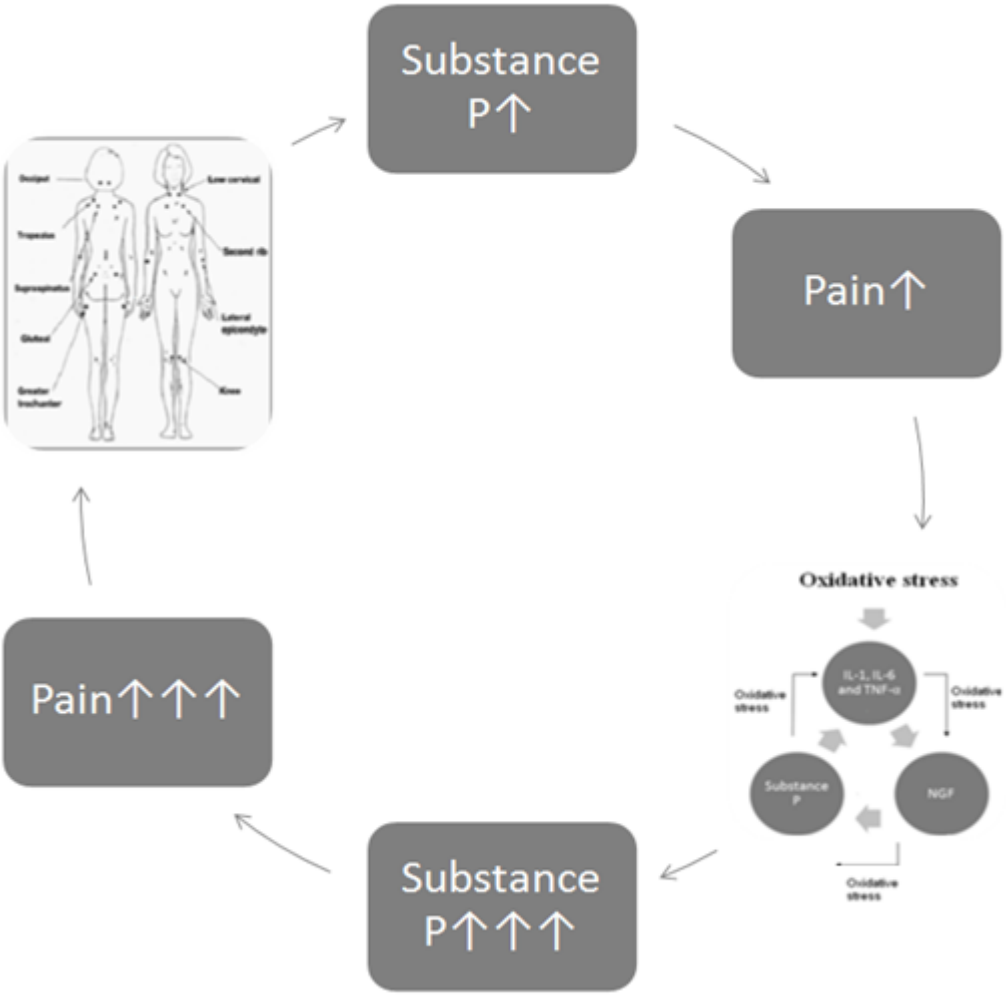
What are the roles of oxidative stress and pro-inflammatory cytokines in fibromyalgia?



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Abstract

Fibromyalgia (FM) is a common disorder of unclear aetiology, characterized by chronic widespread pain and painful tender points (body pressure points). Several researchers suggest that elevated levels of the pronociceptive substance substance P enhance pain experience (sensitization of ascending pain pathways). FM is often accompanied by several non-specific symptoms including fatigue, stiffness, disordered sleep, cognitive dysfunction, dysesthesia, psychological distress, headaches, and poor balance. These non-specific symptoms are also observed in 'sickness behavior' which is induced by infectious and inflammatory processes, characterized by cytokines. A literary search for cytokines in FM patients suggested several upregulated cytokine levels in FM patients, compared with controls. Furthermore, diseases that show overlap with FM are characterized by enhanced oxidative stress levels. Another literary search regarding oxidative stress in FM patients suggested upregulated oxidative stress levels in FM patients, compared with controls. Eventually, this thesis hypothesized that oxidative stress might be responsible for increased substance P levels via cytokine generation, resulting in elevated pain experience. To place this hypothesis in a broad perspective, the physiological effect of several associated co-morbidities (e.g. diabetes) have been analyzed.

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Abbreviations

ACTH-	Adrenal corticotrophe hormone
CFS-	Chronic fatigue syndrome
CNS-	Central nervous system
CR-	Calorie restriction
FM-	Fibromyalgia
GSH-	Glutathione
HPA-	Hypothalamic-pituitary-adrenal
IL-	Interleukin
MDA-	Malonaldehyde
NGF-	Nerve growth factor
NMDA-	N-methyl D-aspartate
NK-	Natural killer
NPY-	Neuropeptide Y
rCBF	Regional cerebral blood flow
ROS-	Reactive oxygen species
SOD-	Superoxide dismutase
Th-	T-helper
TNF-	Tumor necrosis factor

Preliminary survey

My interest for FM was born after a guest lecture on FM, given by a representative of the department 'Clinical & Health Psychology' during my internship at the department 'Rheumatology and Clinical Immunology' at the University Medical Centre Utrecht. After some basic research into FM, I discovered the high incidence of FM in people and a roommate who suffers from FM, which made me even more interested in FM. After an interview with Dr. Prof. R. Geenen, I started my thesis process with a preliminary survey.

During this preliminary survey, I explored that FM is a common disorder with non-specific symptoms (e.g. pain, fatigue, stiffness, disordered sleep, tenderness, cognitive dysfunction, dysesthesia, psychological distress, headaches and poor balance). These non-specific symptoms are also seen in 'sickness behavior' which is induced by infectious and inflammatory processes, characterized by cytokines. Cytokines are proteins that are suggested to be involved in: the modulation of the Hypothalamic-pituitary-adrenal (HPA)-axis, pronociceptive substances, hyperalgesia, allodynia, fatigue, pain and depression, features that are all observed in FM patients. The relation between cytokines and FM symptoms was also shown in a cytokine-based immunotherapy experiment in humans. This cytokine-based immunotherapy caused an induced depression in up to 70% of the patients treated for cancer or hepatitis C. In addition, an increased production of pro-inflammatory cytokines interleukin-1β (IL-1 β), IL-6 and Tumor necrosis factor (TNF-α) were associated with 'sickness behavior' symptoms. Moreover, research suggested that IL-1, TNF-α, IL-6 and IL-12 may promote physiological symptoms and vital exhaustion (e.g. loss of energy, increased irritability, and feelings of demoralization) in chronic diseases such as FM. Additionally, elevated levels of IL-6 and TNF-α have been observed in patients with the Chronic Fatigue Syndrome (CFS), which is a syndrome that shows overlap with FM. Furthermore, in patients with the chronic disease Rheumatoid Arthritis, immediate blockage of TNF-α caused a functional improvement in physical functioning, quality of life and fatigue. Thereby, the administration of IL-1 receptor antagonist (IL-1Ra) was suggested to block the stress-such as effects of IL-1. All together, investigation into the role of cytokines in chronic diseases suggests a

possible influence of certain cytokines on FM such as symptoms in diseases that show overlap with FM.

