Fibromyalgia:
A Psychoneuroimmunological Perspective

Dissertation zur Erlangung der naturwissenschaftlichen Doktorwürde
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vorgelegt von Dipl.-Psych. Judith Heße-Husain

Gutachter:
Prof. Dr. D. H. Hellhammer
Dr. A. C. Gierens

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Abbreviations

ACR	American College of Rheumatology
ACTH	adrenocorticotrophic hormone
ADS	Allgemeine Depressionsskala
AP-1	activator protein 1
APC	antigen presenting cell
AUCi	area under the curve with respect to increase
BBB	blood-brain barrier
BMI	body mass index
cAMP	cyclic adenosine 5'-monophosphate
CFS	chronic fatigue syndrome
Con A	concanavalin A
COX-2	cyclooxygenase 2
CRH	corticotrophin releasing hormone
CVO	circumventricular organ
EDTA	ethylenediamine tetraacetic acid
EGF	endothelial growth factor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthease
FIQ-G	Fibromyalgia Impact Questionnaire, German version
FMS	fibromyalgia syndrome
FS	Fatigue Scale
GH	growth hormone
GLM	general linear model
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
HCV	hepatitis C virus
HPA	hypothalamic-pituitary-adrenal
HPLC	high pressure liquid chromatography
IBS	irritable bowel syndrome
IFN	interferon
Ig	immunoglobulin
IGF-1	insuline-like growth factor-1
IL	interleukin

iNOS	inducible nitric oxide synthase
LC	locus coeruleus
LPS	lipopolysaccharide
MANCOVA	multivariate analysis of covariance
MANOVA	multivariate analysis of variance
MAPK	mitogen-activated protein kinase
MCS	multiple chemical sensitivity
MIF	macrophage migration inhibitory factor
MKP-1	mitogen-activated protein kinase phosphatase-1
MPI	Multidimensional Pain Inventory
NE	norepinephrine
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK cells	natural killer cells
NMDA	N-methyl-D-asparate
NTS	nucleus tractus solitarius
PBMC	peripheral blood mononuclear cells
PDI	Pain Disability Index
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PKA	protein kinase A
PMA	phorbol myristate acetate
PNI	psychoneuroimmunology
PSQI	Pittsburgh Sleep Quality Index
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
SD	standard deviation
SEM	standard error of the mean
SES	socioeconomic status
SNS	sympathetic nervous system
STAI-S/STAI-T	State-Trait-Anxiety-Inventory (measuring State/ Trait anxiety)
Th cell	T helper cell
TLR	Toll-like receptor
TNF	tumor-necrosis-factor

TSST	Trier Social Stress Test
VEGF	vascular endothelial growth factor
WHR	waist-to-hip ratio
5-HTT	serotonin transporter gene
5-HTTLPR	serotonin transporter gene promotor region

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Chapter 1

1 Introduction, Objectives, and Outline of the Thesis

1.1 Introduction

In 1903, Charles Eucharist de Medici Sajous (1852-1929) published a book named "*The internal secretions and the principles of medicine*", which contained a list of 96 features illustrating the failings of physiology, number 96 being: "*That the most fatal and distressing diseases of mankind have not been mastered because the cardinal role of the adrenal system in their pathogenesis, prevention and cure, has been overlooked*." He concluded that "*what are now considered as symptoms of infection or poisoning are all manifestations, more or less severe, of activity or insufficiency of the adrenal system*" (Sajous, 1903). In the 4th edition of his book Sajous described three clinical forms of *hypoadrenia*, among those the so-called "*functional hypoadrenia*", a form in which the adrenals, though not the seat of organic lesions, are functionally deficient because of tardy development, debilitating influences such as fatigue and starvation, and old age. In adults it developed when "*as a result of the vicissitudes of our existence*" the adrenals were exhausted by the strain of exercise or labour. The main symptoms were fatigue and increased susceptibility to infection (Sajous, 1911). At first, the concept of hypoadrenia was based on the premise that through exhaustion the adrenals did not produce enough epinephrine. Consequently, Addison's disease was thought to equal epinephrine deficiency. However, the attention of scientists shifted from the adrenal medulla to the cortex in the late 1920s when Swingle and Pfiffner reported that they had extracted "the cortical hormone" using organic lipid solvent and that it could keep adrenalectomized cats indefinitely alive (see Tattersall, 1999). Nowadays it is well-established that a lack of the steroid hormone cortisol that is produced in the adrenal cortex plays a major role in Addison's disease.

The symptoms of "functional hypoadrenia" as described by Sajous early in the last century resemble a lot those symptoms observed in patients with stress-related disorders of unexplained medical origin such as fibromyalgia syndrome (FMS), chronic fatigue syndrome (CFS), and post-traumatic stress disorder (PTSD). Interestingly, a mild, subclinical form of cortisol deficiency, often termed *hypocortisolism*, has been observed in a subgroup of patients suffering from these disorders (Fries, Hesse, Hellhammer & Hellhammer, 2005; Heim, Ehlert & Hellhammer, 2000). Accordingly, "functional hypoadrenia" most likely represents cortisol deficiency rather than epinephrine deficiency as assumed by Sajous.

Sajous' observation of fatigue and an increased susceptibility to infection as main symptoms of "functional hypoadrenia" entail important implications with regard to the consequences of altered cortisol and/or catecholamine secretion for other bodily systems. For example, while the immune system was long thought to be an auto-regulatory system, accumulating evidence over the last decades has affirmed that other bodily systems such as the hypothalamic-pituitary-adrenal (HPA) axis with its end-product cortisol and the sympathetic nervous system (SNS) via epinephrine and norepinephrine are able to control the outcome of immune responses (e.g., Elenkov, Wilder, Chrousos & Vizi, 2000; Rhen & Cidlowski, 2005). The immune system, in turn, is able to signal the brain and trigger a cascade of events that leads to physiological and behavioral changes (e.g., Dantzer, 2004; Kelley, Bluthe, Dantzer, Zhou, Shen, Johnson & Broussard, 2003).

Based on the fact that the interaction between bodily systems is essential in understanding biological processes, a new research field has emerged that was coined *Psychoneuroimmunology* (PNI) by Ader and Cohen in 1981. PNI encompasses measurable interactions between psychological and physiological processes and the subsequent effects of these interactions upon disease development and progression. In this context, the impact of acute/chronic stress and glucocorticoids on cells of the immune system has been extensively studied in animal models, healthy individuals, and in a multitude of patients suffering from diseases such as cancer and acquired immunodeficiency syndrome (Dhabhar & McEwen, 2001; Glaser & Kiecolt-Glaser, 2005; Segerstrom & Miller, 2004). In stress-related disorders, changes in immune system activity have been investigated with regard to lymphocyte and cytokine levels (Glover, Steele, Stuber & Fahey, 2005; Patarca-Montero, Antoni, Fletcher & Klimas, 2001; Russell, Vipraio, Michalek, Craig, Kang & Richards, 1999), mitogen-induced cytokine production (Gaab, Rohleder, Heitz, Engert, Schad, Schurmeyer & Ehlert, 2005; Rohleder, Joksimovic, Wolf & Kirschbaum, 2004), and natural killer cell activity (Kawamura, Kim & Asukai, 2001; Landis, Lentz, Tsuji, Buchwald & Shaver, 2004).

However, despite the intent to shed light on neuro-endocrine-immune interactions in stress-related disorders, this branch of psychoneuroimmunologic research is still in its infancy. This is reflected by the fact that study results are often inconsistent; in addition, there is a lack of studies concomitantly investigating endocrine, sympathetic and immune system alterations in the same patient cohort.

The present work attempted to elicit associations between potential HPA, SNS, and immune changes in patients with FMS in an effort to contribute to the understanding of neuro-endocrine-immune interactions in stress-related disorders.

1.2 Scope of the thesis

In the present work, results are presented addressing psychosocial, endocrine, sympathetic, and immunological aspects and their potential associations in female FMS patients and healthy women. The study is based on previous findings in animals and humans that reported on neuro-endocrine-immune interactions in health and disease.

1.2.1 Objectives

Within the framework of developing a psychoneuroimmunological model for FMS, which is regarded as a stress-related disorder in the present work, the more precise objectives are as follows:

1. to investigate whether changes in HPA axis (re)activity are accompanied by alterations in norepinephrine and immune cell levels;
2. to examine whether reduced cortisol levels result in a disequilibrium between the hormone and the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF), which functions as a potent counter-regulator of glucocorticoid activity and as a strong inducer of inflammatory agents;
3. to explore whether FMS is accompanied by an increased pro-inflammatory and/or reduced anti-inflammatory cytokine activity and to what extent this is related to hypocortisolemic features and the degree of FMS symptoms in the patients.

1.2.2 Hypotheses

Hypothesis 1

Previous studies have reported reduced basal and stress-induced glucocorticoid activity in FMS patients (e.g., Calis, Gokce, Ates, Ulker, Izgi, Demir, Kirnap et al., 2004; Griep, Boersma, Lentjes, Prins, van der Korst & de Kloet, 1998; Gur, Cevik, Nas, Colpan & Sarac, 2004a; Gur, Cevik, Sarac, Colpan & Em, 2004b; Kirnap, Colak,

Eser, Ozsoy, Tutus & Kelestimur, 2001). Based on the observation that the effects of glucocorticoids on catecholamine release are inhibitory (Kvetnansky, Fukuhara, Pacak, Cizza, Goldstein & Kopin, 1993; Pacak, Kvetnansky, Palkovits, Fukuhara, Yadid, Kopin & Goldstein, 1993), a mild, chronic form of hypocortisolism in FMS patients might result in reduced inhibitory feedback of glucocorticoids on SNS activity. Therefore, the following hypothesis was tested:

Hypothesis 1a: Reduced cortisol levels before and after a psychosocial stress test in female FMS patients are accompanied by elevated norepinephrine levels.

Data indicates that acute elevations of catecholamines result in transient increases in lymphocyte numbers, which is mostly pronounced by elevated natural killer (NK) cell numbers (Benschop, Rodriguez-Feuerhahn & Schedlowski, 1996; Elenkov et al., 2000). However, chronic increases of SNS activity might have an opposite effect on NK cell numbers in terms of decreased levels in the peripheral blood (Elenkov et al., 2000; Maisel, Knowlton, Fowler, Rearden, Ziegler, Motulsky, Insel et al., 1990b). Based on the assumption that chronic adrenal hypoactivity is accompanied by persistently elevated norepinephrine levels, the following hypothesis was developed:

Hypothesis 1b: Elevated norepinephrine levels before and after a psychosocial stress test in female FMS patients that might be the result of chronic adrenal hypoactivity are accompanied by decreased NK cell levels.

Hypothesis 2

MIF is a pro-inflammatory cytokine with exceptional features in that it acts as a potent physiological counter-regulator of the anti-inflammatory effects of glucocorticoids (Bucala, 1996). While MIF is considered a critical mediator of a number of diseases such as septic shock, cancer, atopic dermatitis, atherosclerosis, rheumatoid arthritis, and obesity (see Calandra & Roger, 2003; Lue, Kleemann, Calandra, Roger & Bernhagen, 2002; Ohkawara, Nishihira, Takeda, Asaka & Sugiyama, 2005), little is known about possible MIF alterations in stress-related disorders that are characterized by blunted cortisol levels. In addition, MIF responses to a psychosocial stress test causing elevations in cortisol concentrations have never been tested in previous studies. Therefore, the following hypothesis was mounted:

Hypothesis 2: Increases in the pro-inflammatory cytokine MIF that are expected to occur in response to a psychosocial stress test are more pronounced in female FMS patients than in healthy women, resulting in a lower cortisol/MIF ratio in the patient group, possibly indicating higher inflammatory activity.

Hypothesis 3

Glucocorticoids are the most potent anti-inflammatory agents in the body. Consequently, a chronic, mild reduction of cortisol levels as repeatedly observed in FMS patients might result in the suppression of anti-inflammatory activity, whereas inflammatory agents, such as pro-inflammatory cytokines, might be increasingly released. Results from animal and human studies have shown that elevations in pro-inflammatory cytokine levels are related to the induction of the so-called sickness response, which is characterized by physiological and behavioral changes that occur in infected animals and humans (Larson & Dunn, 2001; Lee, Dantzer, Langley, Bennett, Dougherty, Dunn, Meyers et al., 2004; Maier & Watkins, 1998).

Numerous symptoms of FMS resemble those of the sickness response, including pain, fatigue, and concentration difficulties, which has led to the assumption that typical FMS symptoms are linked to immune activation, actually comprising symptoms of the sickness response (Van Houdenhove & Egle, 2004). However, several studies have investigated the role of cytokines in FMS revealing mixed results (Amel Kashipaz, Swinden, Todd & Powell, 2003; Gur, Karakoc, Erdogan, Nas, Cevik & Sarac, 2002a; Gur, Karakoc, Nas, Remzi, Cevik, Denli & Sarac, 2002b; Hader, Rimon, Kinarty & Lahat, 1991; Pay, Calguneri, Caliskaner, Dinc, Apras, Ertenli, Kiraz et al., 2000; Salemi, Rethage, Wollina, Michel, Gay, Gay & Sprott, 2003; Uceyler, Valenza, Stock, Schedel, Sprotte & Sommer, 2006; Wallace, Bowman, Wormsley & Peter, 1989; Wallace, Peter, Bowman, Wormsley & Silverman, 1990; Wallace, Linker-Israeli, Hallegua, Silverman, Silver & Weisman, 2001). But there is a lack of studies with FMS patients exploring potential cytokine changes in the context of cortisol alterations. Therefore, the following hypothesis was tested:

Hypothesis 3: Compared to a healthy control group, female FMS patients display an increased pro-inflammatory and a decreased anti-inflammatory activity as reflected by lymphocyte production of cytokines before and after a psychosocial stress test. These differences are especially pronounced in patients with high sickness-response-like symptoms and in those with hypocortisolemic features.

