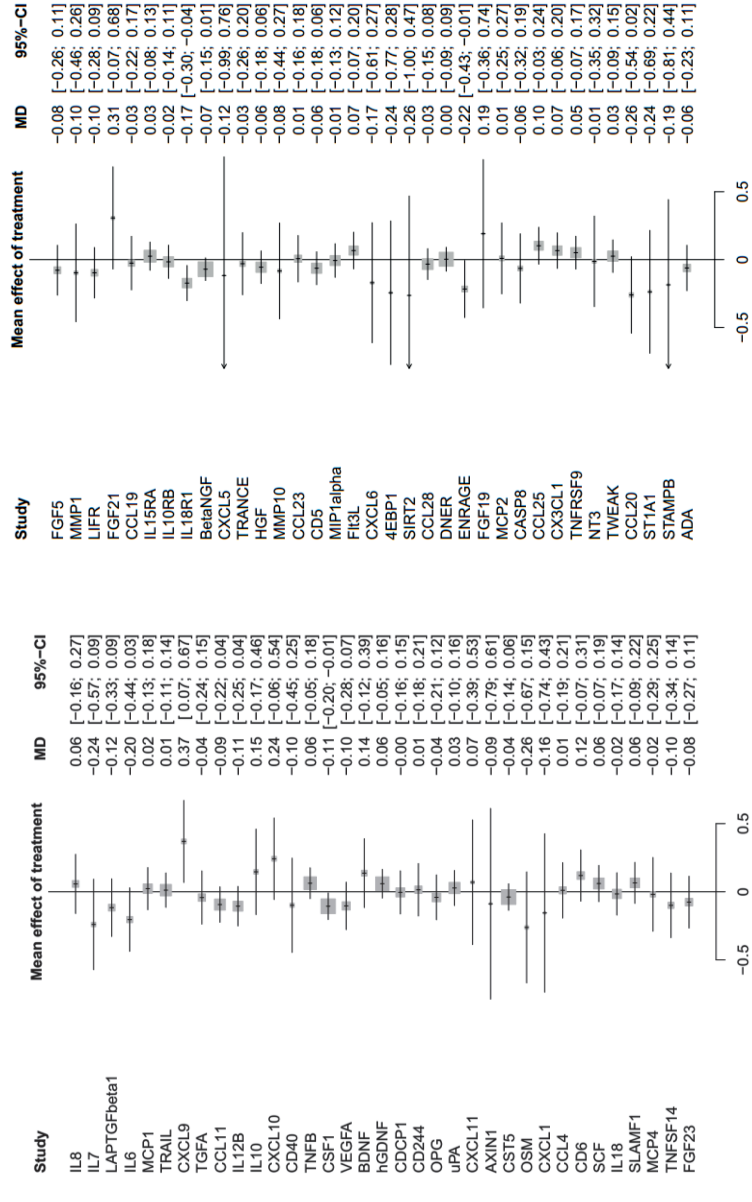


Figure 2 Displays the difference and 95% confidence intervals between study arms for protein values after treatment (anakinra: controls).

Figure 3 Concentration of IL-1Ra for patients treated with either anakinra or placebo

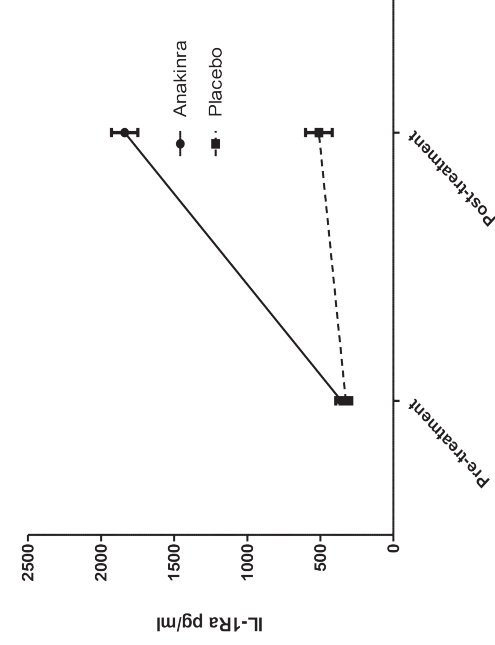


Figure 3 Displays IL-1Ra concentrations±SEM in patients treated with either anakinra or placebo before and after treatment.

Discussion

In this study differences in plasma cytokine profiles of 50 CFS patients compared to 48 healthy age-matched neighborhood controls were evaluated using a PEA protein assay. The concentration of IL-12p40 and CSF-1 were significantly higher in CFS patients. For the remaining cytokines of interest based on the current CFS literature, no differences could be found comparing them individually. However, using the complete inflammatory profile, patients and controls could be discriminated using 22 variables with a positive association and 24 variables with a negative association.

IL-12p40 was significantly higher in CFS patients compared to healthy controls ($p=0.005$). The IL-12p40 subunit is expressed by activated dendritic cells (DC) and combines with either subunit p35 or p19, to form IL-12 or IL-23⁽³⁴⁾. IL-12 targets T-cells and NK-cells, in which it induces IFN- γ production⁽³⁵⁾. IL-23 has an important role in Th17 production, has an effect on memory T-cells, and appears to be critical in cerebral autoimmune inflammation⁽³⁶⁾. Previous studies in CFS patient also found increased concentration of both IL-12^(17, 37, 38), IL-12p40⁽⁹⁾ and IL23⁽³⁹⁾. A relationship between IL-12 and fatigue has been established in studies investigating the effect of administration of human recombinant IL-12 to treat ovarian cancer, and head and neck cancer^(40, 41). A proportion of treated patients developed fatigue, and combined with other side effects such as fever and chills, this toxicity had dose-limiting consequences. Furthermore, increased concentrations of IL-12 and IL-23 have especially been associated with multiple sclerosis, in addition to psoriasis, inflammatory bowel disease, cancer and rheumatoid arthritis⁽³⁴⁾. However, in the light of the explorative nature of the current analysis, replication of this finding is important.

CSF-1 or macrophage colony-stimulating factor (M-CSF) is a hematopoietic growth factor involved in proliferation and differentiation of monocytes and macrophages. Targeting CSF-1 has predominantly been described in cancer, where there appears to be an advantage when it is used in combination with other immune therapies⁽⁴²⁾. Interestingly, the role of CSF-1 in the development of sickness behavior has recently been assessed by Müller et al.⁽⁴³⁾. In this study, neutralization of the CSF-1 receptor prevented the development of sickness behavior in mice treated with an inflammatory stimulus. This behavioral response was mediated through increased IL-10 production. Increased IL-10 concentrations were also demonstrated in the hypothalamus, where the behavioral effect is most likely to be effected.

In contrast to the majority of previous studies measuring circulating cytokines, in this study there were no differences in TGF- β concentrations⁽¹⁴⁾. TGF- β influences cell proliferation, migration, and differentiation, and is known for its dual role in cancer⁽⁴⁴⁾. TGF- β is released in large quantities by activated platelets⁽⁴⁵⁾. Several factors, such as blood

sampling procedures⁽⁴⁶⁾ and use of medication, influence the extent of platelet activation. A possible explanation for the increased TGF β levels previously reported might be caused by differences in sample handling and drug use, which has recently been demonstrated⁽⁴⁷⁾. For example, oral contraceptives induce platelet activation in humans⁽⁴⁸⁾, and directly cause increased TGF- β concentrations in rats⁽⁴⁹⁾.

These differences regarding sample handling might also be the explanation for the fact that previous studies found more cytokines to be differentially expressed in CFS patients. A substantial proportion of studies included multiple cohorts in their analyses^(9, 29), and although this gives the opportunity to investigate a larger number of patients, including patients coming from geographically different regions results in an inherent danger of inaccurate conclusions. This is because sample handling may have been different, for example use of different centrifuges, differences in ambient temperature, variations in collection time, and storage. Such differences in pre-analytic sample handling and storage, were part of the explanation for the spurious reports on the role of murine retroviruses in CFS⁽⁵⁰⁾. In addition, cytokine concentrations are known to be influenced by drug use. The use of non-steroidal anti-inflammatory drugs (NSAIDs), which are frequently used by CFS patients, increase the production of various cytokines^(51, 52). Differences in use of NSAIDs and other medication between patients and controls, might have explained the reported increased levels of pro-inflammatory cytokines in previous studies.

Interestingly, combining all inflammatory markers yields a prediction model containing 47 markers with a corrected AUC of 0.73. To control for optimism, a bootstrapping method was used, which yielded a high correction factor of 0.265. Some of the measured proteins have a positive association with CFS (IL-6, CSF-1), whereas for others there was a negative association (BetaNGF, CXCL-6). This selection of inflammatory markers could be a starting point for further studies investigating potential diagnostic markers in CFS.

According to the 95% confidence intervals, there was an inhibiting effect of anakinra on circulating CSF-1, IL-18R1 and ENRAGE and a stimulating effect on CXCL-9. Given the large numbers of cytokines tested, these findings have to be interpreted with caution. It is important to mention that IL-1 β was not included in the analysis, and IL- α was already below the detection limit before treatment in 82% of samples. It was expected that there would be a significant decrease of IL-6 in the anakinra group, as IL-1 induces IL-6 production, and is frequently used as a readout for IL-1 activity⁽⁵³⁾. In previous studies, there was also a significant reduction of IL-6 concentrations after anakinra treatment. This was investigated in patients intravenously treated after stroke⁽⁵⁴⁾ and patients treated with subcutaneous injections for heart failure⁽⁵⁵⁾. A possible explanation for the lack of a decrease in IL-6 concentrations is that in comparison to the situation in stroke patients

where it has an important prognostic role ⁽⁵⁶⁾, CFS patients exhibit no increase of this cytokine at baseline. Since drug adherence was excellent, which is also reflected by the significant increase of IL-1Ra in the anakinra treated group, it is unlikely that lack of compliance is an explanation for this result.

This study has several strengths. To our knowledge, PEA-based assays have not previously been performed in CFS patients. Over the past few years, there has been a search for sensitive methods to measure multiple inflammatory markers simultaneously in order to find potential biomarkers for CFS. However, this is most commonly performed using multiplex bead-based immunoassays, that have limited sensitivity and specificity ⁽²⁰⁾. This is not the case for the PEA method, which has a much higher specificity as the signals can only be elicited by the cognate antibody pairs, while cross reactions between irrelevant antibody pairs are ignored ⁽²¹⁾. Another asset of this study is the inclusion of neighborhood controls. Each patient was asked to bring a healthy, sex- and age-matched control, and blood withdrawal of both patient and control took place at the same time. Pre-analytic processes were identical, which was not the case in most of the previously published biomarker studies in CFS ^(29, 38). The third advantage of the current study is exclusion of patients who use medication, with the exception of oral contraceptives and paracetamol. CFS patients frequently use a significant amount of medication, in a recent study 64% of patients used complementary and alternative medicine ⁽⁵⁷⁾. Another study found that >90% of CFS patients use at least one drug or supplement, especially antidepressants, sedatives and muscle relaxants ⁽⁵⁸⁾.

A limitation of this study is the relatively small number of patients included measuring a large number of variables. However, this has been accounted for using the LASSO method for logistic regression, which is an elegant variable reduction method ⁽⁵⁹⁾. However, considering the large factor for optimism, the prediction model has to be interpreted with caution. Another limitation of the current study is the fact that IL-1 β was not measured in plasma samples, although the value of this measurement is limited as IL-1 β is usually undetectable in peripheral blood, even with the PEA method.

In conclusion, this study demonstrated increased IL-12p40 and CSF-1 concentrations in CFS patients in addition to a set of predictive biomarkers. There was no effect of anakinra on circulating cytokines other than IL-1Ra. As emphasized in this study, sample handling and diagnostic procedures are very important when measuring cytokines. Future studies should take this into account and in order to replicate findings, methods should be extensively reported.

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SI List of proteins included in the PEA analysis.