Another feature that is apparently involved in symptoms of diseases that show overlap with FM is oxidative stress. For example, CFS patients showed elevated oxidative stress levels. In addition, because oxidative stress is suggested to damage the HPA-axis, and to play a role in cognitive deficits, also oxidative stress may be associated with diseases that show overlap with FM.

To sum up, cytokines and oxidative stress are suggested to be involved in symptoms of diseases that show overlap with FM. Subsequently, cytokines and oxidative stress levels appear to be involved in FM as well. To examine if cytokines and oxidative stress play a role in FM it is needed to analyze the abundance of cytokines and oxidative stress in FM patients. Hence, this review starts with an overview of all the studies that concerned altered cytokine levels in FM patients. Thereafter, an overview of all the studies that concerned altered oxidative stress levels in FM patients will be presented. Subsequently, this thesis will explore if cytokines and oxidative stress are associated. Thereafter, as Nerve growth factor (NGF) and substance P are suggested to play an important role in FM pathology, the possible association between oxidative stress and cytokines and NGF and substance P is examined. In the discussion, the influence of possible FM associated situations on oxidative stress- and cytokine-levels will be analyzed. Finally, oxidative stress based treatments, cytokine based treatments and FM research suggestions for the future will be proposed. But first, an introduction into FM will be given.

Introduction

FM is characterized by chronic widespread pain and painful body pressure points (tender points) *(Fig.1)*. Approximately two percent of the general population suffers from FM, which is mostly diagnosed during midlife. However, FM may be diagnosed at any age [8, 9]. The symptoms involved in FM patients are suggested to be based on a pro- and an anti-nociception-pathway *(Fig.2)* [1]. The pronociception pathway is responsible for the transfer of chronic pain, and is initiated with a sensory signal which is generated by a stimulus from the peripheral tissue. When a synapse including a NMDA receptor receives glutamate and substance P, a neuron with a wide dynamic range will be triggered. The signal is conducted through the spinothalamic track, the thalamus, the somatosensory cortex, and to other associated brain locations, resulting in chronic pain.

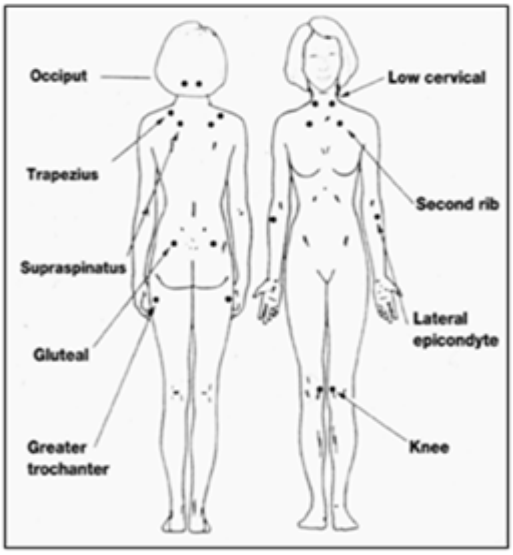

Fig.1) **The 18 tenderpoints of a FM patient.** Suffering from 11 or more of the 18 internally determined tender points; pressure on the indicated tender points results in an abnormal and elevated pain stimulus, is one of the classification criteria for FM.

Besides the pronociception pathway, an anti-nociception pathway is involved as well *(Fig.2)*. In this anti-nociception process, signals from the cortex and the brainstem are transferred descendedly and caudally to the relevant segments of the dorsal horn. Signals interact mainly preganglionically in the dorsal horn and function by counterbalancing, inhibiting, or determining the degree of the pronociceptive signal [56].

As for several biological processes, an ideal balance between pro- and anti-nociception is

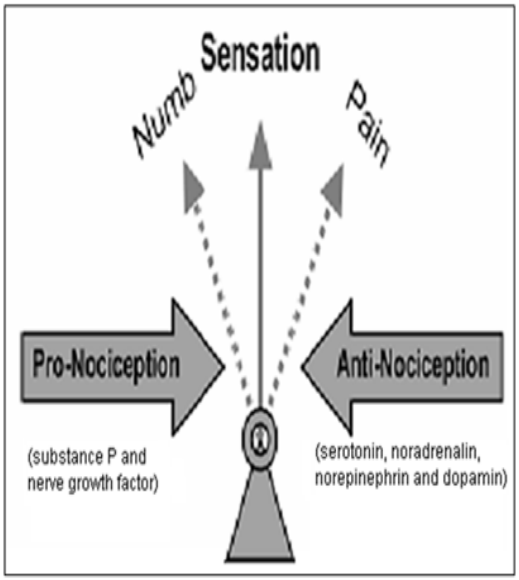

Fig.2) The suggested interplay between the pro-nociception pathway and the anti-nociception pathway in FM patients

preferred so that normal perception of sensitivity can be maintained. In case of FM, increased pronociception levels or decreased anti-nociception levels have been suggested to lead to chronic allodynia (pain due to a stimulus which does not normally provoke pain).

However, other researchers suggest that elevated levels of the pronociceptive substance Nerve Growth Factor (NGF), which is elevated in FM patients, may lead to increased substance P levels, thereby elevating pain experience. Because several researchers suggested this hypothesis as the underlying cause of FM, it will also be the basis of this thesis [4]. Although, the neurochemical mediators of the pronociception pathway such as excitatory glutamate are amplified by increased levels of substance P, the influence of neurochemical mediators on FM symptoms will only be discussed shortly in the discussion. In addition, biogenic amine levels (e.g. serotonin, dopamine and norepinephrin), which are the neurochemicals of the anti-nociception pathway are suggested to be downregulated by substance P. However, most important, high levels of NGF will establish a new, higher steady state concentration of substance P, especially in the brain and in the cerebrospinal fluid, resulting in enhanced pain experience.

A FM diagnosis is required to start treatment. However, due to the lack of objective measurements to diagnose FM, only classification criteria are available. The ACR 1990 classification criteria are developed to classify FM patients. The first criterion of the ACR 1990 diagnostic criteria is the presence of chronic pain and/or stiffness at 3 or more locations in the body. Secondly, pain and stiffness have to be abundant for more than 3 months. Thirdly, suffering from 11 or more of the 18 internally determined tender points; pressure on the indicated tender points results in an abnormal and elevated pain stimulus, completes the diagnosis *(Fig.1)* [5]. Some patients can not be diagnosed by these tender points so new FM diagnostics are needed. Since a couple of months, new neuroimaging techniques are used to examine brain abnormalities in FM patients. These methods infer neural activity in the brain and evaluate the time course of highly localized changes in regional cerebral blood flow (rCBF), occurring in response to changes in neuronal metabolic demand. Other neuroimaging methods that are used in FM research are: the single photon emission

computed tomography technique which measures rCBF, the voxel based method which quantifies focal macro structural changes, and the diffusion tensor imaging method which quantifies micro structural changes. Other neuroimaging methods are the Magnetic resonance spectroscopy which analyses brain biochemistry by measuring glutamate, and the positron emission tomography method which functions via activation maps [6].