1.2.3 Approach

1. HPA axis activity in terms of ACTH and cortisol levels, SNS activity in terms of catecholamine concentrations, and immune cell levels with special regard to NK cells were investigated before and after a psychosocial stress test in women with FMS and healthy women. Potential confounding factors were controlled by including them as covariates in the statistical models. Within the FMS and control groups, subgroups were built based on changes in free cortisol levels in response to the stress test thus obtaining “responder” and “non-responder” groups. These subgroups were also taken into consideration in the context of cytokine alterations as presented in Chapter 5.
2. Cortisol and MIF levels were assessed and analysed before and after a psychosocial stress test in the FMS and control groups. The potential influence of body mass on MIF levels was taken into account by entering the body mass index (BMI) as covariate in the statistical models. The cortisol/MIF ratio served as an index of inflammatory activity.
3. A cytokine biochip array was used for the simultaneous quantification of different pro-and anti-inflammatory cytokine levels in women with and without FMS before and after a psychosocial stress test. Pro- and anti-inflammatory cytokine production was related to the degree of FMS- and sickness-response-like symptoms as assessed by questionnaires in the patient group, and to the cortisol reactivity after the stress test.

1.3 Outline of the thesis

The present **Chapter 1** provides an introduction and a description of the objectives and hypotheses of the thesis. This chapter is followed by a presentation of the theoretical background of the topic in **Chapter 2**, which comprises an overview of the clinical picture of FMS including its definition, prevalence, etiology, and its comorbidity/overlap with other (stress-related) disorders. In the second half of Chapter 2, some basics of neuro-endocrine-immune interactions are introduced that are essential for the understanding of the present work. Chapters 3-5 describe and discuss study results with regard to concomitant HPA, SNS and immune cell alterations in FMS patients as compared to healthy subjects **(Chapter 3)**, MIF and cortisol levels and the cortisol/MIF ratio in women with and without FMS **(Chapter 4)**, and the role of pro- and anti-inflammatory cytokines in FMS **(Chapter 5)**. Since chapters 3-5 are written in a format that allows independent publication of each, abbreviations are introduced anew in each of the three chapters. As part of the general discussion in **Chapter 6**, the hypotheses are resumed, and an attempt is made to integrate the findings from the previous three Chapters into a proposed model of neuro-endocrine-immune interactions in health and disease as exemplified for FMS.

Chapter 2

2 Theoretical Background

2.1 Fibromyalgia

2.1.1 Definition

The fibromyalgia syndrome (FMS) is a disorder of unknown etiology characterized by widespread, chronic musculoskeletal pain and pressure hyperalgesia. FMS is often accompanied by additional symptoms such as irritable bowel syndrome (IBS), irritable bladder, fatigue, morning stiffness, sleep difficulties, a swollen feeling in the soft tissues, paresthesia, cognitive dysfunctions, lightheadedness, and by an increased incidence of depressive symptoms (Thompson & Barkhuizen, 2003; Yunus, 2002). According to the American College of Rheumatology (ACR) 1990 Criteria for the Classification of Fibromyalgia (Wolfe, Smythe, Yunus, Bennett, Bombardier, Goldenberg, Tugwell et al., 1990), chronic widespread pain of at least three months duration in combination with mild or greater tenderness at 11 or more of 18 specific tender points constitute the principal criteria for the diagnosis of FMS. Wolfe and colleagues define pain as widespread when *"all of the following are present: pain in the left side of the body, pain in the right side of the body, pain above the waist, and pain below the waist. In addition, axial skeletal pain (cervical spine or anterior chest or thoracic spine or low back) must be present..."* (Wolfe et al., 1990). Thus, widespread pain involves all four quadrants of the body and the axial skeleton. A tender point is defined as an anatomic site where an individual complains of pain when approximately 4 kg of digital palpation is applied. The determined 18 tender point sites are illustrated in Figure 2.1.

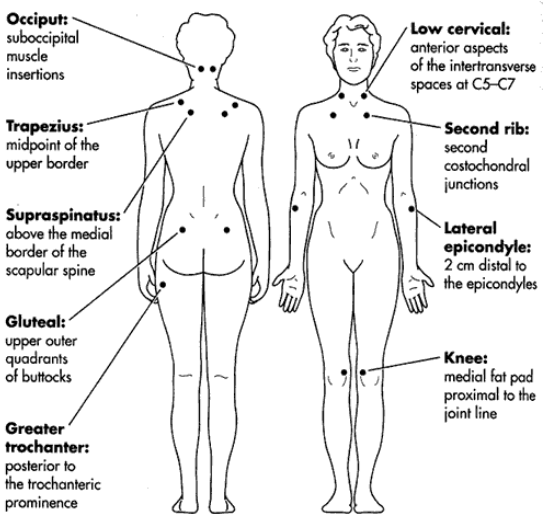

Figure 2.1: Location of the specific tender points in FMS

The ACR study of Wolfe and colleagues (1990) included 558 patients of whom 293 had FMS. The authors found that a combination of widespread pain and tender points in the diagnosis of FMS resulted in a sensitivity of 88.4% and a specificity of 81.8% when used to identify FMS patients from among other rheumatic conditions. However, the criteria of defined tender points has repeatedly been questioned by studies reporting that tenderness does not only occur in these discrete regions but that patients with FMS display increased sensitivity to pain throughout the entire body (Granges & Littlejohn, 1993; Mikkelsson, Latikka, Kautiainen, Isomeri & Isomaki, 1992; Yunus, Dailey, Aldag, Masi & Jobe, 1992). In addition, it has been demonstrated that tender point counts are positively correlated with a person's level of daily stress (Urrows, Affleck, Tennen & Higgins, 1994) and that tenderness is influenced by numerous factors such as female gender, increasing age, poor aerobic fitness, non-specific bodily complaints, mood disorders, distress, and fatigue, which all tend to increase cutaneous pressure sensitivity (Croft, Schollum & Silman, 1994; McBeth, Macfarlane, Benjamin, Morris & Silman, 1999; Petzke, Gracely, Park, Ambrose & Clauw, 2003; Schochat & Raspe, 2003). A recent review has summarized and discussed the problems that arise when tender points are taken into consideration in the diagnosis of FMS (Clauw & Crofford, 2003). Clauw and Crofford point out that while chronic widespread pain as one of the crucial criteria for FMS occurs in 11% of the general population in the USA and the UK (Croft, Rigby, Boswell, Schollum & Silman, 1993; Wolfe, Ross, Anderson, Russell & Hebert, 1995b) with women being about 1.5 times more affected than men, the inclusion of tender points in the diagnosis of FMS leads to lower prevalence rates and to a shift towards women being up to ten times more affected than men. The authors also address the impact of distress on tender points referring to a study conducted by Wolfe (1997) who found a linear relationship between tender points and distress levels, which made him call tender points a "sedimentation rate for distress" (Wolfe, 1997). Based on these findings, it becomes clear that the abandonment of tender points as a requirement for the diagnosis of FMS would lead to a totally different disorder: one affecting more men, with a group displaying considerably lower levels of distress. On the other hand, in support of the maintenance of tender points in the diagnosis of FMS, they ensure that FMS patients experience not just chronic widespread pain but also hyperalgesia and allodynia, which is pain in response to normally non-painful stimuli (Clauw & Crofford, 2003). In addition, the ACR criteria for the classification of

fibromyalgia have allowed a standardization of research in this area, which makes a direct comparison of prevalence in different countries possible, as outlined in the next section.

2.1.2 Prevalence

In the USA, FMS constitutes the second most common rheumatologic disorder behind osteoarthritis with a prevalence between 0.5% and 3.4% in the general population (Wolfe et al., 1995b) and 5% to 20% in patients in medical clinics (Wolfe, 1994). Women comprise 80% to 90% of the affected population (White, Speechley, Harth & Ostbye, 1999; Yunus, 2002). Population studies of FMS using the ACR 1990 criteria have shown a fairly constant prevalence of FMS across several countries throughout the Western world, ranging from 0.7% to 5.5% (see Table 2.1). In all of these studies, an increase in prevalence could be observed with age (e.g., peak prevalence at 55-64 years in Makela's study, at 60-79 years in Wolfe's study), and females were 4 to 8 times more likely to be affected than men. Moreover, factors such as level of education (inverse gradient), physical stress at work, lower household income, being divorced, and being disabled were associated with increased odds of having FMS in these studies. Schochat and colleagues (2003) found that women with FMS had a 3.6-fold risk of having a lower social level compared to healthy control subjects.

Table 2.1: Prevalence of FMS across countries throughout the Western world

Reference	Country	Age group (Years)	Number of subjects	Prevalence (%)		
				Men	Women	All
Makela & Heliovaara (1991)	Finland	≥ 30	8000	0.5	1.0	0.8
Prescott et al. (1993)	Denmark	18-79	6000	0.0	1.3	0.7
Wolfe et al. (1995b)	USA	≥ 18	3006	0.5	3.4	2.0
White et al. (1999)	Canada	≥ 18	3395	1.6	4.9	3.3
McNally et al. (2006)		≥ 12	131535	0.3	1.8	1.1
Lindell et al. (2000)	Sweden	20-74	2425	0.0	2.4	1.3
Schochat et al. (2003)	Germany	35-74	3174	-	5.5	

Several studies have reported gender differences not only in the prevalence but also in the clinical features of FMS. In a community-based study, Wolfe and colleagues (Wolfe, Ross, Anderson & Russell, 1995a) found that women were almost 10 times more likely to have 11 tender points than men. Moreover, women were more likely to have symptoms like sleep disturbance, fatigue, irritable bowel syndrome, and “pain all over”. Similar results were found in another study with 536 FMS patients, where men with FMS had significantly less frequent “hurt all over”, fatigue, morning fatigue and IBS. The number of tender points was also higher in women than in men and constituted the most powerful discriminator between men and women with FMS in a stepwise logistic regression analysis (Yunus, Inanici, Aldag & Mangold, 2000). In contrast to the findings of fewer or less frequent symptoms and tender points among men, a study conducted in an Israeli rheumatology clinic matched 40 men and 40 women with FMS, and found that men had worse health outcomes than women in terms of physical functioning, quality of life, and severity of FMS-related symptoms such as fatigue, morning stiffness and IBS (Buskila, Neumann, Alhoashle & Abu-Shakra, 2000). According to Yunus (2002), the opposite findings on gender differences indicate that socio-cultural factors in the determination of gender differences in FMS may be of great importance. It should be noted at this point that Yunus and colleagues could not find any gender differences in psychosocial parameters in a recent study with FMS patients (Yunus, Celiker & Aldag, 2004).

2.1.3 Etiology

The exact etiology of FMS is mostly unknown. In patient surveys, more than half of the patients cannot associate a causative event with their FMS (Wolfe, 1989), while others experience stressful or traumatic life events, physical trauma or viral infections in the forefront of disease onset. In addition, a genetic component to the disease has been discussed. In the following, studies are presented reporting on the different causes that might contribute to the development of FMS.

2.1.3.1 Stressful life events and chronic work stress

There is growing evidence that life stress in child- and/or adulthood confers an increased risk of chronic pain conditions such as FMS (Goldberg, Pachas & Keith, 1999). For example, one study compared the rate and the impact of life events

experienced in childhood, adolescence and adulthood between FMS patients and healthy volunteers (Anderberg, Marteinsdottir, Theorell & von Knorring, 2000). Results revealed that during childhood or adolescence, half of the participating 40 FMS patients (51%) had experienced life events that they rated as very negative as compared to 28% of the 38 healthy controls. Conflict with parents was most common in both groups; however, FMS patients experienced significantly more bullying. Before disease onset, 65% of the patients faced some negative life events including financial problems (45%), conflict with the partner (42.5%), death of a close relative or friend (37.5%), and physical or psychological abuse (25%). While 24.5% of the healthy volunteers reported very negative life events during the last year, the rate was significantly higher in the patient group (51%), which was especially due to events such as physical or psychological abuse and disease/accident in a close relative. These findings are supported by other studies reporting higher rates of daily hassles (Hauser, Bernardy & Arnold, 2005) that often focus on personal-dependent problems such as dissatisfaction with oneself, insecurity, and lack of social recognition (Van Houdenhove, Neerinckx, Onghena, Vingerhoets, Lysens & Vertommen, 2002), as well as a higher prevalence of emotional neglect and physical and/or sexual abuse throughout the lifespan of FMS patients (Imbierowicz & Egle, 2003; Van Houdenhove, Neerinckx, Lysens, Vertommen, Van Houdenhove, Onghena, Westhovens et al., 2001; Walker, Keegan, Gardner, Sullivan, Bernstein & Katon, 1997). However, as Van Houdenhove and Egle (2004) stated, evidence for a traumatic history is not found in all FMS patients. Therefore, this factor might have variable etiological weight and might as well be buffered by protective factors, such as supportive relationships in later life. In addition, there is a lack of prospective studies in non-selected populations that could confirm the etiological relevance of victimization for the development of FMS (Van Houdenhove & Egle, 2004).

Recently, a prospective cohort study has investigated the role of work stress in the incidence of newly diagnosed FMS (Kivimaki, Leino-Arjas, Virtanen, Elovainio, Keltikangas-Jarvinen, Puttonen, Vartia et al., 2004). A cohort of 4791 Finnish hospital employees (540 male, 4251 female) completed two sets of questionnaires in 1998 and 2000 addressing stress at work with regard to workload, decision latitude and workplace bullying. In addition, a self-administered checklist of common chronic diseases was given to the employees, and those reporting to have FMS in the first survey or who did not respond to the question on FMS were excluded from the study.

After two years, the incidence of newly diagnosed FMS was 1%. Covariate-adjusted results revealed an odds ratio of incident diagnosed FMS for workplace bullying of 4.1, and for both high workload and low decision latitude of 2.1. The authors concluded that stress at work might contribute to the development of FMS. However, as pointed out by the authors themselves and by other researchers commenting on the study (Cleare, 2004; Zurowski & Shapiro, 2004), one should be wary in assigning causation because undiagnosed or developing FMS might have affected reported stress at work. In addition, as Cleare (2004) stated, stressors outside of the workplace were not assessed in Kivimaki's study, implying that cause to the measured work stress cannot firmly be attributed.