Full name	Abbreviation
Adenosine Deaminase	ADA
Artemin	ARTN
Axin-1	AXIN1
Beta-nerve growth factor	Beta-NGF
Brain-derived neurotrophic factor	BDNF
C-C motif chemokine 19	CCL19
C-C motif chemokine 20	CCL20
C-C motif chemokine 23	CCL23
C-C motif chemokine 25	CCL25
C-C motif chemokine 28	CCL28
C-C motif chemokine 3	CCL3
C-C motif chemokine 4	CCL4
C-X-C motif chemokine 1	CXCL1
C-X-C motif chemokine 10	CXCL10
C-X-C motif chemokine 11	CXCL11
C-X-C motif chemokine 5	CXCL5
C-X-C motif chemokine 6	CXCL6
C-X-C motif chemokine 9	CXCL9
Caspase-8	CAPS-8
CD40L receptor	CD40
CUB domain-containing protein 1	CDCP1
Cystatin D	CST5
Delta and Notch-like epidermal growth factor-related receptor	DNER
Eotaxin	CCL11
Eukaryotic translation initiation factor 4E-binding protein 1	4E-BP1
Fibroblast growth factor 19	FGF-19
Fibroblast growth factor 21	FGF-21
Fibroblast growth factor 23	FGF-23
Fibroblast growth factor 5	FGF-5
Fms-related tyrosine kinase 3 ligand	Flt3L
Fractalkine	CX3CL1
Glial cell line-derived neurotrophic factor	GDNF
Hepatocyte growth factor	HGF
Interferon gamma	IFN-gamma
Interleukin-1 alpha	IL-1 alpha

SI Continued

Interleukin-10	IL10
Interleukin-10 receptor subunit alpha	IL-10RA
Interleukin-10 receptor subunit beta	IL-10RB
Interleukin-12 subunit beta	IL-12B
Interleukin-13	IL-13
Interleukin-15 receptor subunit alpha	IL-15RA
Interleukin-17A	IL-17A
Interleukin-17C	IL-17C
Interleukin-18	IL-18
Interleukin-18 receptor 1	IL-18R1
Interleukin-2	IL-2
Interleukin-2 receptor subunit beta	IL-2RB
Interleukin-20	IL-20
Interleukin-20 receptor subunit alpha	IL-20RA
Interleukin-22 receptor subunit alpha-1	IL-22 RA1
Interleukin-24	IL-24
Interleukin-33	IL-33
Interleukin-4	IL-4
Interleukin-5	IL-5
Interleukin-6	IL-6
Interleukin-7	IL-7
Interleukin-8	IL-8
Latency-associated peptide transforming growth factor beta-1	LAP TGF-beta-1
Leukemia inhibitory factor	LIF
Leukemia inhibitory factor receptor	LIF-R
Macrophage colony-stimulating factor 1	CSF-1
Matrix metalloproteinase-1	MMP-1
Matrix metalloproteinase-10	MMP-10
Monocyte chemotactic protein 1	MCP-1
Monocyte chemotactic protein 2	MCP-2
Monocyte chemotactic protein 3	MCP-3
Monocyte chemotactic protein 4	MCP-4
Natural killer cell receptor 2B4	CD244
Neurotrophin-3	NT-3
Neurturin	NRTN
Oncostatin-M	OSM

SI Continued

Osteoprotegerin	OPG
Programmed cell death 1 ligand 1	PD-L1
Protein S100-A12	EN-RAGE
Signaling lymphocytic activation molecule	SLAMF1
SIR2-like protein 2	SIRT2
STAM-binding protein	STAMPB
Stem cell factor	SCF
Sulfotransferase 1A1	ST1A1
T cell surface glycoprotein CD6 isoform	CD6
T-cell surface glycoprotein CD5	CD5
Thymic stromal lymphopoietin	TSLP
TNF-beta	TNFB
TNF-related activation-induced cytokine	TRANCE
TNF-related apoptosis-inducing ligand	TRAIL
Transforming growth factor alpha	TGF-alpha
Tumor necrosis factor (Ligand) superfamily, member 12	TWEAK
Tumor necrosis factor	TNF
Tumor necrosis factor ligand superfamily member 14	TNFSF14
Tumor necrosis factor receptor superfamily member 9	TNFRSF9
Urokinase-type plasminogen activator	uPA
Vascular endothelial growth factor A	VEGF-A

Chapter 7

Pitfalls in Cytokine Measurements – Assessing Circulating TGF- β 1 in Chronic Fatigue Syndrome

Submitted for publication

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Abstract

Serum TGF- β 1 concentrations are reported to be elevated in chronic fatigue syndrome (CFS). However, measurement of circulating cytokines is a complex procedure and control of pre-analytical procedures is essential. The objective of the current study was to measure circulating TGF- β 1 concentrations in CFS patients compared to healthy controls, taking into account differences in pre-analytical procedures. Two cohorts of female CFS patients were included. In both studies patients were asked to bring a healthy, age-matched control. At baseline, TGF- β 1 levels were measured in plasma and additionally P-selectin, a marker of platelet activity, was determined in a subgroup of participants. 50 patients and 48 controls were included in cohort I, and 91 patients and 29 controls in cohort II. Within the cohorts there were no differences in TGF- β 1 concentrations. However, between the cohorts there was a large discrepancy, which appeared to be caused by differences in g-force of the centrifuges used. The lower g-force used in cohort II (1361g) caused more platelet activation, reflected by higher p-selectin concentrations, compared to cohort I ($p < 0.0001$). There was a correlation between TGF- β 1 and p-selectin concentrations ($r = 0.79$, $p < 0.0001$). These results demonstrate that control of pre-analytical procedures is an essential aspect when measuring circulating cytokines.

Introduction

Chronic fatigue syndrome (CFS) is an enigmatic disorder, in which patients suffer from incapacitating fatigue, pain and a series of associated symptoms⁽¹⁾. The complaints are not due to a known underlying disease, and the pathophysiology has not been elucidated. CFS may be provoked by infections (such as infectious mononucleosis), or by any of a series of other triggers. Because of the association with preceding infection, and the similarities in symptomatology with sickness behaviour that can be induced by proinflammatory cytokines⁽²⁾, many studies aiming to find abnormal cytokine regulation have been performed in CFS⁽³⁾. The picture that emerges from these studies is by no means uniform. The reasons for these discrepant findings are often obscure from the publications, but factors like age and gender of the patients, composition of the control groups, the robustness of the pre-analytical procedures (such as sampling, handling, centrifugation and storage) and the kind of assays used, probably play an important role. In a recent systematic review on cytokines in CFS⁽³⁾, the most consistent finding was elevation of the anti-inflammatory cytokine transforming growth factor β (TGF- β).

Recently we performed a large prospective cytokine study in female CFS patients. This study was methodologically robust, as the age- and gender-matched controls from the patient's neighborhood were recruited and bled at the same time and in the same place as the patients. In this way the pre-analytical handling of the samples was exactly the same. The results of this study, in which 92 inflammatory markers were measured simultaneously using the novel Proximity Extension Assay technique, are published elsewhere. In another cohort of CFS patients and similarly matched controls, we also sampled blood to measure inflammatory markers⁽⁴⁾. In the present report we demonstrate the relevance of stringent methods to control for variability that may arise during the pre-analytical phase. To this end we describe the results of TGF- β 1 measurements in these two CFS cohorts.

Methods

Patients

Two cohorts of patients with CFS were enrolled in this study. The first cohort (cohort I) consisted of patients who participated in a double-blind randomized controlled trial (RCT) on the effect of IL-1 inhibition on CFS-related symptoms ⁽⁵⁾. The study was conducted at the Department of Internal Medicine and Expert Centre for Chronic Fatigue (ECCF) of the RadboudUMC, Nijmegen, the Netherlands. Details of the study were described elsewhere ^(5,6). In short, 50 female patients between 18 and 59 years old who fulfilled the Center for Disease Control (CDC) criteria for CFS were enrolled ⁽¹⁾. Use of medication (with the exception of oral contraceptives and paracetamol) was not allowed. Each of the patients was asked to bring a healthy neighborhood control, without complaints of fatigue and within the same age range (± 5 years), to their first study visit.

The second cohort (cohort II) consisted of 91 female patients between 18 and 65 years fulfilling the CDC criteria for CFS. The inclusion and exclusion criteria have been described in detail elsewhere ⁽⁴⁾. Like for Cohort I, a proportion of patients was asked to bring a healthy neighborhood control matched for age and gender at their first study visit.

All participants provided written and oral informed consent before inclusion. The hospitals' ethics committee (Commissie Mensgebonden Onderzoek Regio Arnhem/Nijmegen) approved the study protocols (2014/025 and 2013/113). The study was performed in accordance with the declaration of Helsinki.

Questionnaires

Fatigue was measured in both patients and controls using the fatigue severity subscale of the checklist individual strength (CIS), which has been used frequently in CFS patients ⁽⁷⁾. Scores on the CIS-f can vary between 8 and 56, and a score ≥ 35 reflects severe fatigue.

Blood collection

Blood samples were collected at baseline at the outpatient clinic for Internal Medicine at the RadboudUMC in Nijmegen, the Netherlands from all patients of Cohort I. Samples of controls were collected simultaneously with those of patients. Venous blood was collected in EDTA tubes, and kept on ice until centrifugation which was performed within 2-3 hours. Plasma aliquots were then frozen at -80°C for a maximal duration of 2 years. For Cohort II, the sampling, handling and storage of the blood of patients and controls were done in a similar fashion at the Donders Centre for Neuroimaging in Nijmegen, the Netherlands.

Measurements of TGF- β and P-selectin

Total TGF- β 1 levels were measured by enzyme-linked immunosorbent assay (ELISA), as described in detail previously (R&D systems) ⁽⁸⁾. All samples were acid activated to activate latent TGF- β (1 M hydrochloric acid, 30 min, room temperature, neutralization with 1M NaOH, followed by direct analysis). All assays were performed on the same day using the same reagents.

P-selectin was measured in a proportion of samples by ELISA (R&D systems) according to the instructions of the manufacturer.

Statistical analysis

Study data were analyzed using IBM SPSS statistic package version 22. All continuous variables are presented as means and standard deviations (SD)/ standard error of the mean (SEM). For group comparison a students *t* test was used. Pearson's correlation was used for the correlation between TGF- β and P-selectin.

Results

The results of the TGF- β 1 measurements in the patients and controls from Cohort I are depicted in *figure 1A*. No differences in TGF- β 1 concentrations were found between patients and healthy controls. Likewise, the results of the measurements in Cohort II did not reveal differences between CFS patients and controls (*figure 1B*). However, we were greatly puzzled by the large differences in the TGF- β 1 concentrations found between Cohort I and Cohort II. As it is known that platelets are a rich source of TGF- β (9), we wondered whether the higher TGF- β 1 levels could be caused by higher number of (activated) platelets in Cohort II. To explore whether platelet activation differed between the two groups, we measured P-selectin as a platelet marker in a random selection of samples (10). The P-selectin concentrations differed greatly between the two cohorts ($p < 0.001$; *figure 2*). There was a strong correlation ($r = 0.79$, $p < 0.0001$) between the concentrations of TGF- β 1 and P-selectin. Differences in platelet activation could be explained by differences in the g-force of the centrifuges used at the two study locations. We detected that the g-force of these centrifuges differed greatly: 2959g at the University Medical Centre versus 1361g at the Donders institute.

Table 1 Baseline characteristics of chronic fatigue syndrome patients en healthy controls

	Cohort I		Cohort II	
	CFS (n=50)	HC (n=48)	CFS (n=91)	HC (n=29)
Age, years	31(10)	31(10)	34(11)	33(11)
Fatigue severity (CIS-f)	52(4)	20(11)	51(4)	17(7)

Figure 1 TGF- β 1 concentrations for patients and controls in cohort I and cohort II

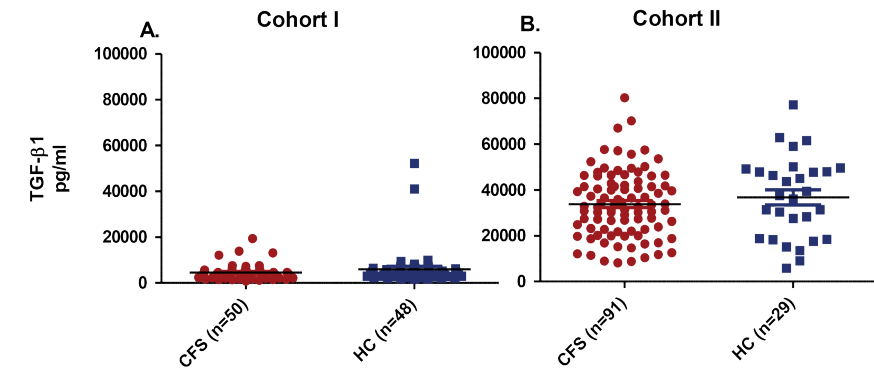
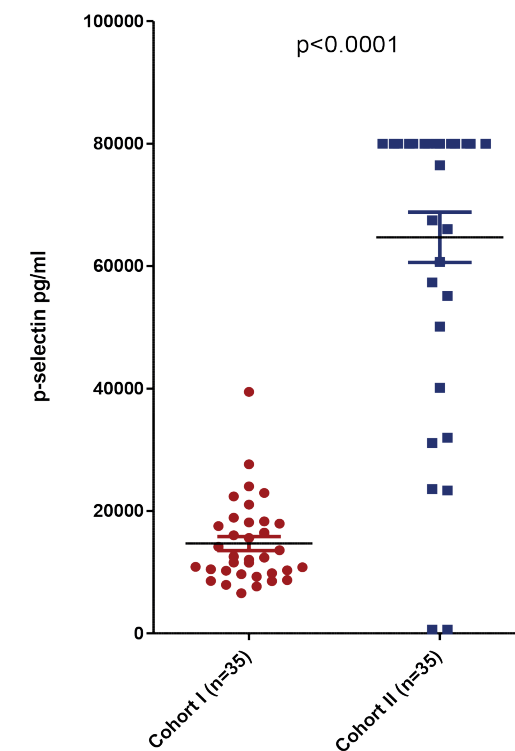


Figure 2 P-selectin concentrations for a proportion of patients of cohort I (n=35) and cohort II (n=35)



Discussion

In this report, we demonstrate that even with scrutinized methodology, inaccurate results may be obtained. It turned out that the different properties of the centrifuges used at the two study locations could be responsible for differences in platelet contamination and platelet activation (as assessed by P-selectin measurements).

Although it is well known that platelets contain considerable amounts of TGF- β ^(9, 11), this is often not taken into account when measuring circulating concentrations of this cytokine. Many cytokine studies do not mention the pre-analytical procedures of patient samples and controls. Our current data show that if for example another cohort of controls would have been used for cohort I, which would have had the sample pretreatment of cohort II, strong differences could have been observed, not due to actual differences caused by the underlying disease but solely by sample handling. The use of different sample collections for patients and controls is fairly common. In fact, in the studies that incriminated the retroviruses XMRV and XMLV ^(12, 13), this was the case and led to results that misled both the scientific community and more sadly, the patients suffering from CFS ⁽¹⁴⁾.

In conclusion, we want to make a plea for better standardisation of pre-analytical sample handling for patient studies, not only restricted to CFS research. The use of neighborhood controls who are bled at the same time and place, and whose samples undergo the exact same procedure as those of the patients is one method to enhance the quality of such research. In addition, precise reporting of the nature of the control group and the pre-analytical procedures of controls and patients is essential in these kinds of studies.