When a person is classified as FM patient, several treatment methods can be considered for the treatment of FM patients. FM treatments are mostly divided in pharmacological treatments and non-pharmacological treatments. Examples of pharmaceutical treatments are Savella® (milnacipran), Cymbalta® (duloxetine) and Lyrica® (pregabalin) [2, 7]. Milnacipran and duloxetine are serotonin-norepinephrin uptake inhibitors and pregabalin causes a reduced release of the neurotransmitters glutamate and substance P [4]. Remarkably, these products are approved in America for the indication FM, but not in Europe for the indication FM. In addition, also non-specific FM pharmacological treatments such as antidepressants, muscle relaxants, local anesthetics and dopamine receptor agonists are available for FM patients. Non-pharmacological treatments for FM patients are exercise, patient education, cognitive-behavioural therapy, relaxation and biofeedback [3]. However, these pharmacological- and non-pharmacological treatments show little benefit of effect. Because FM symptoms appear to be caused by elevated cytokine- and oxidative stress-levels, inhibition of cytokine- and oxidative stress- levels as treatment should be examined. However, before discussing treatments, all the cytokine- and oxidative stress-data of FM studies should be collected and analyzed. Therefore, the following chapter will start with a literary search concerning all the cytokine associated FM studies to obtain an overview of the cytokine environment (serum/plasma/PBMC) in FM patients.

Cytokine levels in fibromyalgia patients

This chapter presents in table 1 all the altered cytokine levels found within FM patients to obtain an overview of cytokine levels in FM patients.

Elevated IL-6 and TNF-α levels have been measured in FM patients compared with controls by *Wallace et al.* (2001) and *Bazzichi et al.* (2007). However, also decreased levels of IL-6 and TNF-α have also been observed in FM patients compared with controls by *Bazzichi et al.* (2007), *Hesse-Husain et al.* (2007) and *Macedo et al.* (2007). Furthermore, anti-inflammatory IL-4 levels were found to be decreased in FM patients compared with controls in studies of *Uceyler et al.* (2006), *Hesse-Husain et al.* (2007) and *Macedo et al.* (2007). In addition, studies of *Macedo et al.* (2007) and *Uceyler et al.* (2006) showed decreased anti-inflammatory IL-10 levels in FM patients compared with controls. However, also increased IL-10 levels have been measured in FM patients by Bazzichi and coworkers (2007). Other studies observed increased IL-2 levels (Hader and coworkers, 1991), but also decreased IL-2 levels in FM patients (Hesse-Husain and coworkers 2007 and by Macedo and colleagues 2007). Also IL-2

First Author/Date	Method	Medium	Cytokine increased	Cytokine decreased
Wallace, 1989	Radioimmunoassay	Serum	a	a
Hader, 1991	Tridated (3H) thymidine	PBMC-stimulated	IL-2	
Maes, 1999	ELISA	Serum	IL-1Ra*	
Wallace, 2001	ELISA	PBMC-stimulated	IL-1Ra*	
			IL-6*	
			IL-8*	
Gür, 2002a	Immunoassay	Serum	IL-2r*	
			IL-8*	
Gür, 2002b	Immunoassay	Serum	IL-2r*	
			IL-8*	
Amel Kashipaz, 2003	Flow cytometry	PBMC-stimulated	b	b
Üçeyler, 2006	ELISA	Serum		IL-4
				IL-10
Ardiç, 2007	ELISA	Serum		IL-1α*
Bazzichi, 2007	ELISA	Plasma	TNF-α	IL-1
			IL-8	IL-6
			IL-10	
Hesse-Husain, 2007	Biochip array	PBMC-stimulated		IL-1α*
				IL-1β*
				IL-2*
				IL-4*
				TNF-α*
Macedo, 2007	Biochip array	PBMC-stimulated	IL-8*	IL-1α*
				IL-1β*
				IL-6*
				TNF-α*
				IL-2*
				IL-4*
				IL-10*

Table 1) Altered cytokine levels found within FM patients. Note: ELISA; immune-linked immune sorbent assay, IL; interleukin, PBMC; peripheral blood mononuclear cells. A) Levels of IL-1β, IL-2, IL-2R, and TNF-α were not significantly different between groups . A) cytokine-producing cells were not statistically different among groups in either unstimulated or IFN-γ condition. P=.05

receptor (IL-2r) levels were upregulated in FM patients in studies of Gur and colleagues (2002), which might indicate a lack of IL-2 or an IL-2r overproduction. Increased IL-8 levels in FM patients were observed by several studies (*Macedo et al. 2007, Bazzichi et al. 2007, Gur et al. 2002 and Wallace et al. 2001*). Furthermore, increased IL-1 receptor antagonist (IL-1Ra) levels were found in FM patients by *Maes et al.* (1999) and *Wallace et al.* (2001). Also IL-1α- and IL-1β-levels were downregulated in FM patients in studies of *Macedo et al* (2007) and *Hesse-Husain et al.* (2007). Finally, IL-1 levels were increased in FM patients in a study of *Bazzichi et al.* (2007). To note, not all the measurements (increases and decreases) of the examined studies showed statistically significance (see table 1) [6,32,36,37,72]. Additionally, a limited amount of FM patients have been enrolled in most of the studies.

Although this data-accumulation is hampered by the amount of studies, limited amount of study subjects in FM studies and contrary findings, it was decided that in combination with previous pro-inflammatory cytokine findings in diseases that show overlap with FM (see: preliminary survey), elevations of pro-inflammatory cytokine levels in FM patients will be the focus of this thesis. In the following chapter, several elevated pro-inflammatory cytokines observed in FM patients will be analyzed.

Characteristics of the FM associated cytokines

To determine the possible influence of pro-inflammatory cytokines on FM features, characteristics of cytokines have to be known. Because IL-4, IL-6 and TNF-α were found to be involved in FM patients (see: cytokine levels in FM patients) and in FM features (see: preliminary survey) it was decided to analyze these cytokines in this thesis [34, 60]. In addition, most information was available for these cytokines. The first cytokine that will be discussed is IL-1.

IL-1

Aside from promoting hyperalgesia and stimulating the release of NGF, IL-1 (IL-1α and IL-1β) is also associated with stress, fatigue, fever, sleep and myalgia. In addition, IL-1 activates the production of IL-6 and IL-8. IL-6 and IL-8 are produced via the NFκβ-pathway, which is a pathway that is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low density lipoprotein and bacterial- or viral- antigens.

IL-1α is suggested to play a role in the maintenance of the skin barrier function which deteriorates with increasing age. Because FM is suggested to be initiated by an infection, IL-1α may be suggested to play a role in the initiation of FM by preventing the entry of an infection via the skin [50]. IL-1β production has been associated with hyperalgesia and fever, which are known features of FM [87].

The gene-expression of IL-1 needs to be regulated properly because over-expression of IL-1β is associated with cell apoptosis which makes the cells susceptible for genotoxic insults and may cause increased reactive oxygen species (ROS) levels [41]. In specific, IL-1 is suggested to be responsible for the release of NO and O_2 free radicals that generate oxidative stress [41]. Moreover, elevated IL-1 levels are associated with cardinal neuropathological changes in Alzheimer patients [40]. Finally, IL-1 gene polymorphisms, which are observed in FM patients, are also associated with chronic age-related diseases such as rheumatoid arthritis and Alzheimer [40].