2.1.3.2 The role of infection and autoimmune disorders

Some investigators have hypothesized that a viral infection might be the triggering event that results in the development of FMS. In an early study, more than half of the patients (55%) reported that their disease had begun suddenly with symptoms that seemed to be associated to a viral syndrome (Buchwald, Goldenberg, Sullivan & Komaroff, 1987). This observation has led researchers to examine associations between FMS and underlying infections such as mycoplasma blood infection and hepatitis C virus (HCV) infection. For example, mycoplasma blood infection has been detected in about 50% of patients with FMS and/or CFS, while such infection is detected in only about 10% of healthy individuals (Endresen, 2003; Nasralla, Haier & Nicolson, 1999). With regard to chronic HCV infection, Rivera and colleagues (1997) found viral antibodies in 15.2% of the 112 FMS patients that were enrolled in their study versus 5.3% of matched rheumatoid arthritis patients. In the same study, 53% of 58 patients with chronic hepatitis C had diffuse musculoskeletal pain and 10% fulfilled FMS criteria, while in a control group of matched surgery clinic patients, diffuse musculoskeletal pain was observed in 22%, and only one patient fulfilled the criteria of FMS. Both differences were statistically significant. Buskila and colleagues reported a prevalence of FMS in 16% of their HCV patients (Buskila, Shnaider, Neumann, Zilberman, Hilzenrat & Sikuler, 1997b; Buskila, Shnaider, Neumann, Lorber, Zilberman, Hilzenrat, Kuperman et al., 1998). This observation was confirmed by Kozanoglu and colleagues (2003) who found FMS in 18.9% of 95 HCV patients as compared to a prevalence of FMS of 5.3% in 95 healthy control subjects. In contrast to these findings, Goulding (2001) observed FMS in only 5% of their 77 HCV

patients. In another study, HCV occurred in only 2.6% of 115 FMS patients (Narvaez, Nolla & Valverde-Garcia, 2005).

Additional studies have focused on the prevalence of FMS in autoimmune disorders. For example, higher prevalence rates of FMS were reported in some studies on systemic lupus erythematosus (Buskila, Press & Abu-Shakra, 2003; Gladman, Urowitz, Gough & MacKinnon, 1997; Tang, Calkins & Petri, 2004), primary Sjogren's syndrome (Dohrenbusch, Gruterich & Genth, 1996; Tishler, Barak, Paran & Yaron, 1997), and rheumatoid arthritis (Goldenberg, 1999; Wolfe & Michaud, 2004). Taken together, these findings indicate that active viral replication or the release of inflammatory modulators might result in symptoms of FMS in a subgroup of patients with accompanying chronic infections and/or autoimmune disorders.

Based on the observation that viral or bacterial infections as well as physical trauma precede the development of FMS in subgroups of patients, Pall (2001) proposed a model in which elevated nitric oxide levels play an essential role in the etiology of FMS and related disorders. Infection and inflammation typically induce increased pro-inflammatory cytokine synthesis and release, including tumor-necrosis-factor (TNF)-α, interleukin (IL)-1, IL-2, IL-6, and interferon (IFN)-γ. These cytokines provoke, in turn, elevated levels of the inducible nitric oxide synthase (iNOS), which then synthesizes increased amounts of nitric oxide that is proposed to be critical for the development and expression of some types of hyperalgesia associated with chronic pain (Haley, Dickenson & Schachter, 1992; Meller, Pechman, Gebhart & Maves, 1992). Similarly, physical trauma known to precede FMS in up to 40% of cases (Al-Allaf, Dunbar, Hallum, Nosratzadeh, Templeton & Pullar, 2002; Buskila, Neumann, Vaisberg, Alkalay & Wolfe, 1997a; Waylonis & Perkins, 1994), has been reported to induce higher levels of nitric oxide in humans (Gebhard, Nussler, Rosch, Pfetsch, Kinzl & Bruckner, 1998; Onuoha, Alpar & Jones, 2001), thus supporting Pall's assumption that elevated nitric oxide might contribute to the etiology of FMS. Indeed, one study measuring arginine, the nitric oxide precursor, and citrulline, the byproduct of nitric oxide synthesis, revealed positive correlations between these two parameters and pain intensity in FMS patients, while no associations between the two markers and pain were found in two control groups consisting of healthy subjects and patients diagnosed with a painful or inflammatory condition other than FMS (Larson, Giovengo, Russell & Michalek, 2000). The study provided indirect evidence of an elevated nitric oxide synthesis in the central nervous system of FMS patients in

association with pain intensity. In addition, some studies have reported increased activity of the N-methyl-D-asparate (NMDA) receptor in FMS patients that is involved in the synthesis of nitric oxide (Graven-Nielsen, Aspegren Kendall, Henriksson, Bengtsson, Sorensen, Johnson, Gerdle et al., 2000; Sorensen, Bengtsson, Backman, Henriksson & Bengtsson, 1995). A recent study has found an increased expression of the NMDA receptor subtype 2D in the skin of FMS patients compared to healthy controls (Kim, Jang & Moon, 2006). Consequently, the use of NMDA receptor antagonists such as ketamine has revealed quite promising results in the treatment of pain in a subgroup of FMS patients (see Henriksson & Sorensen, 2002; Spath, 2003).

2.1.3.3 Genetic predisposition

Studies have indicated a familial component to the development of FMS. For example, one study found FMS in 52% of first-degree relatives of 17 FMS subjects; another 22% had abnormal muscle consistency to palpation, while only 26% of the family members were without any noticeable FMS-related symptoms (Pellegrino, Waylonis & Sommer, 1989). One limitation of the study was that standard criteria for the diagnosis of FMS were not used. In 1996, Buskila and colleagues published a study on the familial occurrence of FMS in 58 children of 20 mothers diagnosed with FMS based on the ACR 1990 criteria (Buskila, Neumann, Hazanov & Carmi, 1996). Of all children, 28% were found to have FMS. Consistent with this finding, Buskila and colleagues reported in another study a prevalence of FMS of 26% among blood relatives (parents, siblings, and children) of 30 FMS patients (Buskila & Neumann, 1997). In a recent study, familial aggregation of FMS was investigated in 533 first-degree relatives of 78 FMS patients and compared to data from 272 first-degree relatives of 40 rheumatoid arthritis patients (Arnold, Hudson, Hess, Ware, Fritz, Auchenbach, Starck et al., 2004). Results demonstrated that the estimated odds of FMS in a relative of an FMS patient were 8.5 times the odds of FMS in a relative of a rheumatoid arthritis patient ($p<0.001$). In addition, relatives of FMS patients had significantly elevated tender point counts compared to relatives of rheumatoid arthritis patients.

Beside the observation of a familial component to the development of FMS, studies have indicated that family members of FMS patients display a higher risk for mood disorders than is seen in the general population. For example, Hudson and

colleagues reported in two studies higher rates of major mood disorder (major depression, bipolar disorders) in families of FMS patients (Hudson, Hudson, Pliner, Goldenberg & Pope, 1985; Hudson, Goldenberg, Pope, Keck & Schlesinger, 1992), which was replicated by Arnold and colleagues describing a higher lifetime prevalence of major mood disorder in relatives of FMS patients than in relatives of rheumatoid arthritis patients (Arnold et al., 2004). The results suggest that FMS and mood disorders share common factors that might potentially be heritable.

In support of this assumption, some studies have reported decreased serum levels of serotonin in FMS patients (Russell, Michalek, Vipraio, Fletcher, Javors & Bowden, 1992; Samborski, Stratz, Schochat, Mennet & Muller, 1996; Wolfe, Russell, Vipraio, Ross & Anderson, 1997). Similarly, disturbances in the serotonergic system have been postulated in patients with depressive disorders (Byerley & Risch, 1985; Maes & Meltzer, 1995; Rosa-Neto, Diksic, Okazawa, Leyton, Ghadirian, Mzengeza, Nakai et al., 2004). Consequently, tricyclic antidepressants and selective serotonin and norepinephrine reuptake inhibitors, both exerting partial effects on the serotonin system, have been successful in the treatment of both depression and FMS. With regard to FMS, symptoms such as pain and fatigue improved during antidepressant treatment independent of the patients' level of distress or depression (Arnold, Rosen, Pritchett, D'Souza, Goldstein, Iyengar & Wernicke, 2005; Gendreau, Thorn, Gendreau, Kranzler, Ribeiro, Gracely, Williams et al., 2005).

Recent genetic studies in FMS patients have addressed potential associations between the disease and polymorphisms in serotonin-related genes. One study investigated a polymorphism in the 5-HT2A receptor gene, which is defined by a T to C transition at position 102 (T102C polymorphism), and found a significantly different genotype distribution in FMS patients compared to controls with a decrease in T/T-homozygotes and an increase in C/T and C/C carriers (Bondy, Spaeth, Offenbaecher, Glatzeder, Stratz, Schwarz, de Jonge et al., 1999). Another study could not confirm a significant association of the T102C polymorphism with FMS, but the authors reported that the T/T genotype was positively related to the lowest pain threshold and to the degree of psychiatric symptoms in the patients (Gursoy, Erdal, Herken, Madenci & Alasehirli, 2001). Offenbaecher and colleagues examined the genotypes of the serotonin transporter gene (5-HTT) promoter region (5-HTTLPR) in FMS patients and found a higher frequency of the short/short (S/S) genotype in the patients, which was related to higher levels of depression and psychological distress

(Offenbaecher, Bondy, de Jonge, Glatzeder, Kruger, Schoeps & Ackenheil, 1999). The association between the short genotype of the 5-HTTLPR and FMS was confirmed by another study with 99 female FMS patients and 559 female control subjects (Cohen, Buskila, Neumann & Ebstein, 2002). In that study, the percentage of FMS patients showing the S/S genotype was twice that observed in the control population (35% vs. 17%). In contrast to these findings, Gursoy (2002) did not find a correlation between a 5-HTTLPR polymorphism and FMS in one study with 53 mentally healthy FMS patients and 60 healthy subjects. These findings indicate that the relationship between FMS and polymorphisms in serotonin-related genes needs further elucidation and might be mediated by psychiatric comorbidity.

Taken together, both genetic and environmental factors seem to play a prominent role in triggering the development of FMS. Therefore, instead of overemphasizing a single factor, one should be aware that FMS constitutes a multifactorial condition in which a number of psychosocial and biological factors seem to interact – factors predisposing a person to develop this condition but also factors tending to perpetuate it (Cleare, 2004).

2.1.4 Comorbidity and symptom overlap

2.1.4.1 Comorbid depression and anxiety

Numerous studies have indicated that prevalence rates of lifetime depression and anxiety disorders are elevated in FMS patients. With regard to depression, some researchers have even hypothesized that FMS might be part of an affective spectrum disorder (Hudson, Arnold, Keck, Auchenbach & Pope, 2004); however, despite high comorbidity rates, FMS symptoms should not be considered a mere expression of depression (Van Houdenhove & Egle, 2004). In the present section, some exemplary studies are cited that have reported increased rates of depression and anxiety in FMS patients mainly from tertiary-care centers but also from a community sample.

Epstein and colleagues (1999) conducted a multi-center investigation at four study sites and revealed lifetime rates for mood disorders of 69% and for anxiety disorders of 35% in FMS patients. Current rate for mood disorders was 29% and for anxiety disorders 27%. In their study, the most common disorders among FMS patients were major depression, dysthymia, panic disorder, and simple phobia. Higher lifetime rates of anxiety and depression were also found in a study with 38 female FMS patients

compared to age-matched healthy women (Anderberg, Forsgren, Ekselius, Marteinsdottir & Hallman, 1999). In that study, 66% of the patients had a lifetime Axis I diagnosis including major depression, minor depression, dysthymia, panic disorder and/or generalized anxiety disorder. An earlier study by Hudson and colleagues confirmed the observation of higher lifetime rates of major depression and panic disorder (Hudson et al., 1992). Thieme and colleagues (2004) evaluated the prevalence of psychiatric disorders in 115 FMS patients using a structured clinical interview based on DSM-IV criteria. Results revealed that Axis I diagnoses were present in 75% of the patients. Based on responses to additional questionnaires, patient subgroups emerged of whom one mainly reported anxiety disorders, another one mainly mood disorders. The authors concluded that FMS should not be viewed as a homogeneous diagnosis but rather as a heterogeneous disorder with patients showing varying proportions of comorbid anxiety and depression dependent on their psychosocial characteristics (Thieme, Turk & Flor, 2004). In a Brazilian sample of 47 female FMS patients and 25 control patients without chronic muscle pain, depression was observable in 80% of the FMS patients compared to 12% of the control subjects. Anxiety occurred in 64% of the FMS group, but only in 16% of the control group (Martinez, Ferraz, Fontana & Atra, 1995). Walker and colleagues (1997) compared prevalence rates of mood and anxiety disorders between 36 FMS patients and 33 patients with rheumatoid arthritis and found significantly higher lifetime rates of both mood and anxiety disorders in the FMS patients. While less than half of the rheumatoid arthritis patients had a prior psychiatric diagnosis, it was reported in 90% of the FMS patients (Walker, Keegan, Gardner, Sullivan, Katon & Bernstein, 1997). In a community sample study (White, Nielson, Harth, Ostbye & Speechley, 2002), the severity of depression and anxiety as assessed by questionnaires was compared between FMS patients and adults with chronic widespread pain who did not meet the 1990 ACR criteria. Results revealed higher scores on the majority of the psychological distress scales for the FMS patients compared to persons with chronic widespread pain only, confirming the assumption of a link between FMS characterized by chronic widespread pain plus increased tenderness and mood disorders.

These findings illustrate that depression and anxiety disorders seem to be not only more prevalent in FMS patients when compared to healthy individuals but also in comparison to patients suffering from other chronic painful conditions such as

rheumatoid arthritis and chronic widespread pain. It is difficult if not impossible to determine if FMS causes mood disorders or if mood disorders are responsible for the development of FMS. Since both the lifetime prevalence and the current prevalence of mood and anxiety disorders have been found to be elevated in some studies, one might assume that on the one hand, depression and anxiety might play a perpetuating role in FMS, while on the other hand, the stress, uncertain prognosis and chronic course of the disease might lead to depressive comorbidities and cause feelings of anxiety and worrying, thus suggesting a bi-directional relationship.