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Chapter 8

Hair and Salivary Cortisol in a Large Cohort of Chronic Fatigue Syndrome Patients

Submitted for publication

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Abstract

Hypocortisolism has been found in chronic fatigue syndrome (CFS) patients in blood, urine, and saliva. It is unclear if hypocortisolism can also be demonstrated using long-term cortisol measurements, such as cortisol in hair. In addition, the interaction between the HPA axis and the immune system, both expected to play an important role in CFS, is unclear. The objective of the current study was to compare hair and salivary cortisol concentrations in a large cohort of female CFS patients to those in healthy controls, and to test the effect of an interleukin-1 receptor antagonist (anakinra) on the HPA axis. Salivary cortisol concentrations of 107 CFS patients were compared to 59 healthy controls, with CFS patients showing a decreased cortisol awakening response (4.2 ± 5.4 nmol/L vs 6.1 ± 6.3 nmol/L, $p=0.036$). Total cortisol output during the day did not differ significantly in saliva, there was a trend to lower hair cortisol in a subset of 46 patients compared to 46 controls (3.8 ± 2.1 pg/mg vs 4.3 ± 1.8 pg/mg, $p=0.062$). After four weeks of treatment with either daily anakinra (100mg/day) or placebo, there was a slight decrease of hair cortisol concentrations in the anakinra group compared to an increase in the placebo group ($p=0.022$). This study confirms the altered dynamics of the HPA axis in a large group of CFS patients, and for the first time shows that this might also be present for long-term cortisol measures.

Introduction

Since the first publication by Demitrack and colleagues in 1991⁽¹⁾, numerous studies have been performed investigating alterations of the hypothalamic-pituitary-adrenal (HPA) axis in patients with chronic fatigue syndrome (CFS). This interest is predominantly caused by the overlap of CFS symptoms with diseases characterized by hypocortisolism such as Addison's disease, where 95% of patients report fatigue at diagnosis⁽²⁾. Several case definitions for CFS exist and, according to most of them, severe and persisting fatigue is its central feature⁽³⁾. The Center for Disease Control (CDC) criteria for CFS are most often used in research and require that at least four out of eight accompanying symptoms have to be present in addition to fatigue (e.g., joint pain, concentration problems, unrefreshing sleep, and post-exertional malaise)⁽⁴⁾.

Studies investigating the relationship between levels of cortisol, the end product of the stress-responsive HPA axis, and CFS thus far have been summarized in a review by Papadopoulos and Cleare⁽⁵⁾. Despite the large heterogeneity between studies, it was concluded that there is substantial evidence for hypocortisolism in CFS. This was also described in a meta-analysis by Tak et al.⁽⁶⁾, where a significantly decreased activity of the HPA axis was found, which was more frequent in studies predominantly including women. A potential mechanism underlying the pathophysiology of hypocortisolism in CFS is an heightened negative feedback response⁽⁷⁾, although it is unclear whether this is of etiological importance or is rather a consequence of for example the use of medication⁽⁶⁾, widespread pain⁽⁸⁾, or illness duration⁽⁹⁾.

Limitations of studies previously performed are the relatively small samples sizes and differences in the type of cortisol measurement. HPA axis deregulation can be manifested in disturbed short term dynamics of cortisol or alterations of the chronic cortisol secretion. Cortisol can be measured in various media, for example blood, saliva, urine, and hair. Saliva is usually depicted as the preferred method, as it offers insight into short term cortisol fluctuations (diurnal rhythm, response to a stimulus), which makes it a useful method to investigate the functional dynamics of the HPA axis. On the other hand, the hair cortisol concentration (HCC) offers information on long-term activity of the HPA axis⁽¹⁰⁾. An advantage of this measurement is that it is less influenced by situational factors, and may thus constitute a more stable measure⁽¹⁰⁾.

Another important issue when investigating the HPA axis is its interaction with other bodily systems, for example the immune system, where increased activity of the HPA axis has an inhibitory effect on the production of pro-inflammatory cytokines⁽¹¹⁾. In the context of CFS this is of particular interest, as alterations of inflammatory activity have been described

in CFS for many years ⁽¹²⁾. It is suspected that increased activity of pro-inflammatory cytokines, for example interleukin-1 (IL-1) and tumor necrosis factor (TNF), lead to the experience of typical CFS symptoms often described as sickness behavior ⁽¹³⁾. The attention for this subject is reflected by the large quantity of studies measuring circulating cytokines in CFS ⁽¹⁴⁾. In a complex illness, like CFS, it is likely that the interaction between the neuroendocrine system and the immune system is altered. A decreased inhibitory effect of dexamethasone on the immune system has already been established in adolescent CFS patients ⁽¹⁵⁾, but in adults the opposite has been found ⁽¹⁶⁾. A decreased signaling at the level of the glucocorticoid receptor (GR) on immune cells can eventually lead to a low-grade inflammatory state ⁽¹⁷⁾. Understanding the interaction between these systems in CFS, might lead to more targeted intervention strategies.

To expand the knowledge on HPA axis alterations in CFS patients, the aim of the current study was two-fold: the first aim was to investigate both dynamic (saliva) and long-term (hair) cortisol outcome measures in a large group of CFS patients as compared to controls, where it was expected to replicate earlier findings on salivary cortisol and for the first time investigate if this can be found in HCC as well. The second aim was to investigate the interaction between the HPA axis and pro-inflammatory cytokines. Therefore, the effect of the interleukin-1 receptor antagonist (IL-1Ra) anakinra on salivary and hair cortisol as compared to placebo treatment was assessed. It was hypothesized that decreasing inflammation would lead to less fatigue in CFS, improving activity and physical functioning, eventually leading to normalization of cortisol.

Methods

Patients

Patients included in the current study were participating in two separate trials that investigated the effect of two distinct interventions on fatigue severity, which were described in detail elsewhere ^(12, 18). Results on the behavioral effects of these therapies will be reported separately ⁽¹⁹⁾. In the study by van der Schaaf et al. ⁽¹⁸⁾, female patients, between 18 and 59 years old, were included when meeting the CDC consensus criteria for CFS ⁽²⁰⁾. In this study, the effect of cognitive behavioral therapy (CBT) on neuronal processes was assessed ('CBT study'). Main exclusion criteria were the presence of a psychiatric disorder (e.g. depression, anxiety), presence of a somatic disease that could explain severe fatigue, or the use of psychotropic medication. In the second study, inclusion and exclusion criteria were largely in accordance with the CBT study. In addition, patients were excluded when using any medication (with the exception of oral contraceptives and paracetamol). In both studies, patients were asked to be accompanied by a healthy female peer who served as a control at baseline. In the study by Roerink et al., patients were treated with either daily subcutaneous anakinra (100mg/day) or placebo injection for a duration of four weeks (anakinra study) ⁽¹²⁾.

For the current analysis, additional exclusion criteria were use of corticosteroids in any application form, the presence of fever on the days of saliva collection, and not adhering to the saliva collection protocol. For HCC, which was determined in patients participating in the anakinra study, samples were excluded when a patient had been sick or used antibiotics during the preceding month.

All patients provided written informed consent prior to participation. Both studies were approved by the local ethics committee.

Questionnaires

In both studies patients and controls were asked to fill out web-based questionnaires on fatigue severity and accompanying symptoms at baseline. Fatigue was measured with the fatigue subscale of the Checklist Individual Strength (CIS), which is a validated questionnaire used frequently in CFS research ⁽²¹⁾. The score can range between 0 and 56, with a score ≥ 35 reflecting severe fatigue ⁽²²⁾.

Impairment as a consequence of fatigue was measured with the Sickness Impact Profile (SIP8 total score), a score ≥ 700 reflects severe disability ⁽²³⁾. The presence of depressive symptoms was evaluated using the Becks Depression Inventory (BDI) primary care version ⁽²⁴⁾. Scores ≥ 4 indicate the presence of clinically relevant levels of depressive symptoms. Only patients

participating in the anakinra study were asked to complete a visual analog scale (VAS) on pain severity, with a range between 0 (no pain) and 10 (worst pain ever).

In addition to the questionnaires, the following information was collected from all patients: age, height, weight, duration of symptoms, use of medication, and menopausal state.

Salivary cortisol

Participants were instructed to collect saliva on two consecutive working days using the passive drool method by using Salicap® devices (IBL, Hamburg, Germany) consisting of a collection tube and a straw. Thirty minutes before collection, patients were asked to refrain from eating, drinking, and taking medication. During the day, four saliva samples were collected; at awakening, 30 minutes after awakening (± 15 min), at noon (between 11 a.m. and 1.15 p.m.), and in the evening (between 7 p.m. and 9.30 p.m.). Participants were asked to note date and exact time of sampling on the label of the respective Salicap® tube. Participants were instructed to store samples in their home freezer before bringing them to the hospital (after several days-weeks). At the hospital, all samples were stored at least at a temperature of -20°C until analysis. Salivary cortisol concentrations were determined by using commercially available enzyme-linked immunosorbent assays (ELISA; IBL, Hamburg, Germany). After completion of therapy in the anakinra study, the collection procedure was repeated.

Hair sampling

Participants in the anakinra study provided hair samples for determination of HCC at baseline (patients and controls) and after treatment (patients only). Small hair strands were taken from the posterior vertex region. As the average hair growth rate is 1cm/month⁽²⁵⁾, cortisol was measured in 1cm before and after treatment to evaluate the effect of the intervention. All hair samples were kept at room temperature at a dark place until analysis. For HCC analysis, washing and extraction procedures were applied according to the laboratory protocol originally described by Stalder et al.⁽²⁶⁾, using 10mg finely cut hair for cortisol extraction. HCC concentrations were determined by using a commercially available luminescence immunoassay (LIA; IBL, Hamburg, Germany), using 50 μL sample and standards for analysis⁽²⁷⁾.

Statistical analysis

All statistical analyses were conducted using SPSS version 22.0 (IBM Corp., Armonk, NY). For baseline variables, continuous variables are displayed as mean \pm standard deviation or median [range] and categorical variables as number (percentage).

For each saliva collection day, three cortisol output measures were calculated:

- I. Cortisol awakening response (CAR), which reflects the increase in cortisol concentrations in the first 30-45 minutes post-awakening⁽²⁸⁾. The CAR was calculated subtracting the awakening sample from the +30 minutes sample.
- II. Area under the curve with respect to ground (AUCg), which was calculated as described earlier by Pruessner et al.⁽²⁹⁾.
- III. Area under the curve with respect to increase (AUCi), which reflects cortisol alterations during the day. AUCi was also calculated using a formula earlier described⁽²⁹⁾.

All samples not collected in the predefined time-range were excluded. In case of missing values, data from the other collection day of the same time-point were imputed whenever possible. The AUCg and AUCi could only be calculated when all four measurements were available. The average of the two collection days for each time-point was used for comparison between groups (CFS patients vs. healthy controls).

To compare patients and controls at baseline, a student's *t* test was used for continuous variables. Since salivary cortisol and HCC showed a skewed distribution, a non-parametric Mann-Whitney test was used. For categorical variables, Pearson's chi-square test was applied. The correlation between cortisol outcomes and fatigue severity, illness duration, and pain was calculated using Pearson's correlation. For evaluation of the effect of anakinra or placebo on salivary and HCC levels, analysis of covariance was used (ANCOVA) for each outcome measure. The outcome measure at four weeks was entered as the dependent variable, treatment as fixed factor, and baseline measurement as covariate.

Results

Subject characteristics

A total of 136 patients and 72 healthy controls collected saliva samples. After exclusion of those patients that used corticosteroids (n=15), had fever while collecting saliva (n=2), did not adhere to the collection protocol (n=14), did not provide baseline samples (n=6), or did not provide enough data to calculate the CAR or AUC (n=5), complete information was available for 107 CFS patients and 59 healthy controls. Between trials, there were no differences with respect to age, BMI, fatigue severity, disabilities or depressive symptoms (data not shown). In the CBT study, participating patients had a significantly longer illness duration compared to those participating in the anakinra study (60[12-276] months vs. 42[7-109] months, $p=0.029$). Baseline characteristics for patients and controls are displayed in **table 1**. CFS patients had significantly higher CIS-fatigue scores (52 ± 4 vs. 20 ± 10 , $p<0.001$) and a higher BDI score (4 ± 3 vs. 1 ± 2 , $p<0.001$) than controls. There were no differences with respect to age, BMI, and menopausal state. In addition, use of oral contraceptives was not different between groups.

Table 1 Baseline characteristics of chronic fatigue syndrome patients (CFS) and healthy controls (HC)

	CFS (n=107)	HC (n=59)
Age, years	33±11	31±10
Body mass index, kg/m ²	25±5	25±4
Illness duration, months	47[7-276]*	N/A
Use of oral contraceptives, n (%)	46 (43)	22 (37)
Postmenopausal, n (%)		
Yes	4 (4)	3 (5)
No	100 (93)	55 (93)
Unknown	3 (3)	1 (2)
Fatigue severity (CIS-fatigue)	52±4	20±10
Disabilities (SIP)	1741±588	N/A
Depressive symptoms (BDI)	4±3	1±2

Table 1 Data are number (%), mean (SD), or median [range]. BDI= Becks depression inventory. CIS= checklist individual strength. N/A= not applicable. SIP= Sickness impact profile. *illness duration missing n=2

Baseline hair and salivary cortisol

In CFS patients non-adherence to the protocol, and thus exclusion of that particular sample, was present for 10% of the saliva samples. The value from the other day could be imputed in 73% of these samples. For controls these numbers were 8% and 72%, respectively. In **figure 1**, baseline cortisol values are displayed for patients and controls. In CFS patients, there was a significantly lower CAR (4.2 ± 5.4 nmol/L vs. 6.1 ± 6.3 nmol/L, $p=0.036$). For the AUCg and AUCi there were no differences between groups.