IL-6

IL-6 is known as myokine, which is a cytokine that is produced by the patient's muscles. This myokine is elevated in response to muscle contraction [84]. Furthermore, IL-6 is also considered to promote fatigue, hyperalgesia, pain and depression, which are all features of FM. Moreover, IL-6 is suggested to stimulate the release of NGF. IL-6 is also thought to be involved in the pathogenesis of numerous chronic age-related diseases such as atherosclerosis, osteoporosis, Alzheimer, multiple sclerosis, systemic lupus erythematosus, CNS trauma, and viral- and bacterial-meningitis [85, 86]. IL-6 also activates B- and T- lymphocytes which form a part of the immune defense. This biological effect might be very important considering infections to be FM initiators. [34]. IL-6 also modulates haematopoiesis which may affect oxygen-levels inblood, and may consequently influence oxidative stress levels. IL-6 is also associated with cell-to-cell signaling, neuronal differentiation, neuronal growth, neuronal survival, coordination of neuroimmune responses and protection of neurons. These processes may all have their role in FM features [44]. IL-6 is also suggested to be a mediator of the peripheral analgesic action of opiods. Finally, it was suggested that local administration of IL-6 is related with anti-nociception [33].

IL-6 gene-expression must be regulated properly because chronic over-expression of IL-6 in transgenic mice suggested changes of the neuroanatomical and neurophysiologic parts of the central nervous system (CNS). Finally, IL-6 is suggested to induce the release of O_2 free radicals that might generate oxidative stress [43, 45].

TNF-α

TNF-α is associated with stress, rapid eye movement sleep and allodynia. Additionally, TNF-α stimulates the production of IL-1, it coordinates the inflammatory response by activating the cytokine cascade and it induces death signaling [52].

Also TNF-α gene expression must be regulated correctly as hemodynamic abnormalities have been observed after TNF-α overexpression. These hemodynamic

abnormalities might affect oxidative stress levels [21, 22]. Finally, TNF-α is suggested to be responsible for the release of NO and O_2 free radicals that eventually generate oxidative stress.

In summary, besides their association with FM features (see: preliminary survey) and their abundance in FM patients, background information about IL-1, IL-6 and TNF-α showed a possible association with FM features. Remarkably, all the cytokines were suggested to have the capacity to generate oxidative stress which might indicate an association between oxidative stress and pro-inflammatory cytokines in FM patients. To obtain more knowledge about this possible association, this thesis will analyze this association later on. Before analyzing this association the existence of oxidative stress levels within FM patients should be known. Therefore, the following chapter will discuss all the oxidative stress characteristics measured in FM patients.

Oxidative stress in fibromyalgia patients

To interpret these oxidative stress characteristics in FM studies, a short introduction into oxidative stress will be given first.

ROSs are products of the aerobic metabolism, which is driven by the mitochondrion (intracellular energy cell source). These ROSs play a role in the oxidative stress levels. ROSs are alkoxyl (RO•); superoxide anion (O_2•); peroxyl (RO_2•); and hydroxyl (HO•)) [1]. Beneficial ROS levels are used by phagocytes to develop the immune-defense. In contrast, an ROS excess is harmful and might: 1) inhibit protein functioning, modify cellular macromolecules and promote cell death resulting in oxidative stress [51]. To prevent excessive oxidative stress levels, several redox systems are developed to restore the imbalance between the anti-oxidant levels and the free radical levels. The Glutathione (GSH) redox system attacks oxidative stress by protecting the mitochondria against chemical and environmental stressors. Having discussed the basics of oxidative stress, all the oxidative stress levels measured in FM patients can be discussed.

The first study observed a decrease in the anti-oxidants Magnesium and Selium in FM patients compared with controls [38]. Another study suggested the downregulation of superoxide dismutase (SOD)-levels. SOD normally functions as an anti-oxidant defender by removing O_2 radicals, which decreases oxidative stress levels [39]. Also downregulated GSH levels have been observed in FM patients [10]. Furthermore, also upregulated levels of the oxidative stress marker Malonaldehyde (MDA) were observed in FM patients [38]. Finally, a study measured elevated oxidative stress indexes in FM patients [11]. In summary, several studies showed elevated oxidative stress characteristics in FM patients. These findings support the suggested oxidative stress state in FM patients.

To sum up, a literary search showed upregulated pro-inflammatory cytokine levels, compared to controls. IL-1, IL-6 and TNF-a were selected for further analysis because of their association with FM features. Thereby, it was observed that these cytokines

may enhance oxidative stress levels. Another literary search suggested that FM patients suffer from an oxidative stress state, compared to controls. All these findings triggered the suggestion that oxidative stress and cytokines may be associated in FM patients. Because the association between cytokines and oxidative stress in FM is not analyzed yet, it was decided to examine this association in a FM like condition. Hence, the model of Chung and coworkers (2009) is used which describes the effect of oxidative stress on cytokine generation in age-related diseases.

The association between oxidative stress and cytokines

The most recent and relevant model showing the association between oxidative stress and cytokines within age-related diseases is the model of Chung and co-workers (2009) *(Fig.3)*. This model is based on the induction of pro-inflammatory cytokines which is characteristic for the ageing process. The redox imbalance becomes more and more important in this ageing process. This redox imbalance could lead to chronic inflammation which is a characteristic of aging, and is observed in arthritis, obesity, diabetes, and cardiovascular diseases that are all diseases that show overlap with FM and are mostly seen in elderly. This increased inflammatory status is based on the age-related inflammatory hypothesis which consists of: (1) a dysregulation of the immune system with age, and (2) an altered redox status during aging. Both processes cause an increased inflammatory status due to an oxidative stress-induced redox imbalance. This redox imbalance is generated by a weakened anti-oxidative defense system and an upregulation of ROS production, leading to immune system activation which proceeds in a vicious circle [28]. These upregulated cytokine levels sustain sufficient through an auto-activation loop, mediated by the transcription factor NF-κβ. NF-κβ is initiated by oxidative stimuli [53]. The following chapter will confirm the suggested association between oxidative stress and cytokines via direct evidence and indirect evidence.

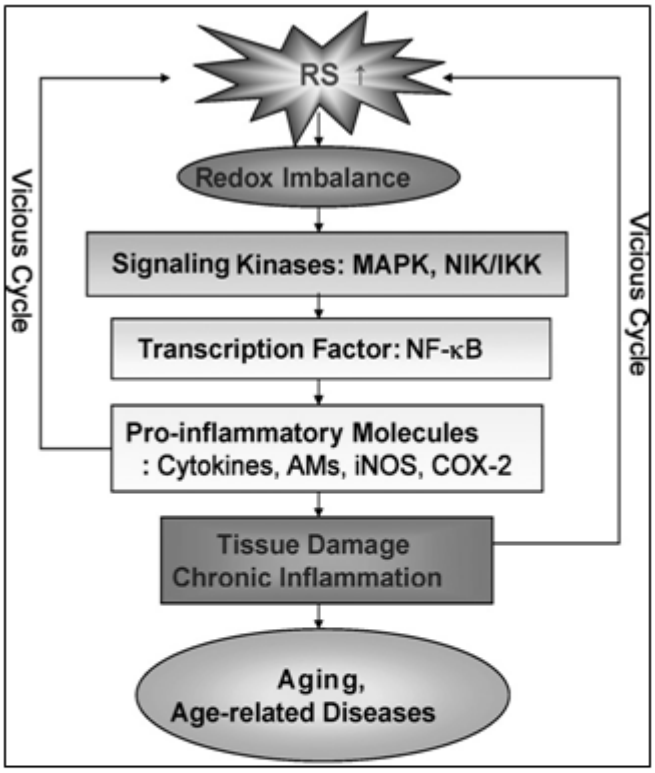

Fig.3) Oxidative stress induced redox imbalance as the patho-physiological basic of systemic inflammation, proposed as the converging link between normal aging and pathological processes. RS, reactive species; MAPK, mitogen-activated protein kinases, NIK, NFκ-B-induced kinase; IKK, IκB kinase; AMs, adhesion molecules; iNOS, inducible NO synthase; COX-2, cyclooxygenase.