2.1.4.2 Comorbidity and symptom overlap with other pain and fatigue syndromes and PTSD

High comorbidity rates and a striking symptom overlap have been reported for pain and fatigue syndromes such as FMS, CFS, and multiple chemical sensitivity (MCS). MCS is characterized by sensitivity to multiple chemicals at levels that are usually tolerated by healthy individuals resulting in unexplained symptoms in multiple organ systems. As extensively reviewed by Clauw and colleagues (Clauw & Chrousos, 1997; Clauw & Crofford, 2003), the above listed syndromes share a lot of common features such as widespread and/or regional pain, fatigue, and cognitive dysfunctions. In addition to these “systemic” conditions, regional syndromes including IBS, temporomandibular disorders, migraine, and tension headaches belong to the same spectrum of overlapping disorders that often present simultaneously in the same patient. In the early 1990s, Hudson and colleagues were amongst the first who reported on the high comorbidity between FMS and disorders such as migraine, irritable bowel syndrome, and CFS (Hudson et al., 1992). Two years later, Buchwald and Garrison compared 30 FMS patients with 30 CFS and 30 MCS patients from a university-based referral clinic and revealed a high frequency of MCS-compatible symptoms in the FMS and CFS groups. Likewise, 70% of the FMS patients and 30% of the MCS patients met CFS criteria (Buchwald & Garrity, 1994). Aaron and colleagues (2001) investigated 127 twin pairs in which one member of the pair experienced chronic fatigue of at least six months duration while the co-twin reported to be healthy and free of fatigue symptoms. Higher rates of FMS were found in the fatigued twins than in the non-fatigued co-twins (>70% vs. <10%). In addition, IBS was more common in the fatigued than in the non-fatigued twins (>50% vs. <15%). The calculation of odds ratios revealed strongest associations between chronic

fatigue and FMS (odds ratios >20), and fatigued twins were 4 to 10 times more likely to be diagnosed with IBS, MCS, chronic pelvic pain, and temporomandibular disorder (Aaron, Herrell, Ashton, Belcourt, Schmaling, Goldberg & Buchwald, 2001). In one study, 42% of FMS patients had IBS-like symptoms as compared to 16% of healthy subjects (Sivri, Cindas, Dincer & Sivri, 1996). This finding was supported by a recent study reporting a prevalence of IBS in FMS patients between 63% and 81%, while the prevalence rate of IBS in rheumatologic controls ranged between 15% and 24%, depending on the type of criteria used for the diagnosis of IBS (Kurland, Coyle, Winkler & Zable, 2006). A review on the profile of patients with MCS addressed the substantial overlap between MCS, FMS and CFS suggesting that FMS and CFS might in fact constitute the same disorder as MCS, given that the two conditions often involve chemical sensitivity (Ziem & McTamney, 1997). Similarly, Wessely and colleagues (1999) stated that the similarities between disorders such as FMS, CFS, and MCS outweigh the differences. The authors suggested that a dimensional classification or symptom clusters would be more favorable than the existing definitions in terms of specific symptoms.

Another line of evidence suggests that medically unexplained pain and fatigue syndromes such as FMS and CFS might be related to chronic or traumatic stress and PTSD. Consequently, elevated rates of emotional neglect, abuse, and PTSD have been found in CFS patients (Boscarino, 2004; Van Houdenhove et al., 2001). Additionally, as outlined in Section 2.1.3.1, an association between FMS and emotional neglect as well as psychological, physical and/or sexual abuse has frequently been reported. In support of a link between PTSD and FMS, two studies described PTSD-like symptoms in 56% and 57% of FMS patients, respectively (Cohen, Neumann, Haiman, Matar, Press & Buskila, 2002; Sherman, Turk & Okifuji, 2000). A recent community-based study has confirmed the observation of a marked comorbidity between FMS and PTSD symptoms (Raphael, Janal & Nayak, 2004). Likewise, Amir and colleagues (1997) found that 21% of their 29 PTSD patients met FMS criteria compared to 0% of the 37 control subjects. Last but not least, studies on male and female war veterans have reported an association between PTSD and physical symptoms including fatigue, joint pain and muscle pain (Asmundson, Wright & Stein, 2004; Dobie, Kivlahan, Maynard, Bush, Davis & Bradley, 2004; Engel, Liu, McCarthy, Miller & Ursano, 2000; Ford, Campbell, Storzbach, Binder, Anger & Rohlman, 2001). Interestingly, it has been observed that chronic pain patients

including those with FMS report higher levels of pain when co-diagnosed with PTSD than pain patients without a PTSD diagnosis (Geisser, Roth, Bachman & Eckert, 1996; Sherman et al., 2000).

2.1.4.3 Proposed biological mechanisms underlying the spectrum of overlapping pain and fatigue syndromes and PTSD

The pronounced symptom overlap in chronic pain and fatigue syndromes and the observation of an increased prevalence of these syndromes in PTSD and vice versa suggest that these conditions might represent a family of disorders with an underlying common physiologic abnormality. Different approaches have been undertaken in order to explain the potential physiological mechanisms that might be responsible for the development and co-occurrence of such medically unexplained conditions. According to Pall (2001), an elevated nitric oxide/peroxynitrite mechanism might be central to the etiology of FMS, CFS, MCS, and PTSD. As described in Section 2.1.3.2, Pall suggested that physical trauma, infections, and chemical or other stress leads to increased nitric oxide levels. This, in turn, results in elevated concentrations of its oxidant product peroxynitrite that are sustained through different proposed positive feedback loops (for a detailed description see Pall, 2000; Pall, 2001). Peroxynitrite reacts with and inactivates several of the enzymes in mitochondria. Thus, mitochondrial and energy metabolism dysfunction is one of the most important consequences of elevated peroxynitrite (see Pall, 2000) indicating that it plays an essential role in the induction of oxidative stress. Indeed, some studies have reported that FMS and CFS might be related to oxidative stress, as reviewed by Ozgocmen and colleagues (2005). Further support of Pall's model is provided by the observation of an increased activity of the NMDA receptor in FMS, CFS, MCS, and PTSD patients, which is involved in nitric oxide and peroxynitrite synthesis (see Pall, 2001). Beside their role in oxidative stress, elevations in nitric oxide and peroxynitrite result in an increased production of pro-inflammatory cytokines. These, in turn, play an essential role in another model on the pathogenesis of medically unexplained disorders focusing on the cytokine-induced response to sickness (Dantzer, 2005).

Dantzer suggested a renewed biopsychosocial approach to somatization and somatoform disorders that might as well apply to disorders such as FMS, CFS, MCS, and PTSD. According to Dantzer (2005, p 948), *"…somatization might be nothing else than the outward manifestation of sensitization of the brain cytokine system that*

is normally activated in response to activation of the innate immune system and mediates the subjective, behavioral and physiological components of sickness". The induction of sickness behavior in response to activation of the innate immune system is usually reversible, implying that sickness with its subjective, behavioral and physiological components ultimately disappears after the organism's successful defense of invading microorganisms (see Section 2.2.5). However, as Dantzer (2005) points out, the brain cytokine system can undergo sensitization in response to stimulation during early stages of development, to repeated activation, or to prior exposure to environmental stressors. Such a sensitized cytokine system is less likely to turn off when the danger is over and is more likely to be triggered by extrinsic non-immune stimuli. Therefore, non-termination of the sickness response, which includes typical symptoms of pain, fatigue, concentration difficulties and depression, might explain a lot of the symptoms that are experienced by patients with FMS, CFS, MCS, and PTSD.

Hellhammer and his research group proposed that low activity of the HPA axis might play an essential role in the pathophysiology of the overlapping disorders (Fries et al., 2005; Heim et al., 2000). This *hypocortisolism* has indeed been observed in patients with FMS, CFS, PTSD, IBS and further syndromes that belong to the spectrum of overlapping disorders (e.g., Gold & Chrousos, 2002; Griep et al., 1998; Gur et al., 2004a; Heim, Ehlert, Hanker & Hellhammer, 1998; Heim et al., 2000; Pruessner, Hellhammer & Kirschbaum, 1999; Roberts, Wessely, Chalder, Papadopoulos & Cleare, 2004; Rohleder et al., 2004). In 1993, Hellhammer and Wade postulated that hypocortisolism might develop after prolonged periods of stress that are at first characterized by a hyperactivity of the HPA axis and excessive glucocorticoid release (Hellhammer & Wade, 1993). After a while, the stress-induced continuous release of corticotrophin releasing hormone (CRH) from the hypothalamus might result in receptor downregulation at the pituitary level. Hellhammer and Wade suggested that if the insensitivity of the pituitary receptors persists even after cessation of the chronic stressor, one would expect only little adrenocorticotropic hormone (ACTH) release by CRH under unstimulated conditions. As a consequence of low ACTH levels, the baseline production of cortisol in the adrenal glands could also be reduced thus explaining the hypocortisolemic features observed in the above described disorders. Alternatively, a receptor down-regulation at the adrenal level due to ACTH hypersecretion, a reduced biosynthesis or release of the respective releasing

hormone on different levels of the HPA axis followed by decreased stimulation of the respective target receptors, an enhanced negative feedback sensitivity of the HPA axis to cortisol, a decreased availability of free, biologically active cortisol, and/or reduced effects of cortisol at target cells might play an important role in understanding possible diverse patterns of hypocortisolism that might develop in different disorders or even in different patient subgroups within the same disorder (see Fries et al., 2005; Heim et al., 2000). Consequences of a disturbed HPA axis activity on other bodily systems including the sympathetic nervous system and the immune system will be discussed in Section 2.2.

Apparently, the three approaches proposed by Pall, Dantzer, and Hellhammer and their research groups do not necessarily exclude but rather complement each other. For example, Pall (2000) stated that low levels of glucocorticoids might play a substantial role in the proposed sustained elevation of nitric oxide/peroxynitrite levels. Glucocorticoids have been shown to inhibit the induction of iNOS in multiple tissues indicating that lowered cortisol levels in FMS and other pain, fatigue and trauma patients might result in a reduced ability to downregulate iNOS, thus sustaining the elevation of nitric oxide and peroxynitrite when they might otherwise decline toward normal levels (Pall, 2000). In addition, as will be described in more detail in Section 2.2.2, glucocorticoids have been shown to inhibit pro-inflammatory cytokine synthesis such as IL-1, TNF-α and IL-6. Consequently, decreased cortisol levels might result in an increased production of pro-inflammatory cytokines that constitute an important component of both Pall's and Dantzer's model.

To conclude, stress-induced disturbances in one bodily system are inevitably accompanied by alterations in other bodily systems. Since the present work focused on disturbances of HPA, SNS and immune system activity in FMS patients, a brief overview on some of the basics of neuro-endocrine-immune interactions will be given in the following section.

2.2 Neuro-endocrine-immune interactions: an overview

The HPA axis, the SNS and the immune system are complex systems that interact with each other in many different ways. For example, immune-modulating effects of the HPA axis are not only exerted by cortisol but also by CRH and ACTH both expressing receptors on immune cells such as spleenic macrophages with regard to CRH (Schedlowski & Tewes, 1996), and B cells, T cells and peritoneal macrophages in terms of ACTH (Johnson, Hughes & Smith, 2001). This overview will be limited to interactions that are most relevant for the present work.

2.2.1 HPA-SNS interactions

In 1932, Walter Cannon coined the term *"homeostasis"* from the Greek *homoios* (same, resembling) and *stasis* (to stand, posture). It refers to the property of living organisms to survive by maintaining a complex dynamic equilibrium of the internal milieu controlled by interrelated regulation mechanisms. The HPA axis and the SNS constitute the peripheral limbs of the stress system, whose main function is to maintain basal and stress-related homeostasis. Both systems become activated when homeostasis is disturbed or threatened by external or internal challenges - which include challenges of the immune system - resulting in increased peripheral glucocorticoid and catecholamine levels that act in concert to keep the steady state of the internal milieu (Elenkov et al., 2000). In the 1930s, Hans Selye referred to this reaction as "general adaptation or stress syndrome". The two principal components of the general adaptation response are the CRH and the locus coeruleus-norepinephrine (LC-NE)/SNS systems. While the CRH system that is widespread throughout the brain is best characterized in the paraventricular nucleus (PVN) of the hypothalamus, the LC-NE/SNS system is located in the brain stem (reviewed by Chrousos & Gold, 1992). Functionally, the two systems seem to participate in a positive, reverberatory feedback loop implying that activation of one system tends to activate the other as well. In support of this assumption, several studies have shown that CRH increases the spontaneous discharge rate of LC neurons and enhances norepinephrine release in the prefrontal cortex (Smagin, Swiergiel & Dunn, 1995; Valentino, 1988; Valentino, Foote & Page, 1993). Conversely, projections of noradrenergic fibers from the LC-NE system to the PVN in the hypothalamus have

been reported indicating activating effects of central norepinephrine on the HPA axis (see Elenkov et al., 2000; Lehnert, Schulz & Hiemke, 1999).

While CRH exerts stimulating effects on the LC-NE/SNS system, there is some evidence suggesting that the effects of glucocorticoids on catecholamine release are inhibitory. Results from animal studies with adrenalectomized rats have shown that endogenous glucocorticoids restrain responses such as catecholamine turnover, synthesis, and release in sympathetic nerves during immobilization stress (Kvetnansky et al., 1993; Pacak et al., 1993). In addition, findings of enhanced tonic and stress-induced CRH release within the LC in adrenalectomized rats (Pavcovich & Valentino, 1997) supports the role of glucocorticoids in the negative feedback regulation of both the CRH and the LC-NE/SNS systems. Studies in humans have also confirmed a negative association between glucocorticoid concentrations and SNS responses. For example, a 1-week treatment of healthy subjects with 20 mg of the synthetic glucocorticoid prednisone reduced SNS activity and plasma norepinephrine levels by 23% and 27%, respectively (Golczynska, Lenders & Goldstein, 1995).