Figure 1 Baseline salivary cortisol outcome measures of CFS patients and healthy controls

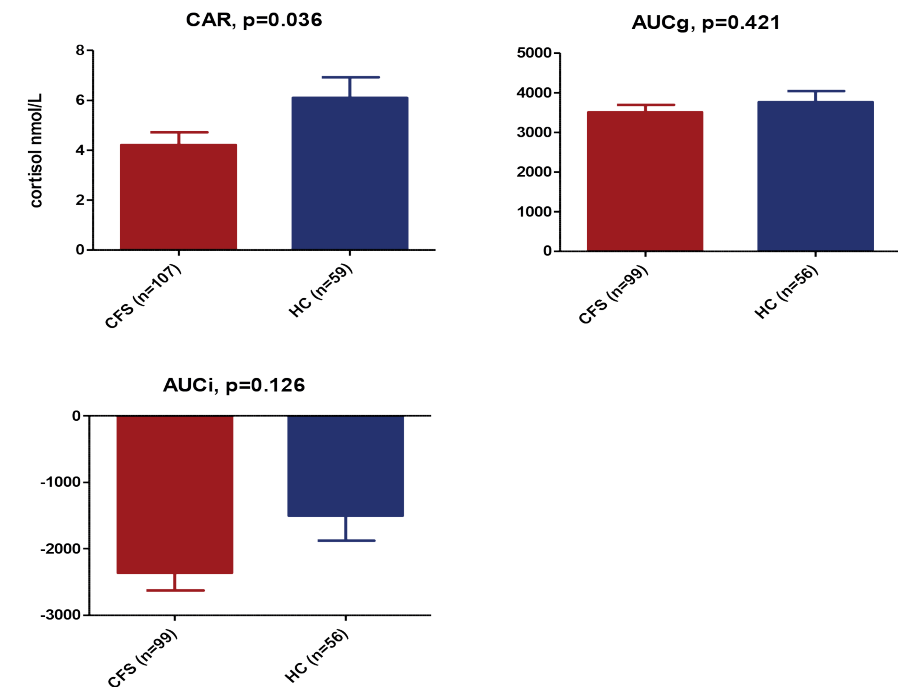


Figure 1 The upper panel displays mean concentrations±SEM (nmol/L) of the cortisol awakening response for CFS patients and controls. The lower two panels display mean ±SEM values for the ‘area under the curve with respect to ground’ (AUCg) and ‘area under the curve with respect to increase’ (AUCi) for both groups.

In patients participating in the anakinra trial HCC reflecting the past month was analyzed. After exclusion of patients who were ill (n=2) or used medication (n=2) in the month preceding hair collection and those patients that provided an insufficient amount of hair to perform the analysis (n=2), results were available for 46 patients and 46 controls. In CFS patients, there was a non-significant trend for lower HCC (3.8 ± 2.1 pg/mg vs 4.3 ± 1.8 pg/mg, $p=0.062$, *figure 2*).

Correlation cortisol with fatigue severity, illness duration, and pain severity

A negative correlation was present between basal pain levels and both the CAR ($r=-0.30$, $p=0.036$) and the AUCg ($r=-0.39$, $p=0.010$) in CFS patients. There was no significant correlation between the AUCi or HCC and pain (respectively $r=0.06$, $p=0.69$ and $r=-0.05$, $p=0.73$). Fatigue severity and duration of symptoms did not correlate with any of the cortisol measures (data not shown).

Influence of anakinra on hair and salivary cortisol

The interaction between the HPA axis and inflammation was assessed in patients participating in the anakinra study. *Table 2* displays changes in cortisol concentrations in saliva and hair after four weeks of treatment with either anakinra or placebo. For salivary cortisol there were no differences between groups. In the anakinra group there was a slight decrease in HCC (-0.18 ± 0.95 pg/mg) compared to patients treated with placebo where there was an increase in HCC (0.49 ± 0.89 pg/mg, $p=0.022$). *Figure 3* displays the change in hair cortisol after treatment for both groups.

Figure 2 Baseline hair cortisol outcome measures of CFS patients and healthy controls

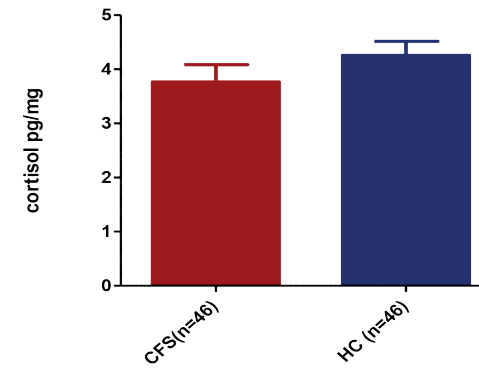


Figure 2 displays mean(±SEM) hair cortisol concentrations (pg/mg) for CFS patients and controls at baseline.

Table 2 Treatment effect of anakinra or placebo on main cortisol outcome measures

Outcome	Anakinra	Placebo	P-value
CAR	-2.38±5.56	-1.22±6.62	0.52
N	22	25	
AUCg	-430.01±1482.14	-483.95±1124.71	0.91
N	17	17	
AUCi	-830.82±3333.07	-995.56±1932.37	0.86
N	17	17	
HCC	-0.18±0.95	0.49±0.89	0.022
N	21	21	

Table 2 displays mean (±SD) change for both groups as compared to baseline.

Figure 3 Change of hair cortisol after four weeks of treatment with either anakinra (n=22) or placebo (n=21).

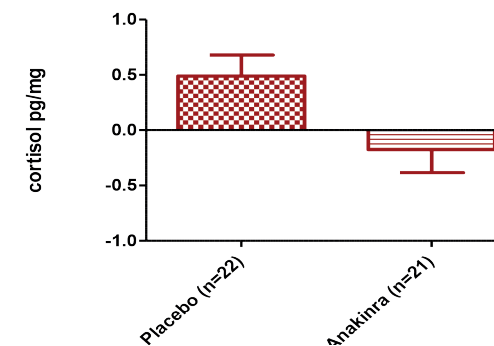


Figure 3 displays mean(±SEM) decrease of hair cortisol concentration (pg/mg) after treatment with either anakinra or placebo for a duration of four weeks.

Discussion

In the current study, hair and salivary cortisol in a large cohort of female CFS patients was compared to healthy controls. To the best of our knowledge, this is the first study that has investigated HCC in CFS. At baseline, CFS patients had a significantly lower CAR and a trend to lower HCC compared to healthy controls. There were no baseline differences for the AUCg and AUCi. In addition, patients treated with anakinra had a slight decrease of HCC after treatment compared to patients treated with placebo where an increase of HCC was observed.

An attenuated cortisol awakening response has been found previously in both adolescents⁽³⁰⁾ and adults⁽³¹⁾ with CFS. Cortisol response after awakening has been described in several studies investigating physical and mental complaints⁽³²⁾ and has been associated with fatigue later during the day in healthy adults⁽³³⁾, and in conditions characterized by (chronic) pain⁽³⁴⁾. In the current study, the correlation between cortisol outcome measures and CFS-related symptoms was assessed, which confirmed that there was a negative correlation between basal pain levels and the CAR. However, CAR was not associated with any CFS-related measures such as fatigue severity or duration of symptoms.

With respect to the AUCg, a measure for total cortisol output during the day, we did not find any differences between groups. This is in contrast with previous studies that found decreased basal cortisol levels using this measure in CFS patients⁽⁶⁾. A possible explanation for the absence of a difference could be the strict exclusion criteria used in the current study.

Despite the absence of a difference in total cortisol output in saliva, there was a trend to lower HCC in CFS patients. It has been described that salivary cortisol is a reliable method to measure free circulating cortisol, but it is subject to day-to-day variation and the patients' compliance to the collection instructions⁽³⁵⁾. HCC might be a more robust method to assess long-term/accumulated cortisol concentrations, and has shown a considerable intra-individual stability/reproducibility⁽³⁶⁾. In CFS, HCC has not been assessed previously but lower concentrations have been found in patients with chronic stress conditions such as generalized anxiety disorder (GAD) and adults with a history of childhood trauma⁽³⁷⁾. The study in GAD patients also found no differences in total saliva cortisol, but did find lower HCC, which stresses the importance of long-term cortisol assessment. A possible explanation for this discrepancy is that stress as a consequence of saliva sampling, that might have a larger influence on patients, influences acute cortisol levels leading to a false-high AUCg⁽³⁸⁾.

With respect to the interaction of the inflammatory system and the HPA axis there are several hypotheses in CFS. One theory within literature focusing on neuro-humoral alterations, is that the combination of decreased circulating cortisol⁽⁶⁾ and decreased cortisol sensitivity of immune cells⁽¹⁵⁾ can lead to a low-grade inflammatory state^(17,39). This is discussed to be a consequence of the known inhibitory effect of cortisol on pro-inflammatory cytokines, which partially acts through decreased 'nuclear factor kappa-light-chain-enhancer of activated B cells' (NFκB) activation⁽¹¹⁾. The anakinra study was aimed at solely intervening at the level of the immune alterations⁽¹²⁾. It was hypothesized that decreasing inflammation with anakinra, an interleukin-1 receptor antagonist (IL-1Ra), would lead to less fatigue, and as a consequence normalization of cortisol concentrations. However, against our initial expectations, a slight decrease in HCC was found in patients treated with anakinra and an increase in placebo treated patients, which makes this theory less likely. This is in line with the known stimulating effect of IL-1 on the HPA axis, a response that is reversible by using IL-1Ra⁽⁴⁰⁾. Thus, the results of this study only provide preliminary insight into the complex interaction between these regulatory mechanisms, and additional research is needed.

This study has several strengths. To our knowledge, this is the largest study measuring salivary cortisol in a cohort of CFS patients and matched controls. Previous studies of similar magnitude were, for example, performed using urine samples⁽⁴¹⁾, or did not include healthy controls⁽⁴²⁾. In addition, cortisol concentrations were determined in hair in a subgroup of patients and controls, a method proven to be of additional value in several various conditions such as mental health (e.g., depression⁽⁴³⁾), and in pregnancy⁽⁴⁴⁾.

A limitation of this study is the inclusion of two separate cohorts of patients. However, in both cohorts there were no important differences in main in- and exclusion criteria, as both studies included female CFS patients of the same age. The only variable that differed between samples, duration of symptoms, did not influence cortisol outcome measures. In addition, the protocol used for saliva collection, storage, and cortisol analysis was identical. Therefore, we do not believe this could have influenced the outcome. Another limitation is non-compliance of patients while performing the saliva sampling at home. Although collection times were recorded and strict criteria were applied, only slight deviations from the protocol can already influence for example the CAR⁽⁴⁵⁾. Fortunately, this is no problem when measuring HCC. Third, as this was no prospective study, it is still unknown if hypocortisolism has a pathophysiological role or is a consequence of CFS. However, a previous prospective study in patients presenting with infectious mononucleosis, could not find a relation between saliva cortisol and the development of fatigue⁽⁴⁶⁾.

In conclusion, in female CFS patients without comorbid depression, a lower CAR was found compared to healthy controls. In addition, there was a trend to lower HCC in patients. Especially the latter has important implications for future studies. When lower HCC can be confirmed in other studies, it might be used as a more stable and reliable tool for the evaluation of the HPA axis in CFS. Earlier studies found that urinary cortisol is able to predict response to CBT⁽⁴⁷⁾, and that salivary cortisol normalizes with adequate treatment⁽⁴⁸⁾. However, in the light of the previously mentioned limitations of salivary cortisol, HCC might be an interesting alternative to predict the effect of treatment. Although the current study did not unravel the exact mechanisms of neuro-humoral and inflammatory interactions in CFS, our findings provide preliminary insight into how an interleukin-1 receptor antagonist might affect endocrine functioning.

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Chapter 9

Postural orthostatic tachycardia is not a useful diagnostic marker for chronic fatigue syndrome

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Abstract

Background: Postural orthostatic tachycardia syndrome (POTS) is considered a diagnostic marker for chronic fatigue syndrome (CFS).

Objectives: The aims of this study were to (i) compare POTS prevalence in a CFS cohort with fatigued patients not meeting CFS criteria, and (ii) assess activity, impairment and response to cognitive behavioral therapy (CBT) in CFS patients with POTS (POTS-CFS) and without POTS (non-POTS-CFS).

Methods: Prospective cohort study at the Radboud University Medical Center in The Netherlands. Between June 2013 and December 2014, 863 consecutive patients with persistent fatigue were screened. Patients underwent an active standing test, filled out questionnaires and wore an activity-sensing device for a period of 12 days.

Results: A total of 419 CFS patients and 341 non-CFS fatigued patients were included in the study. POTS prevalence in adult CFS patients was 5.7% versus 6.9% in non-CFS adults ($P = 0.54$). In adolescents, prevalence rates were 18.2% and 17.4%, respectively ($P = 0.93$). Adult POTS-CFS patients were younger (30 ± 12 vs. 40 ± 13 years, $P = 0.001$) and had a higher supine heart rate (71 ± 11 vs. 65 ± 9 beats/min, $P = 0.009$) compared with non-POTS-CFS patients. Severity and activity patterns did not differ between groups. In CFS patients, criteria for Systemic Exertion Intolerance Disease (SEID) were met in 76% of adults and 67% of adolescents. In these CFS patients fulfilling the SEID criteria, the prevalence of POTS was not different from that in the overall CFS population. POTS-CFS adolescents had less clinically significant improvement after CBT than non-POTS-CFS adolescents (58% vs. 88%, $P = 0.017$).

Conclusion: In adults with CFS, the prevalence of POTS was low, was not different from the rate in non-CFS fatigued patients, and was not related to disease severity or treatment outcome. In POTS-CFS adolescents, CBT was less successful than in non-POTS-CFS patients. The evaluation of POTS appears to be of limited value for the diagnosis of CFS.