Direct influence of oxidative stress on cytokine levels

The first study showed upregulated gene-expression of IL-4 and IL-6 after H_2O_2 administration [13]. Furthermore, elevated lipid peroxidation levels were suggested to cause increased TNF-α, IL-2r, IL-6 and IL-8 levels [12, 16, 20 and 27]. Moreover, a study suggested increased levels of IL-6, IL-1β and TNF-α in a ROS environment [14, 15]. Also the ROS hydroxyl radical and the ROS superoxide anion have been associated with increased levels of IL-1β, IL-6 and TNF-α [17]. Finally, administration of the GSH prodrug oxathizolidine-4 carboxylic acid inhibits the cytokine production of TNF-α, IL-8 and IL-6 [18]. These studies should be performed in FM patients in the future to obtain more insight in the suggested effect of oxidative stress on cytokines in FM patients. Also the indirect influence of oxidative stress on cytokines should be evaluated which will be done in the following chapter.

Indirect influence of oxidative stress on cytokine levels

Kiraly and coworkers found that 10 weeks of exercise resulted in decreased ROS levels, lowered hepatic protein oxidation levels, diminished MDA levels and decreased IL-6 levels [23]. Another study showed that strenuous exercise, which is characterized by oxidative stress generation, resulted in elevated IL-6 levels [55]. Administration of manganese chloride, which induces ROS, triggered the gene expression of IL-6 and IL-8 [24]. Furthermore, a study with Sesamin (anti-oxidant) was suggested to cause increased in SOD activity. Thereby, also a reduction in IL-6 levels was seen [25]. Finally, a study suggested that Omega-3 fatty acids (anti-oxidant) suppress the expression of IL-1β and IL-6 [26].

In summary, the influence of oxidative stress on cytokines was proven in an age-related disease model. The cytokines of this thesis (IL-1, IL-6 and TNF-α) can be applied to this association suggested by *Chung et al* (2009). This oxidative stress-cytokine association was also proven in this thesis in a direct- and indirect-way. Concerning the influence of cytokines on oxidative stress levels, only the oxidative stress production by cytokines described in the chapter 'Cytokine characteristics' was

found. Therefore, it was assumed that oxidative stress affects cytokine expression substantially, and that the production of oxidative stress by cytokines is marginal.

Another thing that has not been discussed yet is the influence of pro-nociceptive substances on oxidative stress. Because NGF and substance P play an important role in FM, it was decided that their association with oxidative stress had to be investigated as well. To note, it is already known that IL-1, IL-6 and TNF-α stimulate the release of NGF, and that substance P stimulates the release of IL-1, IL-6 and TNF-α.

Association between oxidative stress and pronociceptive substances (NGF and substance P)

NGF associated with oxidative stress

The first study showed enhanced O_2 production and suppressed H_2O_2 production in a NGF condition [2]. Another study showed that NGF-gene expression is stimulated by oxidative stress [92]. On a pathological level, Fernyhough and coworkers suggested that oxidative stress is able to elevate NGF expression in diabetics [88].

Substance P associated with oxidative stress

The first study showed that oxidative stress is induced by substance P [4]. Furthermore, Block and coworkers found that substance P activates microglial NADPH oxidase resulting in extracellular- and intracellular- ROS production [5]. In contrast, Huang and colleagues observed that substance P attenuates the production of oxidative stress via the suppression of the JNK pathway is attenuated by substance P [3].

Although a limited amount of studies were analyzed, this thesis hypothesized that oxidative stress induces NGF and vice versa. It was also hypothesized that substance P induces oxidative stress. Evidence supporting the induction of substance P by oxidative stress was not found.

To summarize, enhanced cytokine levels (IL-1, IL-6 and TNF-α) and enhanced oxidative stress have been measured in FM patients. Furthermore, an association between oxidative stress and cytokines was observed in diseases that show overlap with FM. These findings resulted in a hypothesis which is described below.

Hypothesis

Besides the enhanced pro-inflammatory cytokines IL-1, IL-6 and TNF-α, also enhanced oxidative stress levels have been observed in FM patients, compared with controls. Investigating the role between oxidative stress and cytokines showed that most of the studies confirm that oxidative stress stimulates cytokine production (upper part of Fig.4). It was already known that IL-1, IL-6 and TNF-α are suggested to stimulate the release of NGF, resulting in higher substance P levels (middle and right part of Fig.4). Subsequently, substance P is suggested to release IL-1, IL-6 and TNF-α (left and upper part of the Fig.4). This cycle shows that oxidative stress is produced by cytokines and pronociceptive substances which make the cycle proceed continuously. Because ageing is related with increased inflammatory- and oxidative stress levels, the cycle will be activated continuously.

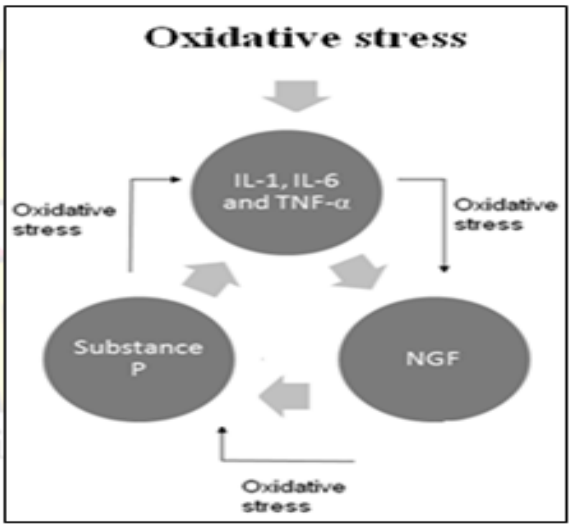

Fig.4) **Hypothetically association between oxidative stress and cytokine production in FM patients.** It is hypothesized that oxidative stress enhances the production of IL-1, IL-6 and TNF-α. This results in the upregulation of NGF, and consequently in the upregulation of substance P which is suggested to be the physiological cause of FM by contributing to the sensitization of ascending pain pathways. Additionally, oxidative stress is produced by substance P, IL-1, IL-6, TNF-α and NGF what also should contribute to elevated levels of IL-1, IL-6 and TNF-α.

This results in elevated substance P (enhanced pain), IL-1 (hyperalgesia), IL-6 (fatigue and depression) and TNF- α (stress and allodynia) *(Fig.5)*. So, the older FM patients are, the more they suffer from FM symptoms. To place this hypothesis in a broader perspective, several suggested FM co-morbidities will be evaluated in the following chapters.