Interestingly, increased catecholamine concentrations have been reported in some studies investigating patients with PTSD, a disorder that has been associated with a mild hypocortisolism (see Section 2.1.4.3). Accordingly, Geracioti and colleagues found augmented norepinephrine levels in the cerebrospinal fluid of male combat veterans with chronic PTSD (Geracioti, Baker, Ekhator, West, Hill, Bruce, Schmidt et al., 2001). In another study with combat veterans, those with PTSD had higher plasma catecholamine levels during experimental exposure to combat sounds than their counterparts without PTSD (Blanchard, Kolb, Prins, Gates & McCoy, 1991).

In sum, a positive feedback loop between the CRH and the LC-NE/SNS systems has been confirmed in several studies. Glucocorticoids, on the other hand, seem to exert negative feedback on the two systems indicating that glucocorticoid deficiency might result in an increased CRH and SNS activity. Implications of a mild hypocortisolism in FMS patients on SNS activity will be addressed in Chapter 3.

2.2.2 Glucocorticoid effects on the immune system

Since the work of Hans Selye (1936), stress has been associated with an activation of the HPA axis resulting in an increased release of cortisol from the adrenal glands. However, while Selye and others posited that glucocorticoids enhance the response

to stress, a revisionist viewpoint postulated that glucocorticoids rather suppress the stress response, thus preventing it from being pathologically overactivated (Munck, Guyre & Holbrook, 1984). This idea first came up in the late 1940ies when glucocorticoids were discovered to be anti-inflammatory agents (Hench, Kendall, Slocumb & Polley, 1949). Based on this observation that did not fit into the concept of glucocorticoids generally enhancing bodily stress responses, Munck and colleagues (1984) proposed that *"(a) the physiological function of stress-induced increases of glucocorticoid levels is to protect not against the source of stress itself, but against the normal defense reactions that are activated by stress; and (b) the glucocorticoids accomplish this function by turning off those defense reactions, thus preventing them from overshooting and themselves threatening homeostasis."* As reviewed by Sapolsky and colleagues (2000), Munck's idea of glucocorticoids turning off defense mechanisms to prevent damage to the organism was already grabbed - but never regularly published - in the early 1950ies by Marius Tausk who metaphorically compared stress to a fire and the role of glucocorticoids to that of preventing water damage rather than putting out the fire (see Sapolsky et al., 2000).

In more recen years the distinguished anti-inflammatory effects of glucocorticoids have been thoroughly investigated. During early, innate immune responses, glucocorticoids promote the survival and proliferation of neutrophils, while they induce apoptosis of eosinophils (Meagher, Cousin, Seckl & Haslett, 1996). They are also capable of stimulating macrophage phagocytotic ability and antigen uptake (Liu, Cousin, Hughes, Van Damme, Seckl, Haslett, Dransfield et al., 1999), thus supporting early termination of inflammation by enhancing the clearance of foreign antigens. Mizobe and colleagues (1997) observed that acute stress in mice exerted suppressive effects on granulocyte and macrophage migration, whereas NK cell migration remained relatively unaffected. Treatment of the animals with the glucocorticoid antagonist RU486 led to recovery from this immunosuppressive state indicating that the stress-related elevations in glucocorticoid levels were responsible for the inhibited immune cell migration (Mizobe, Kishihara, Ezz-Din El-Naggar, Madkour, Kubo & Nomoto, 1997). During a 2-hour stress session, a decrease in T cells, B cells, NK cells and monocytes was observed in rats, which all returned to pre-stress baseline levels within three hours after stress cessation (Dhabhar, Miller, McEwen & Spencer, 1995). Based on the observation that the leukocyte numbers relatively rapidly returned to baseline levels during recovery, Dhabar and colleagues

suggested that the stress-induced decrease in blood leukocyte numbers represents a redistribution of immune cells from the blood to local areas of need, e.g. an inflamed skin, rather than a destruction or loss of leukocytes.

In terms of adaptive immunity, glucocorticoids modulate the differentiation, maturation, and function of dendritic cells thereby decreasing their capacity to present antigen to T lymphocytes and to secrete cytokines (Piemonti, Monti, Allavena, Sironi, Soldini, Leone, Socci et al., 1999). Glucocorticoids also block IL-12 secretion by monocytes (Blotta, DeKruyff & Umetsu, 1997) and suppress IL-12 responsiveness of NK cells and T cells (Franchimont, Galon, Gadina, Visconti, Zhou, Aringer, Frucht et al., 2000; Wu, Wang, McDyer & Seder, 1998), which results in the suppression of cellular [T helper (Th)1] immunity and the promotion of humoral (Th2) immunity (see Section 2.2.4). The latter is further enhanced by an increased anti-inflammatory cytokine expression such as IL-4, IL-10, and IL-13 by T lymphocytes after treatment with glucocorticoids (Ramierz, Fowell, Puklavec, Simmonds & Mason, 1996).

The impact of glucocorticoids on cytokine production is probably the most extensively studied way by which they exert their anti-inflammatory effects. Several studies have shown that glucocorticoids are able to inhibit synthesis, release and efficacy of cytokines and other mediators that promote immune and inflammatory reactions such as IL-1, IL-2, IL-6, IL-12, TNF-α, IFN-γ, IL-8, histamine, nitric oxide, and cyclooxygenase 2 (COX-2) (for an extensive review seeSapolsky et al., 2000).

It has been demonstrated that glucocorticoids apply their anti-inflammatory effects through cytoplasmic, heat shock protein-bound glucocorticoid receptors (GR). There are two splicing variants of the human GR, isoforms GRα and GRβ, depending on the alternative use of exon 9α or 9β, respectively. While GRα is a 777-amino acid protein, GRβ is comprised of 742 amino acids, with the first 727 amino acids from the N-terminal being identical in both isoforms. GRα has an additional 50 amino acids and GRβ an additional 15 that are nonhomologous (Bamberger, Schulte & Chrousos, 1996). Unlike GRα, which binds to glucocorticoids and transduces their biological activities, GRβ does not bind any ligands examined so far and fails to activate transcription (Bamberger, Bamberger, de Castro & Chrousos, 1995). GRβ can also form heterodimers with GRα thus interfering with the function of this protein reflected by much lower transcriptional activity as compared to a GRα homodimer (DeRijk, Schaaf & de Kloet, 2002).

Three mechanisms have been described by which the glucocorticoid-GRα complex acts on target cells: first, after binding to its ligand, GRα dissociates from the heat shock proteins and enters the nucleus where it binds as a homodimer to specific DNA sequences, called glucocorticoid-responsive elements (GREs), that are located in the promoter regions of specific genes. GRα exerts either transactivating or transrepressing effects by stimulating or inhibiting, respectively, the transcription rates of these genes. For this process, ligand-activated GRα forms complexes with co-regulators of transcription, i.e. co-activator or co-repressor proteins, and with several chromatin modulators. The latter alter chromatin structure and enhance or inhibit assembly of the basal transcription machinery and the initiation of transcription by RNA polymerase II (Hebbar & Archer, 2003; McKenna, Lanz & O'Malley, 1999). Second, glucocorticoids exert many of their anti-inflammatory effects through protein-protein interactions known to stimulate inflammation, such as nuclear factor (NF)-κB. The transcription factor NF-κB is a heterodimeric protein composed of the two subunits p65 and p50, which in an inactive form reside in the cytoplasm stabilized by the inhibitory protein IκBα. Once liberated, NF-κB translocates into the nucleus and binds to κB-responsive elements in the promoter region of NF-κB-responsive genes. NF-κB plays a central role in the induction of a large number of immunoregulatory genes, including those encoding IL-1, IL-2, IL-6, IL-8, TNF-α, IFN-γ, iNOS und COX-2 (see McKay & Cidlowski, 1999). Glucocorticoids block NF-κB activation by GRα binding directly to the p65 subunit in the cytoplasm or at κB-responsive elements, respectively, thus inhibiting NF-κB translocation into the nucleus and neutralizing NF-κB's pro-inflammatory transcriptional activity (seeFranchimont, Kino, Galon, Meduri & Chrousos, 2003). Alternatively, glucocorticoids are able to increase IκBα protein synthesis by stimulating its promoter through GREs (transactivation). The inhibitory protein, in turn, segregates active p65/p50 heterodimers from the nucleus and traps them in inactive cytoplasmic complexes thus preventing the induction of immunoregulatory genes by NF-κB (Auphan, DiDonato, Rosette, Helmberg & Karin, 1995). The third mechanism constitutes a non-genomic pathway by which glucocorticoids signal through membrane-associated receptors and second messengers. One such non-genomic mechanism involves the rapid activation of endothelial nitric oxide synthease (eNOS) resulting in the production of nitric oxide. Hafezi-Moghadam and colleagues (2002) observed that acute administration of pharmacological glucocorticoid concentrations in mice led to decreased vascular

inflammation and reduced myocardial infarct size after ischemia and reperfusion injury via this non-genomic mechanism thus exerting protective cardiovascular effects (Hafezi-Moghadam, Simoncini, Yang, Limbourg, Plumier, Rebsamen, Hsieh et al., 2002). This finding is insofar surprising as the production of nitric oxide is generally associated with inflammation and thought to be inhibited by glucocorticoids (see Section 2.1.4.3). Therefore, more research is needed to clarify the role of non-genomic mechanisms in the inhibition of leukocyte migration, vasodilation and vascular permeability (Rhen & Cidlowski, 2005).

Implications of hypocortisolism for immune system functioning

The consequences of glucocorticoid deficiency on immune functions have been extensively investigated in studies with adrenalectomized animals. As early as 1922, Kepinov observed that adrenalectomy sensitizes guinea pigs to bronchial anaphylaxis (severe allergic reaction). Adrenalectomy has also long been known to provoke hypertrophy of the thymus and other lymphoid organs (see Rhen & Cidlowski, 2005). Further studies have shown that adrenalectomy increased fever and mortality after bacterial endotoxin-induced sepsis in rats, while treatment with glucocorticoids reversed these effects (Coelho, Souza & Pela, 1992; Morrow, McClellan, Conn & Kluger, 1993). In addition, adrenalectomized rats as well as intact rats treated with the GR antagonist RU486 had higher levels of plasma IL-6 after endotoxin injection, an effect that was attenuated by glucocorticoid administration (Hawes, Rock, Keogh, Lowry & Calvano, 1992; Morrow et al., 1993).

With regard to clinical reports in humans, Addison already documented in 1855 elevated circulating lymphocyte levels in a patient with adrenal insufficiency. Takasu and colleagues observed an exacerbation of autoimmune thyroid dysfunction after unilateral adrenalectomy in patients with Cushing's syndrome due to an adrenocortical adenoma (Takasu, Komiya, Nagasawa, Asawa & Yamada, 1990). In addition, clinicians have recognized for many years that patients with glucocorticoid deficiency (e.g., Addison's disease) require increased glucocorticoid replacement during episodes of fever, infection, or inflammation to prevent toxic effects of cytokines (Kapcala, Chautard & Eskay, 1995). Sauer and colleagues (1994) reported an elevated phytohemagglutinin (PHA)-stimulated IL-2 secretion by peripheral lymphocytes in patients with adrenal insufficiency compared to healthy individuals and patients with Cushing's syndrome (Sauer, Stalla, Muller & Arzt, 1994).

Taken together, these findings suggest a disinhibition of inflammatory activity, including pro-inflammatory cytokines, due to low glucocorticoid levels in animals and humans. This issue is of special relevance for the present work and will be addressed in the upcoming chapters with regard to hypocortisolemic features in FMS patients and their potential consequences on SNS and immune system activity.

2.2.3 Catecholamine effects on the immune system

The role of the SNS, a major component of the autonomic nervous system, in neuro-endocrine-immune interactions has received much less attention than the role of the HPA axis. However, evidence accumulated over the last three decades indicates that the SNS innervates all lymphoid organs, and catecholamines modulate several immune parameters (Elenkov et al., 2000).

The principal end products of the SNS, epinephrine and norepinephrine, exert their effects by binding to specific adrenergic receptors that are expressed on the surface of leukocytes. These receptors can be divided into α-adrenergic and β-adrenergic receptors, which in turn can be categorized into subtypes depending on their pharmacological, biochemical, and molecular properties (e.g., Hoffmann, Leitz, Oberdorf-Maass, Lohse & Klotz, 2004; Kable, Murrin & Bylund, 2000). Several radioligand binding studies have revealed that virtually all mature lymphocytes with the exception of Th2 clones (Sanders, Baker, Ramer-Quinn, Kasprowicz, Fuchs & Street, 1997) express β-adrenergic receptors that mainly belong to the β_2-subclass. These receptors are differentially expressed on lymphocyte subsets. For example, studies revealed that the density of β_2-adrenergic receptors in B cells is greater than in T cells, NK cells express more β_2-adrenergic receptors than cytotoxic and T helper cells, and receptor density in cytotoxic T cells is higher than in T helper cells (Landmann, 1992; Van Tits, Michel, Grosse-Wilde, Happel, Eigler, Soliman & Brodde, 1990). Maisel and colleagues provided evidence that β_2-adrenergic receptor density is highest in NK cells followed by cytotoxic T and B cells, while lowest receptor density is seen in T helper cells (Maisel, Harris, Rearden & Michel, 1990a).

Stimulation of adrenergic receptors by epinephrine and norepinephrine results in activation of G-proteins that regulate the transduction of transmembrane signals from cell surface receptors to intracellular receptors. In general, β_2-adrenergic receptors couple to a certain subfamily of the α-subunit of G proteins (G_s), thereby stimulating adenylate cyclase, which in turn induces the production of the second messenger

cyclic adenosine 5'-monophosphate (cAMP). In addition to the above mentioned differences in receptor density, the coupling between β_2-adrenergic receptors and adenylate cyclase may also differ in lymphocyte subsets, implying that high β_2-adrenergic receptor density does not necessarily mean high cAMP responses after stimulation of these receptors (Elenkov et al., 2000). Thus, while NK cells and cytotoxic T cells possess high affinity receptors resulting in rapid cAMP accumulation, B cells seem to have a greater prevalence of low affinity β_2-adrenergic receptors with regard to adenylate cyclase activation (Knudsen, Kjaersgaard & Christensen, 1995; Maisel, Fowler, Rearden, Motulsky & Michel, 1989).