Introduction

Chronic fatigue syndrome (CFS) is a debilitating disease with a prevalence ranging between 200 and 1400 per 100,000 persons in both Europe and the USA⁽¹⁻⁴⁾. With fatigue as the most characteristic symptom, CFS patients often report a variety of other symptoms, such as unrefreshing sleep, post-exertional malaise and headaches⁽⁵⁾. More than 25% of patients report having had symptoms more than 5 years before being diagnosed with CFS⁽¹⁾, which illustrates the difficulties encountered in the diagnostic process. Trying to overcome this problem, several research groups have proposed new CFS criteria⁽⁵⁻⁷⁾ since the first case definition published by the Center for Disease Control and Prevention (CDC) in 1988⁽⁸⁾. However, despite the use of different case formulations, none of these diagnostic methods has been sufficiently tested⁽⁹⁾, and substantial heterogeneity remains in addition to difficulties in distinguishing fatigue caused by CFS from other fatigue-causing illnesses and idiopathic fatigue.

In an attempt to improve the diagnostic process, the Institute of Medicine (IOM) recently proposed a new case definition⁽¹⁾, with a new name to describe the illness more accurately: systemic exertion intolerance disease (SEID). The aim of this new definition is a better understanding of CFS, with objective measurements to diagnose patients. In order to fulfill the SEID criteria, a patient must have three core symptoms: (i) severe fatigue that is present for at least 6 months leading to substantial impairment, (ii) post-exertional malaise and (iii) unrefreshing sleep. Additionally, a patient must be diagnosed with cognitive impairment, orthostatic intolerance (OI), or both. The advantage of including OI is that most forms can be diagnosed in an objective manner, by measuring heart rate and blood pressure during an active standing or head-up tilt test.

OI is defined as a syndrome in which patients report symptoms in upright position, with (delayed) orthostatic hypotension, reflex syncope and postural orthostatic tachycardia syndrome (POTS) as the most common clinical manifestations⁽¹⁰⁻¹³⁾; the latter is reported most frequently in the CFS population, and several studies have been conducted to evaluate the prevalence of POTS in CFS⁽¹⁴⁻¹⁶⁾. Based on the literature search conducted by the IOM, the prevalence of POTS is 27% in CFS patients compared to 4% in healthy control subjects. The studies included in the review report a wide variety in duration and type of orthostatic testing. The recommended duration of testing is at least 10 min^(10, 13), although several of the reviewed studies took into account only the first few minutes^(14, 16).

POTS is defined as an increase in heart rate of at least 30 beats/min (bpm) or an increase to 120 bpm within the first 10 min after attaining an upright position in the absence of orthostatic hypotension^(12, 13). POTS is predominantly reported in women⁽¹⁷⁾, and

hypovolemia, deconditioning and increased sympathetic activity have been described as possible pathophysiological mechanisms^(17, 18). Some studies have shown that CFS patients with POTS (POTS-CFS patients) have shorter symptom duration, are younger⁽¹⁴⁾ and report less severe fatigue than CFS patients without POTS (non-POTS-CFS patients)⁽¹⁵⁾, although others did not find differences with respect to symptom severity⁽¹⁹⁾. If POTS in CFS could be explained by deconditioning, POTS-CFS patients would be expected to be less physically active. However, to our knowledge, this has never been investigated. Furthermore it is unknown whether POTS-CFS patients respond differently, compared with non-POTS-CFS patients, to cognitive behavioral therapy (CBT), which is an evidence-based treatment for CFS^(20, 21). Before incorporating OI and POTS into a new case definition, the impact of POTS on CFS-related symptoms and treatment outcome should be known.

To overcome these problems, a large CFS population was studied prospectively to evaluate whether the prevalence of POTS is in accordance with the IOM report. As recommended, testing duration was longer than 10 min in all patients⁽¹³⁾. POTS prevalence was also determined in patients with persistent fatigue who did not meet the CDC consensus for CFS (non-CFS patients), to evaluate the extent to which the presence of POTS discriminates between CFS and non-CFS fatigued patients. The prevalence of POTS in CFS patients fulfilling the SEID criteria was also evaluated. Furthermore, we examined whether POTS-CFS patients report more symptoms associated with POTS (e.g. dizziness), are more fatigued or impaired, have a lower level of physical activity or respond differently to CBT compared with non-POTS-CFS patients.

Materials and methods

Study design and participants

Between June 2013 and December 2014, all patients consecutively referred to the Expert Center for Chronic Fatigue (ECCF) at the Radboud University Medical Center underwent a standardized blood pressure measurement as part of their clinical assessment. Furthermore, patients filled out web-based questionnaires, and during a period of 2 weeks kept a diary recording symptoms and wore a motion-sensing device (actometer)⁽²²⁾. CFS patients were included following diagnosis with CFS according to the CDC criteria⁽⁵⁾. In this patient category, the proportion of patients fulfilling the SEID criteria, omitting the criterion of OI, was also evaluated. Non-CFS fatigued patients were included if they reported persistent fatigue not meeting the CDC consensus criteria for CFS. Patients were excluded if blood pressure measurement was not conducted in accordance with the protocol, or if they had not filled in the questionnaires.

The local institutional ethics committee approved the present study. Informed consent was not required because all measurements were performed as part of routine care.

Blood pressure measurement

Blood pressure measurement and POTS identification were performed using an active standing test. First, heart rate and blood pressure were recorded at 2- to 3-min intervals while the patient was in the supine position for 15 min using an electronic automated device (Mobil-O-Graph, I.E.M., Stolberg, Germany). Next, patients were instructed to stand upright without assistance and remain in this position for the next 14–18 min. After standing for 5–8 min, blood pressure measurement was resumed for the remaining period. POTS was defined as an increase in heart rate of at least 30 bpm compared to the supine position, or a standing heart rate of ≥ 120 bpm for any of the measurements within the first 10 min after attaining an upright position⁽¹³⁾. Supine blood pressure and heart rate were calculated using the mean of the last five measurements before standing. The first three standing measurements were used to determine the mean blood pressure and heart rate in the upright position. Only patients without signs of orthostatic hypotension within the first 10 min, defined as a decrease in systolic blood pressure of ≥ 20 mmHg or diastolic blood pressure of ≥ 10 mmHg, could be identified as having POTS.

Actometer and diary score

The actometer (©Actilog V3.0) is a motion-sensing device, which is worn at the ankle during 12 consecutive days and nights as part of the standard diagnostic work-up at our center. The small, light (26 g) device contains a sensor that is sensitive in three directions^(22, 23). When an acceleration passes the predefined threshold, the motion is registered as

activity. The acquired information can be used to calculate a mean activity score during the time awake; this score is expressed as the average number of accelerations per 5-min period. The mean activity score over 12 days for CFS patients has been reported to be 66 ± 22 ⁽²³⁾. When patients scored above 66 on not more than 1 day out of the 12-days, they are labeled as low active; a score of >66 on 2–9 days and on 10–12 days is considered relatively active and highly active, respectively.

During the same 12-day period, patients kept a diary recording symptoms four times daily. Patients recorded the presence of concentration problems, decreased memory, visual problems, dizziness, shortness of breath, headache, constipation/diarrhoea, feeling tense, irritability and nausea. For each time point, patients reported symptoms to be present or absent. A mean symptom prevalence score per time point over 12 days was calculated using the formula: number of days the symptom was present divided by the number of days the diary was kept, multiplied by 100 ⁽²⁴⁾. The mean of the four different time points was used to calculate the total symptom score.

Questionnaires

Fatigue severity was measured using the subscale fatigue severity of the Checklist Individual Strength (CIS-f). Scores range between 8 and 56 (eight items, 7-point Likert scale). The CIS is often used to measure fatigue in CFS patients and has excellent psychometric characteristics ⁽²⁵⁾. A score of ≥ 35 reflects the presence of severe fatigue ⁽²⁶⁾. CIS-f scores were also collected for patients after completing CBT. The presence of additional CFS symptoms as defined by the CDC case definition during the previous 6 months was also assessed with a questionnaire. The level of functional impairment was measured using the Sickness Impact Profile (SIP8) total score. The SIP evaluates disease-related physical and mental disabilities ⁽²⁷⁾. The presence of depressive symptoms was measured using the Becks Depression Inventory (BDI) primary care version, the score of which can range between 0 and 28 ⁽²⁸⁾.

Other variables collected using questionnaires were age, gender, body mass index, duration of symptoms, use of medication (opioids, sleep medication, and antidepressant, anxiolytic, anticonvulsive, antipsychotic, antihypertensive and stimulant drugs) and presence of comorbid diseases.

Statistical analysis

Blood pressure data, acquired with Hypertension Management software 3.0 (I.E.M.), were exported to an Access database (Microsoft, Redmond, WA, USA) and combined with other relevant variables. All statistical analyses were conducted using IBM SPSS statistics version 22. All continuous variables are presented as means and standard deviations (SD)

or medians and ranges, and categorical variables as percentages. For all reported variables, POTS-CFS patients were compared to non-POTS-CFS patients. Furthermore, CFS patients were compared to non-CFS patients with respect to fatigue severity, disabilities and blood pressure-related characteristics. A final comparison was made between CFS patients and those CFS patients who fulfilled the SEID criteria with respect to POTS prevalence. All categorical variables were compared using Pearson's chi-squared or Fisher's exact tests, depending on the observed frequency within each category. Continuous variables were compared using an independent Student's *t* test. Statistical significance was set at 5% ($P < 0.05$). To correct for multiple testing, a Bonferroni correction was used when comparing use of medication, presence of comorbid disorders and diary scores between groups.

Results

During the study period, 863 fatigued patients underwent blood pressure measurement at the ECCF and filled in questionnaires. Overall, 103 patients were excluded because the blood pressure measurement was not appropriate to determine the presence of POTS: no measurements in the standing position ($n = 34$), first standing measurement after 10 min ($n = 35$), standing measurement <10 min ($n = 33$) or no repeated measurement in the supine position ($n = 1$). Of the 760 remaining patients, 419 were diagnosed with CFS (331 adults, 88 adolescents) and 341 could not be diagnosed with CFS (318 adults, 23 adolescents) due to fatigue as a consequence of a psychiatric illness ($n = 76$) or a chronic somatic illness ($n = 41$), fatigue after cancer treatment ($n = 57$) or fatigue not fulfilling the CDC consensus with respect to severity or accompanying symptoms ($n = 167$). In those patients who fulfilled the CFS criteria, the SEID criteria were met in 75.8% of adults (251/331) and 67.0% of adolescents (59/88).

POTS prevalence in CFS, SEID and non-CFS patients

The prevalence of POTS was 8.4% (35/419) within the total CFS population, 5.7% for adults (19/331) and 18.2% for adolescents (16/88). The POTS prevalence was not different between patients with ($BDI \geq 4$) and without a clinically significant level of depressive symptoms (5.8% vs. 5.7%, $P = 0.96$). In CFS patients fulfilling the SEID criteria, POTS was present in 5.2% of adults (13/251) and 22.0% of adolescents (13/59), which was not significantly different from the POTS prevalence in the overall CFS population ($P = 0.77$ and $P = 0.57$, respectively). In the non-CFS fatigued population, POTS was present in 6.9% (22/318) of adults and 17.4% (4/23) of adolescents, which was not statistically different from CFS patients ($P = 0.54$ and $P = 0.93$, respectively).

Comparison of CFS and non-CFS patients: fatigue, disability and blood pressure

With respect to age, there were no differences between adult CFS and non-CFS patients (40 vs. 40 years, $P = 0.82$), as shown in **table 1**. This could neither be found for adolescent CFS nor for non-CFS patients; mean ages were respectively 16 and 17 years ($P = 0.056$). Although non-CFS patients were severely fatigued with a mean CIS-f of 48, they were less fatigued than CFS patients (mean CIS-f 50, $P < 0.001$). This was also true for adolescent non-CFS and CFS patients (mean CIS-f 44 vs. 50, $P = 0.001$). Furthermore, CFS patients were more impaired than non-CFS patients (mean SIP total score 1548 vs. 1198, $P < 0.001$). Mean blood pressure and heart rate in the supine and upright positions did not differ between groups.

Table 1 Comparison of clinical variables between CFS and non-CFS patients

Characteristic	Adolescent CFS ($n = 88$)	Adolescent non-CFS ($n = 23$)	P-value	Adult CFS ($n = 331$)	Adult non-CFS ($n = 318$)	P-value
Female	79 (90%)	20 (87%)	0.70	224 (68%)	209 (66%)	0.60
Age, years	16 (1)	17 (2)	0.056	40 (13)	40 (13)	0.82
CIS fatigue	50 (4)	44 (8)	0.001	50 (5)	48 (7)	<0.001
SIP	-	-	-	1548 (599)	1198 (699)	<0.001
Blood pressure, mmHg						
Supine position						
Systolic BP	114 (9)	115 (10)	0.87	123 (14)	124 (14)	0.177
Diastolic BP	66 (7)	67 (7)	0.61	78 (11)	78 (11)	0.58
Heart rate, bpm	68 (10)	68 (13)	0.91	65 (9)	67 (12)	0.054
Standing position						
Systolic BP	119 (11)	121 (17)	0.48	126 (14)	127 (14)	0.48
Diastolic BP	75 (8)	75 (8)	0.96	85 (11)	85 (11)	0.72
Heart rate, bpm	88 (12)	88 (16)	0.96	80 (13)	81 (14)	0.46

Table 1 Data were available for all patients, unless stated otherwise, and are shown as number (%) or mean (standard deviation).

CFS, chronic fatigue syndrome; non-CFS, fatigue but not meeting the CDC consensus for CFS; CIS, Checklist Individual Strength; SIP, Sickness Impact Profile; BP, blood pressure; bpm, beats/min.