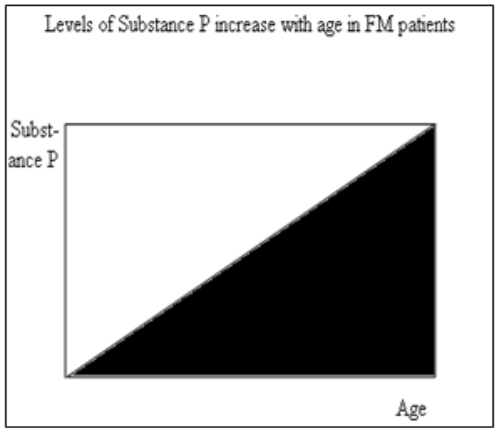

Fig.5 Levels of Substance P increase with age in FM patients. Y-axis represents Substance P levels within FM patients (schematically) and the X-axis represents age of FM patients (schematically)

Discussion

Findings thesis

This thesis started with the generation of all the cytokine data obtained from FM patients, because FM symptoms represent symptoms of the cytokine induced 'sickness syndrome'. Thereby this cytokine generation was performed because altered cytokine levels are also observed in CFS patients. Because IL-1, IL-6 and TNF-α were upregulated in FM patients and are associated with FM features, this thesis focused on IL-1, IL-6 and TNF-α levels. Remarkably, all these cytokines were able to produce oxidative stress. Because chronic ageing diseases with FM features such as CFS, Parkinson and Alzheimer also show oxidative stress levels, another literary search focused on oxidative stress levels in FM patients was performed. Most evidence showed an upregulation of oxidative stress levels within FM patients. These findings triggered another literary search to examine the suggested association between oxidative stress and cytokines. This search showed the effect of oxidative stress on cytokines via an age-related model. Furthermore, direct and indirect evidence showed the association between cytokines and oxidative stress. Another search showed that oxidative stress induces NGF and vice versa. Substance P was suggested to induce oxidative stress.

To place this hypothesis in a broader perspective, several conditions that might appear in FM patients are examined if these conditions affect the hypothesis. The first condition that will be described is the abundance of infections which is proposed by some researchers to be the initial trigger for FM.

The effect of infections on cytokine environment

Although the etiopathogenesis of FM is suggested to be multifactorial (e.g. genetic influence, environmental), some researchers think FM is initiated by an infection. Studies showed that FM patients suffer from Hepatitis C (16%), HIV (29%) and Lyme disease (25%) [93]. Another study showed that 90% of the FM patients in the study population was suffering from the Epstein Bar virus. Furthermore, T-and NK-cell dysfunctioning and decreased intracellular perforin levels were measured in FM patients. These levels might deteriorate infection defense, which subsequently may increase the vulnerability of a patient to get infected with a virus [77].

Some viruses that gain entry produce a specific cytokine or a specific cytokine homolog that may alter the cytokine environment. The Epstein bar virus, an IL-10 cytokine homolog which may possess the available IL-10 cytokine receptors which may disturb IL-10 signaling [76]. A cytokine specific dominated environment can affect cytokine production. For example, an IL-12 dominated environment (e.g. Lyme disease) T helper-1 (Th-1) cells that secrete IFN-γ, IL-2 and TNF-β that have specific physiological effects [94]. For instance, IFN-γ is suggested to decrease the availability of tryptophan leading to lowered serotonin contents in the brain [83]. These lowered serotonin levels in the brain are associated with depressions, which is an important feature of FM. An IL-4 dominated environment (e.g. Hepatitis C) is suggested to trigger the production of T helper-2 (Th-2) cells that secrete IL-5, IL-10 and IL-13 [95].

The balance between Th-1- and Th-2-cells is important as it might determine the outcome of a disease. For example, tuberculoid leprosy is a Th-1 cytokine producing disease which damages skin- and peripheral -nerves, were lepromatous leprosy produces Th-2 cytokines which progresses into infection of the bone and cartilage with nerve and tissue damage [82]. The balance between Th-1- and Th-2 cytokine producing cells is also suggested to play a role in AIDS. Early in the disease, Th-1 cell activity is high, but as AIDS progresses, some researchers have suggested, a shift may occur from a Th-1- to a Th-2-response. Because cytokine levels in FM patients vary a lot as well, transition of the Th-1-Th-2 balance in FM might be worthwhile investigating.

To sum up, it may be that infections affect the cytokine environment, subsequently affecting the hypothesis discussed in this thesis. Besides the influence of infections also the occurrence of co-morbidities may affect cytokine levels and will be evaluated in the following chapter.

Co-morbidities that affect the cytokine environment

Two different pathologies that may occur in FM and affect cytokine levels are sarcopenia and diabetes.

Sarcopenia

Sarcopenia is an age-related disease in which patients suffer from loss of muscle mass, loss of strength and loss of function which are also observed in FM patients. Measurements in sarcopenic patients showed that high levels of TNF-α and IL-6 are associated with reduced muscle strength, reduced muscle mass, poorer physical performance and higher incidences of mobility disability [19].

Diabetes Mellitus

Another disease that may be associated with FM is diabetes mellitus. The inability to mobilize, due to pain or depression may initiate obesitas, consequently leading to diabetes. Normally, adipocytes secrete IL-6 and TNF-α. However, increased adipose tissue in diabetic patients showed elevated IL-6- and TNF-α levels [48]. Pancreatic islet β-cells are affected by these elevated cytokine levels resulting in islet infiltration by T-cells and macrophages that increase local production of IFN-γ, IL-1, TNF-α and oxidative stress [47].

In summary, it is assumable that co-morbidities such as sarcopenia and diabetes influence cytokine levels, subsequently affecting the hypothesized cycle in FM patients. Besides FM associated- infections and -co morbidities, FM associated

substances are also suggested to affect cytokine levels, and hence will be evaluated in the following chapter.

The effect of FM associated changed substances on cytokine levels

Another situation that might affect cytokine environment includes FM associated changed substances. Neuro-endocrin changed substances, autonomic nerve system changed substances and central nerve system changed substances on cytokine levels will be evaluated.

Neuropeptide Y (NPY), which represents sympathic neuronal input, inhibits IFN-γ and IL-4. Diminished NPY levels in FM patients may increase IFN-γ- and IL-4-levels. Consequently, this may trigger a shift in the Th-1-Th-2 cell balance towards the Th-2 cell phenotype, thereby possibly affecting the disease outcome [60]. Reduced growth hormone levels, which are observed in FM patients, are unable to maintain the production of IL-1α, IL-6 and IL-8 [61]. Furthermore, decreased levels of norepinephrin can not stimulate the release of IL-1β, TNF-α and IL-6. Finally, FM patients showed elevated plasma concentrations of cortisol in the evening. Hence, a downregulation of pro-inflammatory cytokines in the evening may be suggested [62,63].

In summary, FM associated-infections, co-morbidities and FM associated substances influence cytokine levels, and subsequently affect the hypothesized cycle in FM patients stated in this thesis. To extend the overview of influences that may affect the hypothesized cycle in FM patients, the effect of FM associated-infections, -co-morbidities and -changed substances should be evaluated on oxidative stress levels as well. However, because it is already known that co-morbidities (e.g. diabetes) and diseases initiated by an infections such as Lyme disease are suggested to enhance oxidative stress levels, only the effect of FM associated changed substances on oxidative stress will be evaluated [10, 96].