The SNS plays an important role in lymphocyte migration and circulation. In this context, several studies have demonstrated that acute stress induces a transient increase in lymphocyte numbers, with the most prominent increase in NK cell numbers (see Benschop et al., 1996). Data indicates that the effects of the SNS on lymphocyte migration and circulation are at least in part modulated by catecholamines via β_2-adrenergic receptors. For example, in one study infusion of epinephrine and norepinephrine in healthy and splenalectomized subjects resulted in a transient increase of lymphocyte subsets with the highest increase in NK cell numbers. These changes in lymphocyte subsets were inhibited by pre-treatment with the non-selective β-adrenergic receptor antagonist propranolol, while the β_1-adrenergic receptor antagonist bisoprolol had no effect on lymphocyte migration, supporting the assumption that β_2-adrenergic receptors are involved in the modulation of lymphocyte migration (Schedlowski, Hosch, Oberbeck, Benschop, Jacobs, Raab & Schmidt, 1996). Interestingly, while acute stress and exercise induce a transient increase in lymphocyte numbers, in particular NK cells, chronic SNS activity as achieved by a 7-day treatment of healthy subjects with the β_2-adrenergic receptor selective agonist terbutaline, resulted in a reduction of cytotoxic T cells and NK cells (Maisel et al., 1990b). The number of B and T helper cells, lymphocyte subtypes that are characterized by low β_2-adrenergic receptor affinity and density, respectively, did not change significantly in that study. With regard to catecholamine effects on NK cells, Maisel's results indicate that an acute increase of SNS activity might have an opposite effect on NK cell numbers than chronic SNS activity: in the short term, catecholamines acutely mobilize NK cells, whereas in the long term, catecholamines chronically decrease lymphocyte numbers, especially of NK cells, in

the peripheral blood. This issue will be further taken into consideration in Chapter 3 in the context of potential chronic SNS hyperactivity in FMS patients.

Further lymphoid cells that express β_2-adrenergic receptors include thymocytes, neutrophils, basophils, and eosinophils (see Elenkov et al., 2000). In addition, dendritic cells express saturable β-adrenergic receptors (Madden, 2001), and IL-12 production from dendritic cells is reduced by β-agonist stimulation (Panina-Bordignon, Mazzeo, Lucia, D'Ambrosio, Lang, Fabbri, Self et al., 1997) indicating that both catecholamines and glucocorticoids can regulate dendritic cell function.

Similar to the anti-inflammatory effects of glucocorticoids, there is a growing body of evidence showing that an increase in cAMP levels via β-adrenergic stimulation results in inhibition of Th1 cytokine production including IL-2 (Sekut, Champion, Page, Menius & Connolly, 1995), IL-12 (Elenkov, Papanicolaou, Wilder & Chrousos, 1996; Panina-Bordignon et al., 1997), IFN-γ (Borger, Hoekstra, Esselink, Postma, Zaagsma, Vellenga & Kauffman, 1998; Sanders et al., 1997) and TNF-α (Severn, Rapson, Hunter & Liew, 1992). In contrast, Th2 type cytokine production remains unchanged or increases, respectively, after catecholamine stimulation as observed for IL-4 (Sanders et al., 1997), IL-10 (Elenkov et al., 1996) and IL-6 (Papanicolaou, Petrides, Tsigos, Bina, Kalogeras, Wilder, Gold et al., 1996; von Patay, Kurz & Mentlein, 1999), the latter possessing both pro- and anti-inflammatory features. Since Th2 cells do not express β_2-adrenergic receptors (Sanders et al., 1997), the effects of catecholamines on Th2 cells are presumably indirect in terms of removing inhibitory restraints on these cells that are otherwise exerted by pro-inflammatory cytokines.

An important pathway in the modulation of cytokine production by catecholamines is the cAMP/protein kinase A (PKA) pathway. Stimulation of cAMP results in activation of PKA, which in turn leads to suppression of pro-inflammatory and enhancement of anti-inflammatory activity. Neumann and colleagues demonstrated that PKA stimulation of T helper cells inhibited transcription of the IL-2 gene by interfering with NF-κB binding to the κB site in the promoter region of the gene, a site that is missing in the IL-4 promoter (Neumann, Grieshammer, Chuvpilo, Kneitz, Lohoff, Schimpl, Franza et al., 1995). In addition, Neumann and colleagues observed an impaired translocation of the NF-κB p65 subunit into the nucleus, which was correlated with a retarded degradation of cytosolic IκBα. Thus, increased PKA activity seems not only to interfere with NF-κB binding to the IL-2 promoter but also with the release of p65 from its cytosolic inhibitor. In another study, binding of the p50/p65 heterodimer to the

κB binding site was also inhibited by cAMP (Tsuruta, Lee, Masuda, Koyano-Nakagawa, Arai, Arai & Yokota, 1995). In addition, cAMP increasing agents are able to modulate further transcription factors that possess binding sites in the IL-2 promoter such as activator protein 1 (AP-1) and nuclear factor of activated T cells (NF-AT). This issue has been comprehensively reviewed by Elenkov and colleagues (2000).

2.2.4 The Th1-Th2 paradigm

As described in Section 2.2.2, glucocorticoids represent the most potent anti-inflammatory hormones in the body provoking a shift from the cellular (Th1) branch of immunity to the humoral branch (Th2). Likewise, catecholamines are able to induce a shift towards Th2 immunity (see Section 2.2.3).

The classification of $CD4^+$ helper cells into Th1- and Th2-type was originally described in the late 1980s among mouse $CD4^+$ T cell clones (Cherwinski, Schumacher, Brown & Mosmann, 1987; Mosmann, Cherwinski, Bond, Giedlin & Coffman, 1986). A few years later, Th1-Th2 patterns were also detected in human T cells (Del Prete, De Carli, Mastromauro, Biagiotti, Macchia, Falagiani, Ricci et al., 1991). Categorization of T helper cells into the two functionally dichotomous subsets usually takes place on the basis of the cytokines they produce. For example, Th1 cells secrete high levels of pro-inflammatory cytokines such as IL-2, TNF-β, and IFN-γ resulting in the activation of macrophages, NK cells, and cytotoxic T cells, which constitute the major components of the cellular immune response against invasive intracellular pathogens. Th2 cells, on the other hand, produce a variety of anti-inflammatory cytokines including IL-4, IL-5, IL-10, and IL-13, thus promoting humoral immune responses against extracellular pathogens by activating eosinophils and mast cells and driving B cells to differentiate and produce immunoglobulins (Ig) including IgM, IgA, and particularly IgE (see Elenkov & Chrousos, 1999; Janeway, Travers, Walport & Shlomchik, 2001). Importantly, mutual cross inhibition between Th1 and Th2 cytokines polarize functional T helper cell responses into cellular or humoral immune responses (Opal & DePalo, 2000). For example, IFN-γ and IL-12 downregulate Th2 activity, whereas IL-4 and IL-10 downregulate Th1 activity (Romagnani, 1997).

Strong evidence suggests that Th1 and Th2 cells do not derive from distinct lineages, but rather develop from the same antigen-naïve T helper cell precursor. Upon antigen

exposure through contact with antigen presenting cells (APCs) of the innate immune system, including monocytes/macrophages, dendritic cells, and B cells, T helper cell precursors undergo differentiation through autocrine stimulation via IL-2 to an uncommitted cell termed Th0. This stage of T cell differentiation has been suggested to represent a population of cells secreting multiple varieties of cytokines (Bucy, Karr, Huang, Li, Carter, Honjo, Lemons et al., 1995; Romagnani, 1997).

A number of factors have been proposed to impact the development of Th1 and Th2 cells from Th0 cells. In this context, cytokines produced by the APCs seem to play an essential role: IL-12 released by activated APCs is a major inducer of cellular immunity and acts in concert with NK-cell-derived IFN-γ to promote Th1 responses (Trinchieri, 1995). In addition, APC-derived IL-12 and TNF-α along with IFN-γ released by NK and Th1 cells, stimulate the functional activity of cytotoxic T cells, NK cells and activated macrophages, as well as the synthesis of nitric oxide and other inflammatory mediators, rendering them the most important pro-inflammatory cytokines (Mosmann & Sad, 1996; Trinchieri, 1995). In contrast, IL-4 and IL-10 as the major anti-inflammatory cytokines, promote humoral immunity by stimulating the growth and activation of mast cells and eosinophils and the differentiation of B cells into primarily IgE-secreting B cells. Importantly, these cytokines inhibit macrophage activation, T cell proliferation and the production of pro-inflammatory cytokines, thus inhibiting Th1 responses (Elenkov & Chrousos, 1999; Mosmann & Sad, 1996).

Beside the impact of certain cytokines on T helper cell differentiation, the presence of further substances has an influence on the Th1-Th2 paradigm. For example, both glucocorticoids and prostaglandin E_2 (PGE_2) induce a down-regulation of pro-inflammatory cytokines while up-regulating anti-inflammatory cytokines, thus triggering a shift towards Th2 immunity (Franchimont et al., 2003; Snijdewint, Kalinski, Wierenga, Bos & Kapsenberg, 1993). In addition, differentiation of Th0 cells into Th1 or Th2 might depend on the kind of APC involved: Schmitz and colleagues (1993) demonstrated that resting T helper cells differentiate into Th1 cells secreting IL-2 and IFN-γ if antigen is presented by macrophages. Antigen presentation by B cells, on the other hand, rather triggers the development of Th2 cells (Schmitz, Assenmacher & Radbruch, 1993). Except for the nature of APCs, T cell differentiation might also depend on the dose of presented antigen. Thus, very low and very high doses of dendritic cells and activated B cells have been shown to promote the development of Th2-like cells producing predominantly IL-4, while moderate antigen

levels predispose Th0 cells to become Th1 cells that generate increased amounts of IFN-γ (Hosken, Shibuya, Heath, Murphy & O'Garra, 1995). The differentiation of Th1 and Th2 cells is illustrated in Figure 2.2.

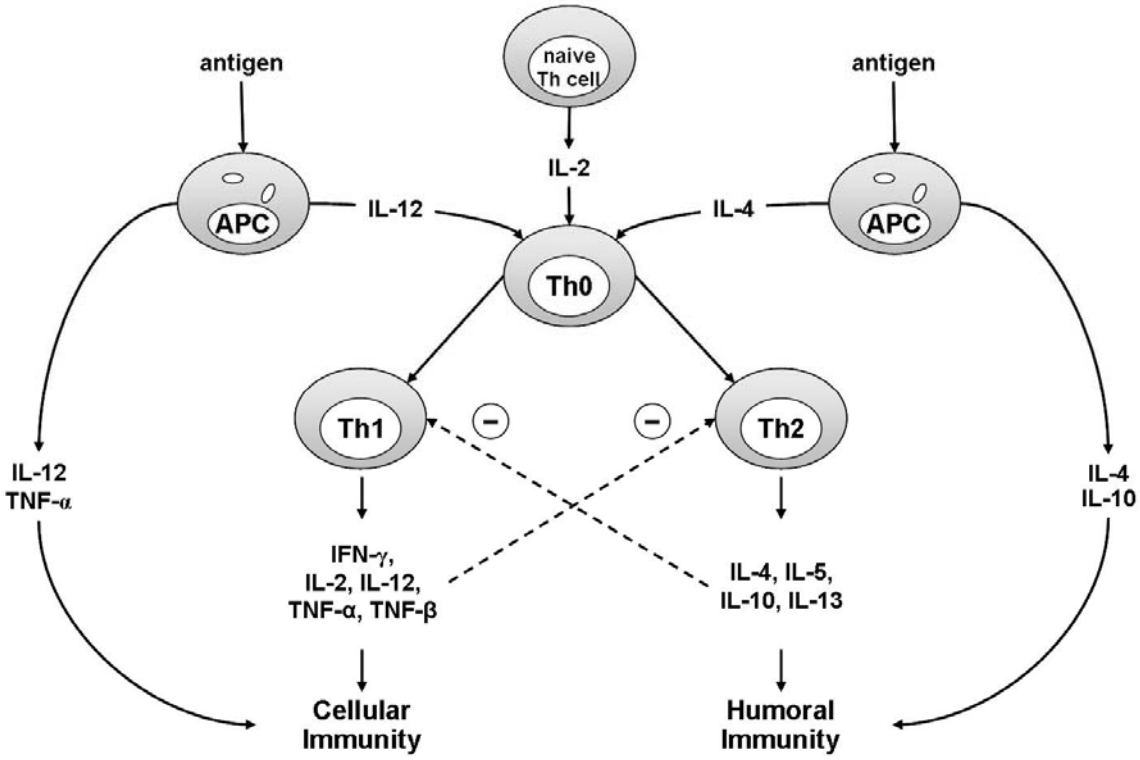

Figure 2.2: Differentiation of Th1 and Th2 cells. Solid lines represent stimulation, dashed lines inhibition. Abbreviations: APC, antigen-presenting cell; IL, interleukin; Th, T helper cell; TNF, tumor necrosis factor.

Importantly, the Th1-Th2 paradigm must be seen as a simplification of an essential part of immune activity that is characterized by an enormous complexity. For example, while IL-10 is considered one of the major anti-inflammatory cytokines, human studies have provided evidence that both Th1 and Th2 subsets secrete IL-10 (Katsikis, Cohen, Londei & Feldmann, 1995). Similarly, IL-6 possesses both pro- and anti-inflammatory features. Altogether, the Th1-Th2 paradigm has been debated and refined many times, and yet it has withstood all criticism due to its straightforwardness in an intricate research field.