Comparison of POTS-CFS and non-POTS-CFS patients: demographics and blood pressure

Table 2 summarizes the comparison of demographic and blood pressure-related characteristics between POTS-CFS and non-POTS-CFS patients. Among adults, female patients were equally distributed between the two groups, and POTS-CFS patients were significantly younger (30 vs. 40 years, $P = 0.001$). Furthermore, heart rate in the supine position was higher in POTS-CFS patients (71 vs. 65 bpm, $P = 0.009$), which as expected was also found while standing (102 vs. 79 bpm, $P < 0.001$). Other variables did not differ between groups, although POTS-CFS patients tended to have a lower blood pressure in the supine position than non-POTS-CFS patients. When comparing adolescents, the only variable that differed between groups was heart rate in the standing position, which was higher in POTS-CFS patients (99 vs. 86 bpm, $P < 0.001$).

Table 2 Comparison of baseline characteristics and blood pressure between POTS-CFS and non-POTS-CFS patients

Characteristic	Adolescent POTS-CFS (n = 16)	Adolescent CFS (n = 72)	P-value	Adult POTS-CFS (n = 19)	Adult CFS (n = 312)	P-value
Female	15 (94%)	64 (89%)	0.56	17 (89%)	207 (66%)	0.179
Age, years	16 (1)	16 (1)	0.60	30 (12)	40 (13)	0.001
BMI, kg/m ²	20.7 (3.2)	20.9 (3.9)	0.88	24.0 (5.6)	25.1 (4.7)	0.31
Fatigue duration*, months	30 [19–46]	24 [11–40]	0.23	43 [30–75]	67 [30–121]	0.117
Blood pressure, mmHg						
Supine position						
Systolic BP	112 (8)	115 (9)	0.28	117 (10)	123 (14)	0.073
Diastolic BP	65 (6)	66 (7)	0.69	73 (9)	78 (11)	0.072
Heart rate, bpm	68 (9)	68 (10)	0.88	71 (11)	65 (9)	0.009
Standing position						
Systolic BP	118 (11)	119 (11)	0.73	125 (15)	126 (14)	0.71
Diastolic BP	74 (8)	75 (8)	0.64	81 (12)	85 (11)	0.169
Heart rate, bpm	99 (8)	86 (11)	<0.001	102 (12)	79 (12)	<0.001

Table 2 Data were available for all patients, unless stated otherwise, and are shown as number (%), mean (standard deviation) or median [interquartile range].

POTS-CFS, chronic fatigue syndrome with postural orthostatic tachycardia syndrome; non-POTS-CFS, chronic fatigue syndrome without postural orthostatic tachycardia syndrome; BMI, body mass index; BP, blood pressure; bpm, beats/min.

*Data missing for one patient.

Comparison of POTS-CFS and non-POTS-CFS patients: symptoms, disability, activity patterns, comorbidity and use of medication

There were no significant differences between POTS-CFS and non-POTS-CFS patients with respect to fatigue severity, number of CDC symptoms, reported disabilities and depressive symptoms (**Table 3**). Mean activity scores also did not differ between groups. The prevalence of all 10 different symptoms assessed with the diary score was not significantly different between groups (data not shown).

A total of 10.5% ($n = 2$) of POTS-CFS adults used antidepressants, compared with 18.6% ($n = 58$) of non-POTS-CFS patients ($P = 0.54$); antihypertensive drugs were respectively used by 5.3% ($n = 1$) and 11.2% ($n = 35$, $P = 0.71$). These drugs were rarely used by adolescents (antidepressants, $n = 1$ CFS patient; antihypertensive drugs, $n = 1$ CFS patient). The presence of other medical conditions in addition to CFS was distributed equally between all groups (data not shown).

Table 3 Comparison of questionnaires and activity patterns between POTS-CFS and non-POTS-CFS patients

	Adolescent POTS-CFS (n = 16)	Adolescent CFS (n = 72)	P-value	Adult POTS-CFS (n = 19)	Adult CFS (n = 312)	P-value
CIS fatigue	51 (3)	50 (4)	0.45	50 (6)	50 (6)	0.71
SIP	-	-	-	1462 (602)	1553 (599)	0.52
BDI	-	-	-	3 (2)	3 (3)	0.71
CDC symptoms (n)*	6 (3)	5 (2)	0.21	7 (2)	7 (2)	0.82
Activity score†	64 (15)	66 (19)	0.83	64 (15)	67 (19)	0.51
Activity group						
Low	4 (25%)	22 (30%)	0.77	6 (33%)	77 (26%)	0.47
Relatively active	10 (63%)	40 (56%)	0.61	10 (56%)	162 (54%)	0.89
Highly active	2 (12%)	10 (14%)	0.88	2 (11%)	62 (20%)	0.54

Table 3 Data were available for all patients, unless stated otherwise, and are shown as number (%) or mean (standard deviation).

POTS-CFS, chronic fatigue syndrome with postural orthostatic tachycardia syndrome; non-POTS-CFS, chronic fatigue syndrome without postural orthostatic tachycardia syndrome; CIS, Checklist Individual Strength; SIP, Sickness Impact Profile; BDI, Becks Depression Inventory.

*Data missing for three patients; †data missing for 11 patients.

Therapy outcome

In December 2015, 62% of adults and 63% of adolescents had completed CBT; patients who did not finish CBT were still following therapy sessions, were referred for treatment outside the ECCF or decided not to start therapy after the diagnostic process. **Figure 1A** shows the mean decrease in CIS-f scores after treatment compared to scores before treatment for patients who completed CBT. **Figure 1B** shows the percentage of patients with a CIS-f score of ≤ 35 , which can be considered as no longer being severely fatigued ⁽²⁹⁾. Among adults, POTS-CFS and non-POTS-CFS patients responded similarly to CBT. However, fewer adolescents with POTS-CFS recovered after CBT compared with non-POTS-CFS patients (58% vs. 88%, $P = 0.017$).

Figure 1 Fatigue severity after completing cognitive behavioral therapy in POTS-CFS and non-POTS-CFS patients

Fig. 1a: mean decrease CIS-f after completing CBT

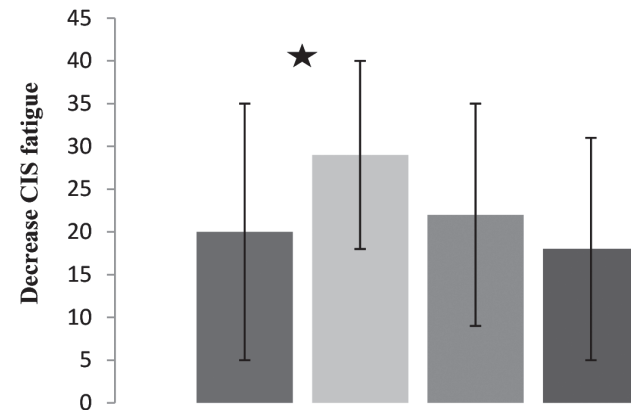


Fig. 1b: % CIS-fatigue ≤35

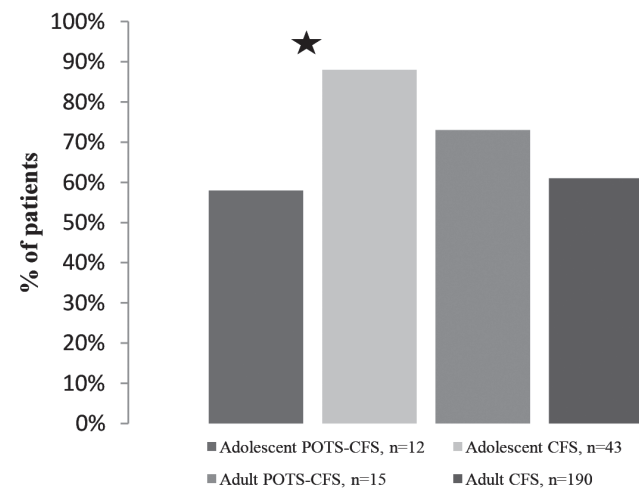


Figure 1 Fatigue severity after completing cognitive behavioral therapy (CBT) in patients with chronic fatigue syndrome (CFS) and postural orthostatic tachycardia syndrome (POTS-CFS) and in patients with CFS alone. a) Decrease in the subscale fatigue severity of the Checklist Individual Strength (CIS-f) scores after completing CBT compared to before treatment are presented as means and standard deviation for all patients who completed therapy. b) Shows the percentage of patients no longer significantly fatigued after treatment. * $P < 0.05$.

Discussion

In this study, the prevalence of POTS in a large CFS cohort of adults and adolescents was 5.7% and 18.2%, respectively, which is much lower than the 27% reported in the review by the IOM. POTS prevalence was not different in CFS patients who fulfilled the SEID criteria. To the best of our knowledge our cohort of 419 CFS patients is the largest population studied thus far. Although the prevalence of POTS in the healthy population is unknown, healthy controls in studies reviewed by the IOM reveal a combined prevalence of 4%, which seems to be more in accordance with results from the present study. This large discrepancy in prevalence could be explained by two methodological issues. First, many of the reported studies were performed in healthcare centers specialized in the assessment of autonomic symptoms, or included patients who were selected based on their symptoms (e.g. dizziness upon standing). This could have led to referral bias and thus an overestimation of the true prevalence^(16, 30, 31). Secondly, in previous studies the age limit of included patients was not reported⁽¹⁴⁻¹⁶⁾, which could mean that adolescents were also included in the CFS sample. This could have led to a higher prevalence rate in these cohorts than in our sample of adults, although we still found a POTS prevalence of only 18.2% among adolescents. As previously reported and found in the present study, orthostatic tachycardia is common in adolescents and therefore it has been suggested that different POTS criteria should be used for these patients^(32, 33).

This is the first study to report the POTS prevalence in patients presenting with persistent fatigue but not meeting CFS criteria according to the CDC. The prevalence of POTS in non-CFS patients was not significantly different from that in the CFS population. Especially in adults, POTS cannot be used to discriminate between CFS and fatigued non-CFS patients. To date, nine different CFS definitions have been proposed, none of which has been proven to discriminate between different causes of fatigue. Other studies even describe 20 different case definitions to be present⁽³⁴⁾. Before a new case definition can be introduced and used in daily clinical practice, this has to be validated in order to add value to the existing definitions. Furthermore, the aims of the SEID criteria proposed by the IOM were a better understanding of CFS and a faster diagnosis, but with the described criteria a large proportion of CFS patients might not even fulfill the SEID criteria. This could lead to an extension of the diagnostic procedure, and unnecessarily withholding effective treatment.

In line with previous studies, we found that adult POTS-CFS patients were younger and had a higher heart rate in the supine position, compared to non-POTS-CFS patients^(14, 15). Increased heart rate in the supine position has been reported previously in CFS patients by our group and could reflect increased sympatho-adrenomedullary activity⁽³⁵⁾. These differences between POTS-CFS and non-POTS-CFS patients were not found in adolescents.

Fatigue severity and CFS-related symptoms were not different between patients with and without POTS, in agreement with some ⁽¹⁶⁾ but not all previously reported studies ⁽¹⁴⁾.

Several pathophysiological mechanisms have previously been described that could be responsible for the development of POTS. Such mechanisms include hypovolaemia ^(17, 36), increased sympathetic activity ⁽³⁷⁾ and cardiovascular deconditioning ⁽¹⁸⁾, although it is not clear whether the latter is the cause of POTS or rather a consequence. However, given the fact that CFS patients are usually less active than healthy control subjects ⁽²²⁾, we assumed deconditioning to be a contributing factor. Although maximum oxygen uptake during exercise is the gold standard to measure deconditioning, actometer scores give an indication of a patient's activity pattern, and both of these methods have not been used previously in POTS-CFS patients. We did not find differences in the level of physical activity between POTS-CFS and non-POTS-CFS patients, which makes it less likely that deconditioning is the cause of POTS in these patients.

The influence of POTS on CFS treatment outcome has not been investigated previously. For adults, no differences could be found in the response to CBT. However, adolescents with POTS did have a higher fatigue score after treatment, although these patients did not report more POTS-related symptoms. Despite this significant difference, 58% of POTS-CFS adolescents were no longer severely fatigued after treatment, which indicates that treatment is still effective in the majority of patients. We propose the use of a stepped-care approach in adolescents, with evaluation for the presence of POTS in those who do not respond to CBT treatment or those who report vasovagal symptoms. Fortunately, treatment of POTS patients is remarkably successful, which means a combined approach could be effective ⁽³⁸⁾. Treatment of POTS mainly includes lifestyle interventions, such as preventing dehydration and avoiding extreme heat; in addition, regular exercise should be promoted ⁽¹³⁾. There is limited evidence to support pharmacological treatment, for example using aldosterone analogues or beta-adrenergic blockers ⁽³⁶⁾.

This study has several limitations. First, blood pressure measurement was resumed after a standing period of 5–8 min. This means that the reported values could be slightly underestimating the true prevalence of POTS. Nevertheless, patients with POTS had persistent tachycardia at least until 18 min in the standing position, which is consistent with the findings of others ⁽³⁹⁾. In previous studies, POTS prevalence was most often based on heart rate after 2 min in the upright position, without taking into account the remaining 8 min. In that situation it should be realized that a transient tachycardia directly after standing is normal ⁽⁴⁰⁾. Thus in daily practice, difficulties are encountered in differentiating those patients with a tachycardia upon standing from those with a normal physiological response, particularly among adolescents. Another limitation of the present study is the fact

that POTS-related symptoms were not recorded during the active standing test. However, these symptoms were recorded in the diary kept by all participants for 12 days.

Conclusion

In conclusion, in adult CFS patients the prevalence of POTS was low and not different from that in the non-CFS fatigued population. In addition, POTS in adult CFS patients was not related to disease severity, physical activity or treatment outcome. POTS-CFS adolescent patients showed a smaller reduction in fatigue after CBT. However, as most patients still benefit from CBT, there is no reason to withhold such treatment. Based on the results of this study, evaluation for POTS appears to be of limited additional value for the diagnosis of CFS; our findings do not support the addition of POTS to a new CFS case definition as proposed by the IOM. With the new SEID criteria, we are concerned that a subset of patients will not be diagnosed with CFS, although they are not different from other patients with respect to fatigue severity or mental and physical disabilities. This eventually could lead to a delay in starting effective treatment, which is in contrast to the goal of the IOM to accelerate the diagnostic process.