The effect of FM associated changed substances on oxidative stress level

NMDA-receptor over activation, which is observed in FM patients, may affect oxidative stress levels [89, 90]. Over activation of NMDA receptors is suggested to induce mitochondrial dysfunction, oxidative stress and apoptosis. To note, this was measured in cultured neonatal rat cardiomyocytes [59]. Thereby, because NMDA receptors function via glutamate, upregulated levels of glutamate can be expected. Because elevated glutamate levels are associated with neuron damage in the hippocampus, it may be suggested that oxidative stress contributes to neuron damage in the hippocampus. To note, substance P levels generate glutamate as well.

So far it was observed that cytokines are suggested to have the most clinical relevance, and oxidative stress is only suggested to enhance cytokines. However, apparently, oxidative stress plays also an initial role because a well balanced oxidative/redox level is important to develop the immune defense.

Is oxidative stress associated with the initiation of FM?

Because infections are suggested to be the initial trigger for FM, immune defense to prevent the entry of infections seems relevant. GSH is essential for maintaining a proper oxidation/reduction balance which is used to develop the immune defense. In specific, GSH is used by the immune system to: 1) enhance killing activity of cytotoxic T cells and Natural killer (NK) cells, (2) regulate apoptosis, thereby controlling the immune response, and (3) modulate antigen presentation to lymphocytes, thereby influencing cytokine production. Because geriatrics show reduced GSH levels that contribute to a high oxidative stress state, it can be suggested that the immune defense is not optimal *(Fig.6)* [10]. So, a lack of GSH may enhance the vulnerability for an

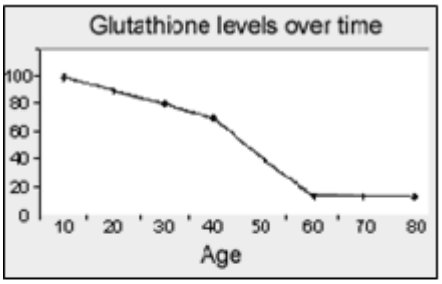

Fig.6) **Glutathione levels decrease over time.** It was found that the decline in GSH levels begins to rapidly occur at age forty in the average population. Y-axis represents GSH levels (%). The X-axis represents age of population (years).

infection that might initiate FM. Also the monthly menstrual cycle, which has an additional demand on GSH levels, appears worthwhile investigating as 90% of the FM population is women [74,79].

In this thesis it is hypothesized that oxidative stress: 1) plays a substance P elevating role, and 2) plays an initial role in FM. All together, it appears that oxidative stress plays a significant role in FM. However, another literary search suggested that the body is aware of this oxidative stress threat as it seems to target oxidative stress at the expense of it's own functioning and will be discussed below.

At the expense of…

Thyroid hormone levels are suggested to be blunted in FM patients. Because thyroid hormone levels are associated with the generation of oxidative stress, it may be suggested that an anticipating system responds to the oxidative stress state of FM patients by decreasing thyroid hormone generation. As a result their own functioning (regulation of neuron maturation) is neglected. This might be suggested as underlying cause for dysregulated neuron maturation in FM patients [30].

Another FM associated substance that appears to function by this suggested anticipating mechanism is the adrenal corticotrope hormone (ACTH), which is involved in cell survival. A mRNA study showed that ACTH induces the SOD-2 gene which protects adrenocortical cells from the cytotoxic damage of ROS's. When ACTH prefers the combat against oxidative stress in an oxidative stress environment, relatively less energy is available for cell survival what might result in the loss of function in several ways [57].

On a central nervous system level, glycine, which is correlated with pain levels in FM is known for its immune stabilizing and tissue generating properties [97]. Studies in Metabolic syndrome patients showed that glycine decreases lipid peroxidation with 25% and SOD activity with 20% [58]. Consequently, relatively less energy can be used for immune stabilization and tissue generating properties, which might cause increased vulnerability for FM triggers (e.g. infections).

In summary, it may be suggested that FM characteristic measurements (e.g. blunted thyroid hormone levels) arise as a result of the defense against oxidative stress. However, the association remains speculation as FM levels in combination oxidative stress were examined in this way. Furthermore, only one study was performed in a disease that shows overlap with FM and some studies were performed *in vitro*. However, hopefully, this literary search triggers researchers to look upon this suggested anticipating system in FM patients.

Having analyzed several possible underlying mechanisms of FM, an applicable treatment for FM should be chosen. Because this thesis hypothesizes that elevated levels of cytokines and elevated oxidative stress levels contribute to the worsening of FM pathology, a proposed treatment should target elevated oxidative stress-levels and elevated cytokine-levels. To note, treatments inhibiting cytokine and oxidative stress levels are only performed in diseases that show overlap with FM, and will be presented below.

Biological treatments (non-pharmaceutical)

Assuming that an increase of cytokine- and oxidative stress- levels contribute to the worsening of FM pathology, treatments should be based on cytokine- and oxidative stress-inhibition [3]. The biological treatments (non-pharmaceutical) calorie restriction and exercise are suggested to diminish cytokine- and oxidative stress-levels, and will therefore be discussed in the following part.

Calorie restriction

The anti-aging effect of calorie restriction (CR) is based on the protective effect against chronic inflammation and oxidative stress during the ageing process *(Fig.7)*. A study showed that CR reduces the age -associated elevation of TNF-α, IL-1β, IL-6 and oxidative stress [28].

Fig.7) Calorie restriction and exercise are suggested to inhibit redox imbalance, thereby inhibiting molecular inflammation which should have beneficial effects on pathological ageing and physiological ageing, eventually resulting in an increased life span.

Exercise

Physical activity is suggested to affect the immune system, depending on the person's endurance capacity, frequency, volume of exercise and intensity. A study showed diminished IL-6 levels during aerobic exercise. However, this could also be caused by the reduction in adipose tissue as adipocytes produce IL-6 [28]. Diminished IL-6- and TNF-α-levels have also been observed in healthy, physical active elderly [28]. Also elevations of anti-oxidant defense systems (e.g. SOD, catalase, and GSH) have been observed during regular exercise [28].

To sum up, calorie restriction- and exercise- treatments target cytokine- and oxidative stress- levels and should

therefore be considered as possible treatment. Besides non-pharmaceutical treatments, also pharmaceutical treatments should be examined.

Biological Treatment (pharmaceutical)

Pharmaceutical biological treatments based on cytokine- and oxidative stress-inhibition should also be examined to determine the cytokine- and oxidative stress-influence more precisely, and on an individual level. Notably, these treatments are only examined in diseases that show overlap with FM.

Cytokine inhibition treatment

Cytokine inhibiting treatments can be divided in non-specific cytokine inhibiting treatments and in specific cytokine inhibiting treatments. This chapter starts with non-specific cytokine treatments.

Non-specific treatment

Glucocorticoid administration inhibits the expression of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 and TNF-α. After glucocorticoid administration 500 CFS patients were followed; 94% of the CFS patients showed general improvement, 75% showed significant improvement and 62% reported substantial improvement of energy levels [64].