2.2.5 Immune-to-brain communication: the cytokine-induced sickness response

In the 1970s, Besedovsky and his research group provided the first evidence that the immune system that was long thought to be self-regulated can signal the brain (Besedovsky & Sorkin, 1977; Besedovsky, Sorkin, Felix & Haas, 1977). A few years later it became clear that immune signals to the brain are mainly mediated by cytokines such as IL-1, IL-6, and TNF-α resulting in HPA axis and SNS activation and consequently in elevated ACTH, glucocorticoid and catecholamine levels in the periphery (e.g., Chrousos, 1995; Dunn, 1988; Dunn, 2000; Sapolsky, Rivier, Yamamoto, Plotsky & Vale, 1987) as part of the so-called *sickness response*. Besides triggering an endocrine and sympathetic stress response, sickness comprises several physiological adjustments such as increased body temperature, a shift in liver metabolism, alterations in plasma iron, zinc, and copper, leucocytosis, i.e. increased white cell counts, more sleep, and increased slow wave sleep (Maier & Watkins, 1998). In addition, behavioral changes in terms of reduced food and water intake, reduced activity, decreased social and sexual behavior, depressed mood, cognitive alterations, and increased pain sensitivity have been observed in infected animals and humans (Larson & Dunn, 2001).

In the late 1980s, Hart formulated the nowadays widely acknowledged hypothesis that the observed changes during sickness constitute a highly organized and adaptive response in combating viral and bacterial infections (Hart, 1988). According to Hart's assumption, the symptoms of sickness are not pathological or a sign of debilitation produced by microbial or viral infections but rather are defensive responses evolved in order to control infection. For example, a reduction in plasma iron during infection can be considered adaptive insofar that microorganisms require iron for effective reproduction (Maier & Watkins, 1998). Fever that occurs not only in mammals but also in birds, amphibians, reptiles, and fish, is another example demonstrating the adaptiveness of the organism to fight infection. As reviewed by Kluger and colleagues (1996), fever can be regarded a "*defended increase*" in core body temperature in that animals choose conditions that allow core body temperature to rise (Kluger, Kozak, Conn, Leon & Soszynski, 1996). As a result, reproduction is much poorer in microbial organisms that grow best at or below the body temperature of the host. In addition, fever can potentiate innate and adaptive immune responses, e.g. in terms of white blood cells dividing more rapidly at elevated temperatures and

phagocytes killing more efficiently because some of the enzymatic processes involved proceed at a greater intensity (reviewed by Maier & Watkins, 1998). With regard to the behavioral components of the sickness response, it has been assumed that they function to reduce the energetic cost of behavior so that available energy stores can be used to produce fever, which requires a noticeable increase in the metabolism rate (Kluger, 1979). In addition, a reduction in activity, social interaction, sexual behavior and mood as well as increased sleep reduces the amount of exposed bodily surface area thus preventing heat loss (Maier & Watkins, 1998). The beneficial effects of increased HPA activity in preventing the immune system from overshooting have already been presented in Section 2.2.2.

Numerous studies have provided evidence that the sickness response seems to be mainly mediated by pro-inflammatory cytokines. In a comprehensive review, Larson and Dunn (2001) depicted results from animal studies demonstrating that cytokine and lipopolysaccharide (LPS) administration, the latter a component of the cell walls of gram-negative bacteria with highly cytokine-stimulating properties, elicit typical sickness responses such as reductions in food intake, exploratory and sexual behavior, learning and cognitive function, as well as an increase in non-rapid eye movement sleep (Larson & Dunn, 2001). These symptoms can be prevented by cytokine receptor antagonists (Bluthe, Dantzer & Kelley, 1992; Bluthe, Laye, Michaud, Combe, Dantzer & Parnet, 2000; Cunha, Cunha, Poole & Ferreira, 2000). Importantly, the behavioral changes of sickness seem to reflect motivational changes and a reorganization of behavioral priorities rather than the result of inevitably occurring debilitation and physical weakness (Aubert, 1999; Dantzer, 2001). This implies that in case of possible adverse effects of sickness- or cytokine-induced behavioral changes, behavior is less likely to be disrupted. In support of this suggestion, Aubert and colleagues (1997) found out that nest building was suppressed by LPS in lactating mice tested at room temperature (22°C). However, when lactating mice were exposed to cold temperature (6°C), nest building activity was no longer suppressed by LPS administration. Since the consequences of failing to build a nest at 6°C are more severe than building a nest at 22°C, motivation for nest building might be greater in a cooler environment. Thus, the behavioral expression of LPS-induced sickness seems to depend on the priority of the behavior under consideration (Aubert, Goodall, Dantzer & Gheusi, 1997). In another study, Aubert and colleagues observed that food hoarding was less disrupted by LPS when

rats received their food exclusively from hoarding as compared to rats that received food supplements in their home cage (Aubert, Kelley & Dantzer, 1997). The results demonstrate that LPS-treated animals still appear to be able to adjust their defensive behavioral strategies with regard to their needs and capacities.

The role of cytokines in eliciting sickness responses in humans has been most clearly illustrated by therapy studies administering cytokines such as IL-2 and IFN-α to patients with cancer and hepatitis B or C in order to stimulate the immune system. In these patients, IFN-α, IL-2 or a combined IFN-α/IL-2 therapy elicits symptoms such as pain, fatigue, cognitive impairment, and depression (e.g., Brenard, 1997; Capuron, Gumnick, Musselman, Lawson, Reemsnyder, Nemeroff & Miller, 2002; Eton, Talpaz, Lee, Rothberg, Brell & Benjamin, 1996; Kurzrock, 2001). The fact that these symptoms usually disappear shortly after termination of the cytokine treatment strongly suggests a causal role for cytokines in producing symptoms of the sickness response (Yirmiya, Pollak, Morag, Reichenberg, Barak, Avitsur, Shavit et al., 2000). Similarly, in healthy subjects, IL-6 administration induced plasma ACTH and cortisol increases, fatigue, inactivity and poor concentration, a slight increase in body temperature and a decrease in rapid eye movement sleep (Spath-Schwalbe, Hansen, Schmidt, Schrezenmeier, Marshall, Burger, Fehm et al., 1998). In another study, LPS-injection in healthy men resulted in significant elevations in circulating TNF-α, IL-6, IL-1 receptor antagonist, and cortisol levels as well as increases in body temperature, anorexia, anxiety, and depression, and in a marked impairment in both verbal and non-verbal memory functions. Significant positive correlations were found between the levels of anxiety, depression, and memory impairments and the level of circulating cytokines (Reichenberg, Yirmiya, Schuld, Kraus, Haack, Morag & Pollmacher, 2001).

2.2.5.1 Humoral and neural transmission of the cytokine message

For the induction of sickness responses, cytokines signal the brain via humoral and neural pathways. In this context, IL-1β seems to be the most extensively studied and most potent and important cytokine; however, the involvement of other pro-inflammatory cytokines such as IL-6 and TNF-α in the sickness response has also been proven (Maier, 2003).

With regard to humoral routes, cytokines most likely enter the brain parenchyma at regions where the blood-brain barrier (BBB) is weak or absent because they are

relatively large, hydrophilic molecules and thus unlikely to enter the brain parenchyma by passive diffusion. Regions with a weak or non-existent BBB comprise the choroid plexus and circumventricular organs (CVOs) such as the subfornical organ, median eminence, area postrema, and organum vasculosum lateralis terminalis (see Maier, 2003). At these regions, cytokines induce PGE_2, a small lipophilic molecule that is able to diffuse freely into the brain parenchyma and to initiate neural signaling (Saper & Breder, 1994). Synthesis of PGE_2 is dependent on the induction of the two enzymes COX-2 and prostaglandin E synthase that are both expressed in endothelial cells of cerebral blood vessels and possibly perivascular macrophages after intravenous IL-1β administration (Rivest, 2001). Studies have shown that pre-treatment with specific COX-2 inhibitors attenuate cytokine-induced fever and HPA activation, symptoms that are mainly PGE_2-mediated (Cao, Matsumura, Yamagata & Watanabe, 1997; Cao, Matsumura, Yamagata & Watanabe, 1998; Parsadaniantz, Lebeau, Duval, Grimaldi, Terlain & Kerdelhue, 2000). PGE_2 acts on neuronal EP_3 or EP_4 receptors in the brainstem and hypothalamic areas involved in the control of HPA and SNS activity and body temperature regulation. HPA and SNS activation by circulating cytokines is achieved by PGE_2-mediated stimulation of PVN neurons containing CRH, and of noradrenergic A1 neurons in the brainstem projecting to the PVN. The fever response to cytokines is attained by PGE_2-mediated activation of the medial preoptic area, which in turn activates descending projections of the PVN (reviewed by Konsman, Parnet & Dantzer, 2002).
In addition to the PGE_2-dependent humoral pathway, a PGE_2-independent pathway has been described mediating further cytokine-induced sickness responses including changes in social behavior and food intake. Studies have shown that in response to peripheral immune stimuli, IL-1β is synthesized by macrophage-like cells in the CVOs and choroid plexus (Konsman, Kelley & Dantzer, 1999; Quan, Whiteside & Herkenham, 1998). IL-1 produced in this way seems to act in a paracrine manner to induce the production of additional central IL-1 in cells further removed from BBB regions. This process can lead to IL-1β production by glial cells – immunocompetent cells of the central nervous system – which can in turn activate further glial cells in a paracrine manner. Thereby the IL-1β signal can be propagated to distant regions, at which the released IL-1β can act on neurons (Maier, 2003). As reviewed by Konsman and colleagues (2002), brain-derived IL-1β might act on IL-1 receptors in the area postrema resulting in the activation of a neuronal pathway that projects to the

parabrachial nucleus and from there to the central amygdala and the bed nucleus of the stria terminalis. Alternatively, IL-1 might also propagate by volume transmission from the choroid plexus into the surrounding brain parenchyma to reach structures such as the basolateral amygdala, which contains IL-1 receptor expressing neurons. While these two pathways might be responsible for the behaviorally depressing effects of IL-1, cytokine-induced reductions in food intake might be mediated by IL-1 diffusing from the median eminence to the arcuate nucleus (Konsman et al., 2002). Furthermore, studies have demonstrated that cytokines – despite their size – can enter the brain via specific saturable transport mechanisms that allow cytokines to cross the BBB (see Banks, 2001). Altogether, cytokines arriving at the BBB via the humoral pathway are able to alter the function of distant brain structures via different mechanisms including PGE_2 induction, glial cell activation, volume transmission, and/or active transport of blood-borne cytokines across the BBB.

The neural transmission of immune signals to the brain proceeds faster than the humoral transmission. It is mainly mediated by the vagus nerve that innervates bodily regions in which immune responses occur, including the gut, spleen, thymus and lymph nodes. Subdiaphragmatic vagal afferents have been shown to mediate at least in part the induction of the sickness response and the neural activation of the brainstem, hypothalamus and limbic structures in response to peripherally administered LPS and IL-1 (Dantzer, Konsman, Bluthe & Kelley, 2000). The role of the vagus nerve in the transmission of information from the periphery to the brain has been assessed by numerous vagotomy experiments. Results from these experiments have revealed that typical physiological sickness symptoms such as fever, increased pain responsivity, changes in brain norepinephrine levels, and glucocorticoid increases no longer occur after cutting the vagus nerve (Fleshner, Goehler, Hermann, Relton, Maier & Watkins, 1995; Fleshner, Goehler, Schwartz, McGorry, Martin, Maier & Watkins, 1998; Hansen, O'Connor, Goehler, Watkins & Maier, 2001; Watkins & Maier, 2000). In addition, vagotomy prevents behavioral changes including reductions in activity, social interactions, and food intake that otherwise occur during infection or cytokine/LPS administration (Bret-Dibat, Bluthe, Kent, Kelley & Dantzer, 1995; Laye, Bluthe, Kent, Combe, Medina, Parnet, Kelley et al., 1995; Luheshi, Bluthe, Rushforth, Mulcahy, Konsman, Goldbach & Dantzer, 2000). Noteworthy, the role of vagal afferents seems to be more important in behavioral depression than in physiologic changes such as fever initiation (Konsman et al., 2002) given that an

increase in body temperature after IL-1 or LPS administration has not been blocked by vagotomy in some studies (Konsman, Luheshi, Bluthe & Dantzer, 2000; Luheshi et al., 2000). All in all, the effect of vagotomy on changes in body temperature after IL-1/LPS administration seems to be dose-dependent in terms of vagotomy suppressing temperature raises at low doses but failing to prevent fever at higher doses (Hansen et al., 2001). This indicates that the mechanisms of cytokine-to-brain communication are not necessarily the same for the different components of the sickness response.

Goehler and colleagues have shown that the vagus nerve and vagal paraganglia, i.e. sensory structures surrounding vagal terminals, contain macrophages and dendritic cells that produce IL-1 in response to LPS injection (Goehler, Gaykema, Nguyen, Lee, Tilders, Maier & Watkins, 1999). Paraganglia seem to contain very dense binding sites for IL-1, whereas no such binding sites could be detected on vagal terminals (Goehler, Relton, Dripps, Kiechle, Tartaglia, Maier & Watkins, 1997). In contrast, Ek and colleagues (1998) were able to show that mRNA for the IL-1 receptor is present in the cell bodies of afferent vagal fibers in the nodose ganglion (Ek, Kurosawa, Lundeberg & Ericsson, 1998), indicating that IL-1 receptors are indeed present on the vagal terminals themselves. Evidence suggests that IL-1 that is released by activated immune cells such as macrophages and dendritic cells binds to IL-1 receptors on paraganglia and vagal terminals in the vicinity. In brief, this leads to transmitter release by the paraganglia onto the vagal terminals, resulting in activation of afferent vagal fibers that terminate in the nucleus tractus solitarius (NTS), a nucleus of the brain stem. The NTS sends projections to other brain regions such as the hypothalamus and hippocampus where the release of glial IL-1 is induced and where other neural events such as PGE_2 release are initiated (see Maier & Watkins, 1998; Maier, 2003; Wieseler-Frank, Maier & Watkins, 2004), finally resulting in sickness responses via central mechanisms that resemble those of the above described humoral pathway.