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Chapter 10

**Summary and future
perspectives**

In this thesis several biological aspects of the chronic fatigue syndrome (CFS) were investigated. Aspects of the immune system, hypothalamic-pituitary-adrenal axis (HPA axis), and cardiovascular system in relation to CFS were studied, with an emphasis on the role of the pro-inflammatory cytokine interleukin-1 (IL-1). As the studies investigating these systems thus far in CFS showed conflicting results, there was a need for strictly controlled studies in well-defined patients and controls.

In **chapter 2** we investigated whether there is a relation between circulating interleukin-1 (α or β), or the interleukin-1 receptor antagonist (IL1-Ra) and fatigue in different inflammatory and non-inflammatory diseases. In addition, we reviewed studies that inhibited IL-1 and investigated the influence on fatigue severity. It was concluded that based on the available literature there is limited evidence for an association between circulating IL-1 α or IL-1 β and fatigue. This for some part can be explained by the use of different methods to measure fatigue and different approaches to measure cytokines. In addition, it is known that circulating concentrations of IL-1 tend to be low and not very informative. IL-1Ra, which is often used as a readout for IL-1 activity, appeared to be correlated with fatigue in some diseases, especially in cancer. Evaluation of the effect of inhibiting IL-1 on fatigue was more informative. This has been done both in inflammatory and non-inflammatory diseases, where it appeared to have a positive effect. For example, a decrease of fatigue after lowering IL-1 was found in patients with rheumatoid arthritis and Sjögren's disease treated with anakinra (IL-1 receptor antagonist) ^(1,2), cryopyrin-associated periodic syndrome (CAPS) patients treated with canakinumab (monoclonal antibody against IL-1 β) ⁽³⁾ and diabetes patients treated with gevokizumab (IL-1 β antibody) ⁽⁴⁾.

It is suspected that cytokine disturbances causing fatigue are predominantly situated in the central nervous system (CNS), where pro-inflammatory cytokines such as IL-1 and TNF are able to influence neural processes (e.g. dopamine synthesis), leading to behavioral alterations ⁽⁵⁾. Therefore, it would be interesting to interfere with cytokines on a central level in addition to inhibition in the periphery. As most cytokine inhibitors are monoclonal antibodies with large molecular weights, passage of the blood-brain barrier is minimal. Therefore, in **chapter 3** a proposed non-invasive method for drug delivery into the CNS was investigated. Nine Sprague-Dawley rats were given a single injection of cetuximab (146 kDa), etanercept (51 kDa) or anakinra (17 kDa) in the cervical perispinal region or dorsal tail vein. After this injection, no intracranial delivery of the drugs could be visualized using a single-positron emission computed tomography in all but one of the animals. Based on these results it was concluded that a single injection in the perispinal region is not a proper method to deliver drugs to the CNS.

In the light of these results, we looked for an alternative method to inhibit IL-1 in CFS patients, aiming at both peripheral and central inhibition of both IL-1 α and IL-1 β . For anakinra, central penetration has been described after intravenous and subcutaneous administration in rodents and humans ⁽⁶⁻⁸⁾. Thus, we performed a randomized-controlled trial to investigate the effect of subcutaneous anakinra for a period of four weeks on fatigue severity in fifty female CFS patients. **Chapter 4** describes the protocol for this study. It was hypothesized that the intervention would lead to a significant decrease of fatigue severity. The results of this study were described in **chapter 5**. In contrast to our hypothesis, there was no benefit of treatment with anakinra. There was a decrease of fatigue severity in both treatment arms directly after treatment, and up to six months after initiation of the treatment. In the anakinra group, 8% of patients no longer had severe fatigue after treatment compared to 20% of patients in the placebo group, which was not significantly different. Also for the secondary outcomes (functional impairment, physical and social functioning, psychological distress, and pain severity) there were no differences between the anakinra and placebo condition.

In **chapter 6** we asked ourselves the question whether we were able to determine distinct cytokine profiles in CFS patients compared to controls, using a new multiplex analysis technique (proximity extension assay). Based on recent CFS studies ⁽⁹⁻¹¹⁾, 20 pro- and anti-inflammatory proteins were first compared individually. In this exploratory analysis, there was an increased concentration of IL-12p40 and CSF-1 in CFS patients. In addition, we aimed at the constitution of a prediction model to discriminate CFS patients from healthy controls, using a combination of 92 inflammatory markers. This led to a combination of markers that reasonably discriminated between CFS patients and controls. As an explorative analysis, the effect of IL-1Ra on circulating cytokines was evaluated. There was a trend to a decrease of CSF-1, IL-18R1 and ENRAGE in patients treated with anakinra, and an increase in CXCL-9, although this has to be interpreted carefully in the light of the number of proteins analyzed. Replication of the prediction model in an independent study cohort might help us to develop an inflammatory profile to distinguish CFS patients more accurately.

TGF- β 1 was the only cytokine that appeared to be elevated in a recent systematic review on the role of circulating cytokines in CFS ⁽¹¹⁾. Therefore, we measured TGF- β 1 concentrations in two different cohorts of female CFS patients and healthy controls of which the results are described in **chapter 7**. We did not find any differences between patients and controls within both cohorts. However, there was a large difference in TGF- β 1 concentration between the two cohorts which could be explained by differences in sample handling. There appeared to be a difference in the g-force of the centrifuges used in the two studies, where the lower g-force in cohort II (1361g) caused more platelet activation which was

reflected by higher p-selectin concentrations. Activated platelets are an important source of TGF- β ⁽¹²⁾, and this finding could explain the differences between the two cohorts. We conclude that it is plausible that elevations of TGF- β reported in the literature, reflect a methodological problem, rather than real differences. An important lesson from this study is that studies like these have to be meticulously controlled, and even then, conclusions may be drawn that reflect artifacts rather than real biological phenomena. In the past, the use of controls that were not simultaneously sampled with the patients misled the scientific community, as well as patients and the general public ⁽¹³⁾.

Next to investigating the immune system in CFS patients, we also focused on the HPA axis. In **chapter 8** we compared saliva and hair cortisol of a large group of CFS patients (n=107) to healthy controls (n=59). Although measurement of cortisol in saliva has been performed repeatedly in CFS, long-term cortisol determinations in hair had not been done before. In CFS, there was a decreased cortisol awakening response and a trend towards lower cortisol levels in hair. Total salivary cortisol during the day was not different between patients and controls. In the subgroup of patients participating in the anakinra trial, the effect of IL-1 inhibition on the HPA axis was assessed. We found a slight decrease of hair cortisol in patients treated with anakinra, compared to an increased concentration in placebo-treated patients. Earlier studies demonstrated that cortisol concentrations can be used as measure to predict treatment response. However, as there is a substantial variance in day-to-day cortisol output, hair cortisol might be a more stable measure to use in this context.

In **chapter 9** we investigated the prevalence of postural orthostatic tachycardia (POTS), one of the most frequent forms of orthostatic intolerance (OI), in a group of 419 CFS and 341 fatigued non-CFS patients. This prevalence was compared to the numbers described in the report published by the 'Institute of Medicine', in which it was claimed that OI should become one of the criteria in the new Systemic Exertion Intolerance Disease definition for CFS. We found a POTS prevalence of 5.7% in adult CFS patients and 6.9% in non-CFS patients; these numbers were not significantly different. In adolescents, this prevalence was 18.2 and 17.4%, respectively. This is substantially lower than the reported prevalence of 27% by the IOM. Disease severity was not significantly different in POTS patients with CFS, compared to those that did not fulfill the POTS criteria. Response to treatment with cognitive behavioral therapy was not different between adult CFS patients with and without POTS, in adolescent CFS patients clinical improvement after treatment was less frequent in those affected with POTS (58% vs. 88%). Based on these results we concluded that there is limited value of diagnosing POTS in CFS patients. Routine measurement in patients without orthostatic complaints does not seem necessary.

General discussion

The primary aim of this thesis was to investigate the role of IL-1 in fatigue, and evaluate the effect of lowering IL-1 on fatigue severity in CFS patients. For the discussion of my thesis, I will therefore predominantly focus on the role of cytokines in CFS and the RCT that we performed.

In contrast to our expectations, administration of IL-1Ra during one month had no benefit in CFS. Considering these results, we wondered about the main reason for the absence of this effect, especially in the light of the positive effect of lowering IL-1 on fatigue severity in other conditions, as we described in **chapter 2**. There are several possible explanations that I would like to discuss.

First of all, patient selection might have played a role. In addition to the selection criteria discussed in **chapter 5**, e.g. not only including patients with post-infectious fatigue, disease severity might have played a role as well. As a consequence of the intensity of the study, with patients having to visit the hospital frequently and inject themselves on a daily basis, only the relatively mobile patients could participate. Some studies report that immunological alterations are different in patients that are housebound compared to those that are still relatively active ^(14, 15). However, these studies are too small and inconclusive to draw conclusions. Additionally, including these patients as well would have resulted in a more heterogeneous study population, an issue that is already a subject of debate in CFS research.

Second, the method we used to administer IL-1Ra should be discussed. In this thesis, we described that IL-1 and other pro-inflammatory cytokines influence the central nervous system, altering behavioral decision making and eventually leading to the development of fatigue as described in **chapter 2** ⁽¹⁶⁾. Therefore, we were intrigued by a study describing the central delivery of monoclonal antibodies within 30 minutes after administration to the brain, after one single injection in the perispinal region ⁽¹⁷⁾. Following the exact same methods used in this study, we could not replicate these results as described in **chapter 3**. Despite that, we decided to proceed with our approach of repeated peripheral IL-1Ra administration in CFS. The main reason to continue with this approach was that, despite a large concentration gradient, central delivery of anakinra has been described after repeated or continuous administration of anakinra in both animals and humans ⁽⁶⁻⁸⁾. Additionally, it was assumed that only peripheral IL-1 inhibition would be effective as well, although this effect is probably more pronounced in illnesses with marked peripheral inflammation as described in **chapter 2**. However, considering the results of our trial, peripheral inhibition of IL-1 does not have an effect in CFS and the central effect of anakinra might have been too small to make a difference.

The third important possible explanation is that frequent injection site reactions contributed to the absence of an effect. Unfortunately, in hindsight, this local skin reaction was not scored in an objective manner, and our records were based on what study participants reported. Therefore, we could not control for this adverse event in our analysis. The possibility that injection site reactions could have influenced the outcome is demonstrated in a study investigating the effect of anakinra on insulin sensitivity in patients with the metabolic syndrome ⁽¹⁸⁾. In this study, patients with severe injection site reactions had more macrophage infiltration of the adipose tissue and were less insulin sensitive. Anakinra-treated patients with a low macrophage influx did reveal a significant improvement of insulin sensitivity. Treating patients for a longer period could also have been a method to avoid this possible confounder, as it is known that injection site reactions evade with prolonged treatment ⁽¹⁹⁾.

Finally, it is possible that in CFS increased inflammation that played a role at the initiation of symptoms, no longer play a role in the chronic phase of the disease. This theory is strengthened by earlier findings of an increased association of IL-1 and fatigue in the acute phase of an infection ⁽²⁰⁾, which was no longer present in the chronic phase ⁽²¹⁾. In the chronic phase it is possible that the upstream effects of IL-1, such as the effect on dopamine synthesis, play a role as well ⁽⁵⁾. This would mean that IL-1 inhibition as a treatment of fatigue is more effective in diseases that are accompanied by (low-grade) inflammation, such as advanced cancer patients ⁽²²⁾.

Future perspectives

General aspects in (CFS) research

One of the most important lessons from this thesis, especially considering the results of **chapter 7**, is that it is of major importance to conduct studies in a tightly controlled setting. Over the years there has been a number of studies performed in CFS patients investigating abnormalities of the immune system, HPA axis and the cardiovascular system. However, a significant proportion of these studies do not control for pre-analytical procedures which can result in wrong conclusions as we have described. It is our hope that future studies in CFS will take this into account and will control for pre-analytical procedures in addition to inclusion of proper controls. This is an important topic, not only in CFS research, and will lead to an increase in reproducibility of findings.

Diagnostic markers

In **chapter 6** of this thesis, we focused on possible diagnostic markers for CFS, investigating alterations in inflammatory profiles. The set of inflammatory proteins selected in **chapter 6** could be an interesting focus of future studies. The number of markers that was selected by LASSO regression, some of which are IL-1 driven (IL-6, TNF β , IL-10), illustrates the complex interaction between different cytokines. Finding a distinct combination of markers in CFS, could lead to a new diagnostic test. However, as we performed a small study with a large number of markers, the performance of these markers can only be evaluated in a second independent cohort of CFS patients.

Therapeutic options

If our hypothesis on the role of IL-1 in CFS is correct, an interesting subject for future studies is to search for drugs that are capable of IL-1 inhibition within the CNS. The primary source of IL-1 in the brain is production by activated microglia. Increased microglia activation has been demonstrated in CFS patients, although in a small group of patients ⁽²³⁾. Our group is currently performing a study to see whether we can replicate these results and additionally investigate if there are differences between CFS patients and patients that have persistent fatigue after acute Q-fever (Q-fever fatigue syndrome, QFS). The asset of including QFS patients is that these patients have an overt inflammatory start of their symptoms, and hence might be more prone to develop neuroinflammation. If the previous findings can be replicated, drugs that can inhibit microglial activity, for example the antimicrobial drug minocycline, could also be worth investigating ⁽²⁴⁾. This drug has a broader effect compared to selective inhibition of IL-1.

Research agenda

At the end of this thesis I would like to shortly elaborate on the future research that needs to be done, based on our findings.