Specific treatment

Two cytokine specific treatments are the anti-IL-6 therapy and the anti-TNF-α therapy that block the functioning of IL-6- and TNF-α levels, respectively. After administration of Tocilizumab® (atlizumab), which inhibits the IL-6 receptor, improved quality of life due to decreased pain was measured in RA patients [70]. Treatment with Humira® (adalimumab), which blocks TNF-α, showed a significant

improvement in disability- and quality of life in psoriatic patients (chronic immune disease) [67]. In addition, administration of Enbrel® (etanercept), which blocks TNF-α, demonstrated improvement in fatigue and depression in psoriatic patients [68]. Furthermore, Remicade® (infliximab), which also blocks TNF-α, showed improvement in pain and fatigue in patients with the primary sjogren's syndrome [69]. Finally, a combination of these specific treatments, Thalomid® (thalidomide), which blocks IL-6 and TNF-α, demonstrated pain reduction in complex regional pain syndrome patients [66]. Notably, treatments that influence TNF-α levels also affect IL-1 and IL-6 production. Therefore, it is difficult to link a specific cytokine to a specific symptom. Finally, pharmaceutical biological treatments based on oxidative stress inhibition will be evaluated.

Oxidative stress inhibition treatment

Injections with vitamin B12, which scavenges nitric oxide, showed alleviations of symptoms in FM patients, CFS patients and multiple complex syndrome patients [74,75]. Furthermore, intra-muscular GSH injections showed positive results after 6 months of treatment; 82% of the patients experienced improvement in fatigue, 71% showed improvement in memory and concentration, and 62% experienced improvement in levels of pain [73]. Finally, the administration of N-acetyl-cysteine, a strong anti-oxidant that supports GSH homeostasis, delays muscle fatigue in exercising humans [82].

In summary, it appears that non-pharmaceutical- and pharmaceutical-treatments based on cytokine- and oxidative stress- inhibition, have clinical effect in diseases that show overlap with FM. However, more research is needed to determine in which way, and to what extend cytokine- and oxidative stress-levels play a role in FM. Other topics can be of interest for FM research as well and will be discussed in the next chapter.

Future fibromyalgia research

Evidence for the abundance, and possible involvement of cytokines and oxidative stress in FM is growing. At the moment, FM studies based on cytokines and oxidative stress are still very limited, show contrary findings, and mostly lack significant results. Furthermore, the suggested associations of oxidative stress and cytokines with infections, co-morbidities, FM associated substances and treatments should also be examined in FM patients. Finally, factors that influence cytokine levels such as gender, length of disease, body mass index, concomitant medications and co-morbidities have not been included in most of the studies. Hence, several clinical criteria are required for future FM research.

Firstly, more FM experiments, cytokine- and oxidative stress- based, are needed. Secondly, because FM is a heterogeneous disease, it might be wise to develop experiments with FM subgroups (different symptoms), because subgroups may differ in pathophysiology resulting in different responses to treatments. FM subgroups were recently created by *Muller et al* and may function as example to follow in future FM research; 1) FM with extreme sensitivity to pain, but no associated psychiatric conditions; 2) FM and co morbid pain-related depression; 3) Depression with concomitant FM syndrome; 4) FM due to somatization. *Muller et al* suggested that mild inflammatory processes are the underlying cause in group 1. This group appears to be a perfect group to investigate cytokine influence in FM patients as chronic inflammation is characterized by cytokines [91]. A study

Biochemical parameter (pg/ml)	Highly pressure sensitive tenderpoints and no depression	Slightly sensitive tenderpoints and depression	Fibromyalgia resulting from a somatization process	New subgroup
TNF-α	34.05	15.51		
IL-1α	337.54	190.82		
IL-1β	15.76	10.88		
IL-10	14.08	6.14		
IL-6	?	?		
IL-4	?	?		
IL-12	?	?		
Pro-inflammatory-cytokines vs. anti-inflammatory	?	?		
Oxidative stress	?	?		
+ Biological treatment (cytokine inhibition/ oxidative stress inhibition)	?	?		

Table 2) Various biochemical parameters in FM patients with highly pressure-sensitive tender points but no signs of depression and patients with slightly pressure-sensitive tender points and depressive symptoms in some cases. Biochemical parameters that are indicated by question marks should be included in future FM research.

performed in 25 FM patients showed 12 patients who exhibited extreme sensitivity to pain without depressions (group 1), and 13 patients who experienced slight sensitivity with depressions (group 2). Because it appears that the upregulated pro-inflammatory cytokines cause sensitive tenderpoints a cytokine-FM symptom association can be suggested (table 2). Also the distinction between primary FM patients (no observed cause) and secondary FM patients (cause known, e.g. mechanical injury) on a cytokine- and an oxidative stress- level may be worthwhile investigating. Furthermore, several biochemical parameters are missing to my opinion, which are indicated by question marks in table 2. Follow-up research should include IL-6 because this cytokine is associated with depressions. Thereby, research should include IL-4 as it is associated with diseases that show overlap with FM (e.g. osteoporosis), and IL-12 as it plays a central role in the initiation and regulation of the cellular immune response, and because IL-12 is associated with CFS [99,100,101]. Because the balance between pro-inflammatory cytokines and anti-inflammatory cytokines possibly determines the outcome of a disease, this should be examined as well. Thereby, because an alteration of cytokine receptors and cytokine receptor antagonists was observed in FM patients, cytokine receptors and cytokine receptor antagonists of all the examined cytokines should be investigated in future FM research. Furthermore, oxidative stress characteristics and biological treatments (pharmaceutical and non-pharmaceutical), based on cytokine- and oxidative stress inhibition should be examined.

Inclusion- and exclusion-criteria regarding for study populations should also be monitored properly. Patients that show altered levels of factors that may increase oxidative stress levels such as metal-decompartimentalization, metal-overload, NADPH oxidase, alcohol, smoking, environmental agents and hyperglycemia (diabetes), should be excluded. In addition, factors that are suggested to inhibit oxidative stress levels such as anti-oxidant enzymes (e.g. SOD), anti-oxidant vitamins (A,C,E) and GSH should be excluded as well. Eventually, when all the study criteria are fulfilled, oxidative stress levels can be measured with a technique based on DNA/RNA damage which measures cerebrospinal fluid samples. Other techniques that are developed to measure the oxidative stress state are based on lipid peroxidation, oxidative protein damage and anti-oxidants (e.g. catalase, GSH, SOD).

All these oxidative stress characteristics can be obtained from blood-, urine-, tissue- and cell-samples.

As described before, factors that influence cytokine levels such as age, gender, length of disease, body mass index, concomitant medications and co-morbidities should be controlled properly as well. Characteristics (e.g. temporal gene expression) from cytokines should be measured across time with multiplex technologies (e.g. Enzyme-Linked-Immuno-Sorbant-Assay, flow cytometric multiplex array). A study investigating temporal cytokine characteristics suggested that biomarkers of oxidative stress and IL-6 were highly correlated in young men but not in old men. Another study showed a decreased production of IL-6 and TNF-α at the end of the spring in contrast to the rest of the year [80].

Fulfilling all these study criteria will enhance our understanding of the complex pathophysiology of FM regarding cytokines and oxidative stress. These future investigations hopefully contribute to the realization of an optimal individual treatment for this 'quality of life destructing' disease. Hopefully, this review makes researchers aware of the fact that FM research in the cytokine area and the oxidative stress area is needed to understand the disease better.

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