- I. Perform studies in a controlled setting with strict regulation of (pre-)analytical procedures and inclusion of proper controls. As demonstrated by the effect of placebo on fatigue severity in our anakinra trial, there is no place for uncontrolled intervention studies.
- II. Investigate the diagnostic performance of the selection of diagnostic markers in an independent CFS cohorts, and ideally search for identifiable subgroups (CBT responders/non-responders, post-infectious/non-infectious, etc.).
- III. Replicate earlier findings of increased microglia activity in CFS to create additional therapeutic options.
- IV. Search for inhibitors of cytokines like IL-1 β that are able to block this cytokine in the brain, to see whether such an intervention ameliorates CFS.

These studies are necessary to find out whether (neuro)inflammation and especially IL-1, play a role in CFS. It is important for our understanding of the syndrome what pathophysiological mechanisms lead to this incapacitating illness. If we can find other therapeutic modalities in addition to behavioral interventions that improve the complaints of CFS patients, this would mean a major step forward. Well controlled studies with a robust methodology (like that applied in our anakinra study) are necessary to achieve this.

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Chapter 11

Nederlandse samenvatting

Dankwoord

Lijst met publicaties

Curriculum Vitae

Nederlandse Samenvatting

Dit proefschrift is gericht op de biologische aspecten van het chronisch vermoeidheidssyndroom (CVS). In dit kader zijn het immuunsysteem, de hypothalamus-hypofyse-bijnier-as (HPA-as), en het cardiovasculaire systeem bestudeerd in relatie tot CVS, waarbij de nadruk werd gelegd op het pro-inflammatoire cytokine interleukine-1 (IL-1). Aangezien de huidige literatuur geen uitsluitsel geeft over de rol van de genoemde systemen in CVS, was het belangrijk om strikt gecontroleerde studies uit te voeren in zorgvuldig geselecteerde patiënten en gezonde controles.

In **hoofdstuk 2** onderzochten we literatuur over de relatie tussen circulerend interleukine-1 (α of β) of de interleukine-1 receptor antagonist (IL-1Ra) en vermoeidheid in verschillende inflammatoire en niet-inflammatoire aandoeningen. Daarnaast bestudeerden we studies die het effect van het remmen van IL-1 op de ernst van de vermoeidheid hebben onderzocht. Op basis van de bestaande literatuur werd geconcludeerd dat er beperkt bewijs is voor een associatie tussen circulerend IL-1 α of IL-1 β en vermoeidheid. Deze bevinding kan deels worden verklaard door de grote verschillen in gebruikte methoden voor het meten van cytokinen en vermoeidheid, waardoor het moeilijk is om de resultaten te vergelijken. Bovendien zijn circulerende concentraties van cytokinen over het algemeen laag, ook in het geval van actieve infecties. IL-1Ra wordt frequent gebruikt als graadmeter voor IL-1 activiteit. In een deel van de studies, voornamelijk de studies verricht bij patiënten met kanker, werd een relatie gevonden tussen IL-1Ra en vermoeidheid. Evaluatie van het effect van het remmen van IL-1 op vermoeidheid was meer informatief. Dit is gedaan in zowel inflammatoire als niet-inflammatoire aandoeningen, waarbij een positief effect wordt beschreven. Zo werd er bijvoorbeeld een afname van vermoeidheid gezien in patiënten met de ziekte van Sjögren behandeld met anakinra (recombinant IL-1Ra), in patiënten met cryopyrine-geassocieerd periodiek syndroom behandeld met canakinumab (monoklonaal antilichaam tegen IL-1 β) en diabetespatiënten behandeld met gevokizumab (IL-1 β antilichaam).

Er wordt vermoed dat de cytokine verstoringen in CVS-patiënten zich vooral afspelen in het centraal zenuwstelsel (CZS). In het CZS kunnen pro-inflammatoire cytokinen zoals IL-1 en TNF neurale processen beïnvloeden (o.a. dopamine synthese) waardoor er gedragsveranderingen optreden. Daarom is naast het remmen van cytokinen in de periferie, ook het centraal remmen van cytokinen belangrijk in het geval van CVS. Echter, het merendeel van de beschikbare cytokine remmers zijn monoklonale antilichamen met een hoge moleculaire massa, waardoor passage door de bloed-hersen-barrière wordt bemoeilijkt. In **hoofdstuk 3** onderzochten we een methode waarmee mogelijk op een niet-invasieve manier centrale afgifte van medicatie kan worden bewerkstelligd. Er werd een injectie met cetuximab molecuul gewicht (146 kDa), etancercept (51 kDa) of anakinra (17 kDa)

toegediend in het perispinale gebied of de dorsale staartvene van negen Sprague-Dawley ratten. Na deze toediening vonden we, op één van de ratten na, geen centrale depositie van het medicijn. Voor het meten van centrale depositie werd gebruik gemaakt van een 'single-positron emission computed tomography' (SPECT). Op basis van deze resultaten concludeerden we dat het toedienen van medicatie in het paraspinale gebied geen geschikte methode is voor de centrale toediening van medicatie.

In het licht van deze resultaten moesten we op zoek naar een andere methode om IL-1 te remmen in CVS-patiënten, waarbij we zowel perifeer als in het CZS IL-1 α en IL-1 β wilden remmen. Centrale depositie van anakinra is beschreven na intraveneuze en subcutane toediening. Om de effectiviteit van anakinra te onderzoeken in CVS, voerden we een gerandomiseerde dubbelblinde studie uit. Vijftig vrouwelijke CVS-patiënten werden gedurende vier weken dagelijks behandeld met subcutane injecties met anakinra of placebo. **Hoofdstuk 4** beschrijft het onderzoeksprotocol voor de uitvoering van deze studie, waarbij de hypothese was dat de interventie tot een afname van vermoeidheid zou leiden. De resultaten van deze studie zijn beschreven in **hoofdstuk 5**. In tegenstelling tot onze verwachting werd er geen voordelig effect gevonden van behandeling met anakinra. In beide behandelgroepen werd een afname van vermoeidheid gezien direct na behandeling en tot zes maanden na het starten van de behandeling. In de anakinra groep was er bij 8% van de patiënten niet langer sprake van ernstige vermoeidheid na behandeling en in de placebogroep was dit 20%, waarbij er geen significante verschillen waren tussen beide groepen. Ook voor de secundaire uitkomstmaten (functionele beperkingen, fysiek en sociaal functioneren, psychische stress en pijn) waren er geen verschillen tussen patiënten behandeld met anakinra of placebo.

In **hoofdstuk 6** onderzochten we met behulp van een multiplex analyse techniek (proximity extension assay) of CVS patiënten een ander cytokine profiel hebben dan gezonde (buurt) controles. Daarnaast vergeleken we 20 cytokinen, geselecteerd op basis van de recente CVS-literatuur, tussen patiënten en controles. In deze exploratieve analyse werd een hogere IL-12p40 en CSF-1 concentratie gevonden bij CVS-patiënten. Daarnaast keken we of het mogelijk was om een predictiemodel op te stellen om CVS-patiënten te kunnen onderscheiden van gezonde controles, gebruik makend van 92 inflammatoire markers. Dit heeft geleid tot een combinatie van markers met een redelijk onderscheidend vermogen. Tot slot onderzochten we het effect van behandeling met anakinra op circulerende cytokinen. Hierbij vonden we een trend tot afname van CSF-1, IL-18R1 en ENRAGE in patiënten behandeld met anakinra, en een toename van CXCL-9. Gezien het grote aantal gemeten markers moeten deze resultaten echter met enige voorzichtigheid worden geïnterpreteerd. Replicatie van het gevonden predictie model in een onafhankelijk studie cohort, kan ons in de toekomst mogelijk helpen een inflammatoir profiel op te stellen waarmee CVS-patiënten kunnen worden onderscheiden van gezonde controles.

TGF- β 1 was in een recente systematische review het enige circulerende cytokine dat bij CVS-patiënten in een hogere concentratie aanwezig was. Derhalve hebben wij TGF- β 1 concentraties gemeten in twee verschillende cohorten van vrouwelijke CVS-patiënten en gezonde controles. De resultaten hiervan worden beschreven in **hoofdstuk 7**. Er werden geen verschillen tussen patiënten en controles gevonden in TGF- β 1 concentraties binnen beide cohorten. We vonden echter grote verschillen tussen de twee cohorten, wat kon worden verklaard door verschillen in het preanalytische proces. Er bleek een verschil te zijn in de g-kracht van de centrifuges gebruikt in de twee studies. Hierbij zorgde de lage g-kracht in cohort II (1361g) voor meer activatie en aanwezigheid van trombocyten, wat werd bevestigd door hogere P-selectine concentraties. Geactiveerde trombocyten zijn een belangrijke bron van TGF- β waardoor deze bevinding de verschillen tussen beide cohorten kan verklaren. Op basis hiervan concludeerden we dat de eerder beschreven verhoogde TGF- β concentraties mogelijk worden verklaard door methodologische verschillen, in plaats van ware verschillen. Een belangrijke les die uit deze studie kan worden getrokken is dat het strikt gecontroleerd uitvoeren van wetenschappelijk onderzoek van groot belang is, omdat de kleinste verschillen in methode tot verkeerde conclusies kan leiden.

Naast het immuunsysteem onderzochten we ook de HPA-as in CVS-patiënten. In **hoofdstuk 8** vergeleken we cortisol concentraties in speeksel en haar tussen CVS-patiënten (n=107) en gezonde controles (n=59). Speeksel cortisol is in eerdere studies frequent gemeten in CVS-patiënten, echter de lange-termijn cortisol concentraties zoals kunnen worden gemeten in haar, werden niet eerder onderzocht. In CVS-patiënten werd een lagere cortisol piek na wakker worden gevonden, en een niet significante trend tot lagere cortisol concentraties in haar. Totale speeksel cortisol output gedurende de dag was niet verschillend tussen patiënten en controles. In een subgroep werd het effect van het remmen van IL-1 op de HPA-as onderzocht. Hierbij werd een minimale afname van haar cortisol gezien in de met anakinra behandelde patiënten, en een stijging in de placebogroep. Eerdere studies lieten zien dat cortisol concentraties in CVS-patiënten mogelijk kunnen worden gebruikt om effect van behandeling (met cognitieve gedragstherapie) te voorspellen. Echter, gezien de grote variatie in speeksel cortisol concentraties is haar-cortisol hier mogelijk een meer stabiele en betrouwbare maat voor.

In **hoofdstuk 9** onderzochten we de prevalentie van het posturale orthostatische tachycardie syndroom (POTS), een van de meest voorkomende vormen van orthostatische intolerantie (OI), in een groep van 419 CVS-patiënten en 341 vermoeide patiënten die niet voldeden aan de CVS criteria. De gevonden prevalentie werd vergeleken met de aantallen beschreven in het in 2015 gepubliceerde rapport van het 'Institute of Medicine' (IOM), waarin werd gepleit voor het opnemen van OI als een van de diagnostische criteria voor CVS. Hierbij werd tevens een nieuwe naam voor CVS voorgesteld, namelijk 'Systemic Exertion Intolerance

Disease'. Wij vonden een POTS prevalentie van 5.7% in volwassen CVS-patiënten, en een prevalentie van 6.9% in vermoeide patiënten zonder CVS. In adolescenten was de prevalentie respectievelijk 18.2% en 17.4%. Deze prevalentiecijfers zijn substantieel lager dan de prevalentie van 27% die wordt genoemd in het IOM rapport. Er was geen verschil in ziektelast tussen CVS-patiënten met en zonder POTS. Cognitieve gedragstherapie was even effectief in volwassenen met of zonder POTS. Echter, behandeling was minder effectief in adolescenten met POTS waarbij POTS patiënten in 58% van de gevallen herstelden, in vergelijking met 88% van de adolescenten zonder POTS. Op basis van deze resultaten concludeerden we dat het stellen van de diagnose POTS in CVS-patiënten van zeer beperkte waarde is. Het routinematig verrichten van onderzoek hiernaar in patiënten zonder orthostatische klachten achten wij niet noodzakelijk.

In **hoofdstuk 10** worden de belangrijkste resultaten en conclusies van dit proefschrift beschreven. Een van de belangrijkste lessen die kan worden geleerd uit dit proefschrift, is dat het verrichten van goed gecontroleerd onderzoek van groot belang is. In de huidige CVS literatuur is sprake van een groot aantal tegenstrijdige bevindingen, wat deels verklaard kan worden door methodologische verschillen. Zeker in het geval van een complexe aandoening als CVS, is gestructureerd onderzoek met aandacht voor het preanalytische proces van groot belang. Wat betreft vervolgonderzoek is met name het centraal beïnvloeden van inflammatoire cytokinen (o.a. IL-1) een interessante therapeutische optie.

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Lijst met publicaties

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Curriculum Vitae

Megan Elisabeth Roerink werd geboren op 27 september 1989 te Breda. In 2007 behaalde zij haar gymnasium diploma op het Stedelijk Gymnasium Breda. In hetzelfde jaar verhuisde zij naar Nijmegen waar zij begon aan de studie Geneeskunde.

Gedurende haar studie verrichte Megan onderzoek naar de kwaliteit van zorg bij patiënten met HIV en diabetes onder begeleiding van dr. van Crevel en dr. de Galan in het Radboudumc. Dit onderzoek leverde haar eerste wetenschappelijke publicatie op.

Van 2013 tot 2016 was Megan werkzaam in het Radboudumc waar zij onderzoek deed naar het effect van interleukine-1 inhibitie bij patiënten met het chronisch vermoeidheidssyndroom. Gedurende dit onderzoek werd zij begeleid door Prof. dr. van der Meer (promotor) en Prof. dr. Knoop (co-promotor). Dit proefschrift is het resultaat van haar promotieonderzoek.

Megan is in 2017 gestart met de opleiding tot internist, hiervoor is zij momenteel als arts-assistent werkzaam in het Rijnstate ziekenhuis in Arnhem (opleider dr. Reichert).

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