2.2.5.2 The symptom of pain

The symptom of pain plays an important role in the context of FMS, constituting the main syndrome of the disease. In addition, increased pain sensitivity is one of the symptoms of the sickness response. Watkins and Maier (2000) even consider pain the main determinant of the sickness response rather than just a component of it.

Under normal conditions, pain protects the organism from harm, triggering immediate behavioral responses to minimize or prevent tissue damage. Therefore, pain seems to have an adaptive function under these conditions (Wieseler-Frank et al., 2004). However, pain can become pathological, with painful pressure, heat and cold being grossly amplified ("hyperalgesia"). Tender point hyperalgesia in FMS patients constitutes a good example for pathological pain that seems to be no longer adaptive.

Recent research has suggested that pro-inflammatory cytokines released by glial cells in the spinal cord during sickness are critically involved in the creation and maintenance of pathological pain (Wieseler-Frank, Maier & Watkins, 2005). In the context of sickness responses and pain in particular, the term "glia" refers to microglia and astrocytes, which upon activation synthesize and release pro-inflammatory cytokines. Both microglia and astrocytes are immunocompetent cells spread throughout the brain and the spinal cord whose roles are similar to those of peripheral macrophages. This implies that, besides inducing the pro-inflammatory cytokine cascade, these glial cells are capable of increasing phagocytosis and present antigen to T cells in the central nervous system (Dong & Benveniste, 2001; Kreutzberg, 1996).

Painful stimuli activate specific receptors expressed on nerve endings of A-delta and C fibers, which are responsible for conveying information about painful stimuli from peripheral tissues to the spinal cord. In the spinal cord, A-delta and C fibers synapse on neurons within the dorsal horns, resulting in the release of glial-excitatory substances such as substance P, glutamate, nitric oxide, and prostaglandins (see Wieseler-Frank et al., 2005). Glial cells express receptors for these substances in a regionally heterogeneous way. For example, the spinal cord is one of the few sites in the central nervous system where glia express receptors for substance P (Marriott, Wilkin & Wood, 1991). Upon activation, glial cells release neuroexcitatory substances such as pro-inflammatory cytokines, nitric oxide, and prostaglandins, resulting in amplified pain sensitivity and finally in hyperalgesia. Importantly, glia are only involved in pathological pain but not in acute normal pain (Watkins & Maier, 2000; Wieseler-Frank et al., 2004; Wieseler-Frank et al., 2005).

The contribution of glial cells to exaggerated pain was first considered by Garrison and colleagues who reported that manipulation creating hyperalgesia in rats also activated astrocytes and that pharmacological treatment blocking the hyperalgesic

state suppressed astrocyte activation (Garrison, Dougherty, Kajander & Carlton, 1991; Garrison, Dougherty & Carlton, 1994). Since then, every animal model of pathological pain has revealed a positive relationship between hyperalgesia and glial activation in the spinal cord (Wieseler-Frank et al., 2004; Wieseler-Frank et al., 2005). Noteworthy, microglia and astrocytes seem to differently contribute to the state of hyperalgesia. Immunohistochemical staining for microglia and astrocyte activation markers revealed that microglia are most activated during the development of hyperalgesia, while astrocytes are most activated during the maintenance of exaggerated pain, with initial astrocyte activation delayed relative to that of microglia (see Wieseler-Frank et al., 2005).

The role of glia-derived pro-inflammatory cytokines in the induction and maintenance of hyperalgesia has been demonstrated in several studies employing spinally-delivered cytokine antagonists. For example, Milligan and colleagues (2003) reported that intrathecal (peri-spinal) inhibition of mitogen-activated kinases that are involved in pro-inflammatory cytokine production, as well as intrathecal IL-1, IL-6 and TNF-α specific antagonists prevented and reversed hyperalgesia in a rat model of sciatic inflammatory neuropathy (Milligan, Twining, Chacur, Biedenkapp, O'Connor, Poole, Tracey et al., 2003). Similarly, intrathecal administration of an IL-1 receptor antagonist in combination with soluble TNF-α receptors significantly reduced exaggerated pain states in a rat model of neuropathic pain (Sweitzer, Martin & DeLeo, 2001). On the other hand, endotoxin or cytokines administered intrathecally produced hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli in the rat (Falchi, Ferrara, Gharib & Dib, 2001; Reeve, Patel, Fox, Walker & Urban, 2000). These results suggest that spinal pro-inflammatory cytokines released upon peripheral cytokine or immune signals and after painful stimuli activating A-delta and C nerve fibers might play an essential role in the induction and maintenance of hyperalgesia.

The similarities between symptoms of the sickness response and those of FMS suggest that pro-inflammatory cytokines as main mediators of the sickness response might well be involved in symptoms experienced by FMS patients. This issue and the potential relevance of pro-inflammatory cytokines in the degree of symptom severity in FMS patients will be addressed in Chapter 5.

Chapter 3

3 Hypocortisolism and Sympathetic Hyperactivity in Fibromyalgia Patients in the Trier Social Stress Test: Potential Association with Blunted Natural Killer Cell Counts

3.1 Abstract

Background: Endocrine and sympathetic abnormalities have frequently been reported in patients with fibromyalgia syndrome (FMS), while only relatively few studies have looked at immune system alterations in this patient group. In addition, despite the well-known interactions between the three bodily systems, there is a lack of studies investigating associations between disease-related changes in these systems. The present study is aimed at detecting simultaneous alterations in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system (SNS) and the immune system that might be related to each other in female FMS patients before and after a psychosocial stress test.

Methods: Saliva and blood samples were collected before and after the Trier Social Stress Test (TSST) in 23 female FMS patients and 26 healthy, age-matched women for the determination of cortisol, adrenocorticotropic hormone (ACTH), norepinephrine, and lymphocyte subsets. FMS impact on functioning, pain, sleep disturbances, fatigue, depression, anxiety, body mass index (BMI), and demographic characteristics were evaluated by questionnaires, interviews, and during a comprehensive medical examination.

Results: Women with FMS had lower cortisol levels before and after the stress test, while ACTH concentrations were similar in the FMS and the control group. The blunted adrenal responses were even more evident when only subgroups of patients and healthy subjects were compared who responded to the TSST in terms of defined salivary cortisol increases after the stress test. Low cortisol levels in the FMS group were accompanied by elevated norepinephrine levels and low natural killer (NK) cell levels. The patients scored higher on all symptom scales applied in the study.

Conclusions: The results indicate concomitant alterations in HPA, SNS and immune system activity in female FMS patients that might be related to each other. One should be aware, however, that the observed overlap in altered activity of the three systems does not allow for the deduction of causal relationships. Further studies are needed that clarify the role of changes in neuro-endocrine-immune interactions in FMS patients and related pain and fatigue syndromes.

3.2 Introduction

In recent years, a phenomenon termed “hypocortisolism” has increasingly attracted the attention of scientists working in the field of psychoneuroendocrinology. This phenomenon is characterized by a hyporesponsiveness on different levels of the hypothalamic-pituitary-adrenal (HPA) axis in a number of stress-related states and has been reported in subgroups of patients with stress-related disorders such as fibromyalgia syndrome (FMS), chronic fatigue syndrome (CFS), irritable bowel syndrome (IBS), and post-traumatic stress disorder (PTSD) (for a comprehensive review see Fries et al., 2005; Heim et al., 2000). One common feature of these stress-related disorders is a striking symptom overlap (e.g., Clauw & Chrousos, 1997; Crofford, 1998; Fries et al., 2005; Hudson et al., 1992) suggesting a shared physiological pathway, in which blunted HPA axis activity might play an essential role.

With regard to the diversified regulatory functions of the HPA axis, hypocortisolism might have considerable effects on other bodily systems such as the sympathetic nervous system (SNS). While corticotrophin releasing hormone (CRH) activates noradrenergic neurons of the locus coeruleus resulting in the release of norepinephrine in the prefrontal cortex (Smagin et al., 1995; Valentino, Foote & Aston-Jones, 1983), glucocorticoids rather exert inhibitory effects on norepinephrine release. For example, one-week treatment of healthy humans with 20 mg of the synthetic glucocorticoid prednisolone resulted in reduced SNS activity and plasma catecholamine levels by 23% and 27%, respectively (Golczynska et al., 1995). In addition, immobilization studies with adrenalectomized rats have found that glucocorticoid elimination resulted in increased catecholamine synthesis and release, which was reversed by cortisol treatment (Kvetnansky et al., 1993; Pacak et al., 1993). Based on these observations, Fries and colleagues have lately hypothesized that hypocortisolemic features as observed in subgroups of patients with stress-related disorders might result in reduced inhibitory feedback activities of cortisol on catecholamine release and synthesis (Fries et al., 2005). One aim of the present study was to investigate this suggestion in a cohort of female FMS patients with expected hypocortisolemic features.

FMS is a disorder characterized by widespread chronic pain of at least three months duration and specific tender points on clinical examination (Wolfe et al., 1990). The

disorder is accompanied by a multitude of additional symptoms such as fatigue, sleep disturbances, morning stiffness, depression, and anxiety (Pagano, Matsutani, Ferreira, Marques & Pereira, 2004; Thompson, Lettich & Takeshita, 2003). In the early 1990s, Hudson and colleagues were amongst the first investigating symptom overlap and comorbidity of FMS with medical and mental disorders (Hudson et al., 1992). They reported a higher prevalence of migraine, IBS, and CFS, as well as higher lifetime rates of depression and panic disorder in patients with FMS, which has been confirmed in several other studies (Aaron et al., 2001; Clauw & Chrousos, 1997; Clauw & Crofford, 2003; Epstein et al., 1999; Thieme et al., 2004).

With regard to HPA axis activity, low cortisol concentrations have been observed in FMS patients in terms of 24-h urinary free cortisol levels (Crofford, Pillemer, Kalogeras, Cash, Michelson, Kling, Sternberg et al., 1994; Griep et al., 1998; Lentjes, Griep, Boersma, Romijn & de Kloet, 1997) and basal blood cortisol levels (Griep et al., 1998; Gur et al., 2004a; Gur et al., 2004b; Lentjes et al., 1997) supporting the assumption of a mild hypocortisolism in this patient group. In addition, pharmacological stimulation tests and exercise studies have revealed hypocortisolemic stress responses on adrenal level in FMS patients. For example, exaggerated adrenocorticotropic hormone (ACTH) responses in the CRH test and insulin tolerance test have been accompanied by unchanged cortisol levels in some studies (Crofford et al., 1994; Crofford, Engleberg & Demitrack, 1996; Griep, Boersma & de Kloet, 1993; Griep et al., 1998). This has been interpreted as a hyperreactivity on pituitary level and concomitant reduced adrenal responsivity to elevated ACTH levels in FMS patients. That assumption has been in part supported by studies reporting diminished cortisol increases in response to both 1µg and 250µg ACTH (Calis et al., 2004; Kirnap et al., 2001). Results from exercise studies have indicated lower post-exercise cortisol levels in FMS patients than in healthy controls (Paiva, Deodhar, Jones & Bennett, 2002; van Denderen, Boersma, Zeinstra, Hollander & van Neerbos, 1992).

It should be noted that some studies have reported contradicting results in terms of unchanged or elevated basal cortisol concentrations in blood, saliva or urine samples in FMS patients when compared to healthy subjects (Adler, Kinsley, Hurwitz, Mossey & Goldenberg, 1999; Catley, Kaell, Kirschbaum & Stone, 2000; Maes, Lin, Bonaccorso, van Hunsel, Van Gastel, Delmeire, Biondi et al., 1998; Riedel, Layka & Neeck, 1998). Additionally, one study has reported reduced ACTH reactivity in

response to insulin injection, while cortisol levels did not differ between FMS patients and healthy controls (Adler et al., 1999). Another study could not find differences in cortisol and ACTH levels in the CRH test when FMS patients and healthy subjects were compared (Riedel, Schlapp, Leck, Netter & Neeck, 2002). However, it seems that the majority of studies investigating HPA reactivity in response to pharmacological and exercise stress in FMS patients has found hypocortisolemic stress responses on adrenal level reflected by blunted cortisol increases or normal increases despite elevated ACTH reactivity, respectively.

Considering the above described inhibitory effects of glucocorticoids on the SNS, one would expect a disinhibited SNS activity in patients with observed hypocortisolism. In support of this assumption, heart rate variability studies including 24-h recordings and recordings under resting conditions have indicated sympathetic hyperactivity in both male and female FMS patients (Cohen, Neumann, Shore, Amir, Cassuto & Buskila, 2000; Cohen, Neumann, Alhosshle, Kotler, Abu-Shakra & Buskila, 2001; Martinez-Lavin, Hermosillo, Rosas & Soto, 1998). In addition, one study reported norepinephrine-evoked pain after injection of 10µg of norepinephrine in 80% of FMS patients, whereas the injection caused pain in only 30% of rheumatoid arthritis patients and 30% of healthy controls. Pain intensity was also higher in the FMS patients than in the other subjects after the norepinephrine injection (Martinez-Lavin, Vidal, Barbosa, Pineda, Casanova & Nava, 2002). Based on their and other findings, Martinez-Lavin and colleagues have hypothesized that FMS constitutes a sympathetically maintained pain syndrome, in which permanent sympathetic hyperactivity might cause sensitization of primary nociceptors resulting in typical FMS symptoms such as widespread pain and allodynia (Martinez-Lavin, 2001; Martinez-Lavin et al., 2002; Martinez-Lavin, 2004). In contrast to the reported elevated basal SNS activity, some studies investigating SNS responses to applied stress have reported a reduced SNS reactivity in FMS patients as observed in a heart rate variability study using an orthostatic and mental stress paradigm (Friederich, Schellberg, Mueller, Bieber, Zipfel & Eich, 2004) and in studies reporting blunted catecholamine responses to exercise and insulin injection (Adler et al., 1999; van Denderen et al., 1992). On the other hand, Torpy and colleagues found not only basally elevated norepinephrine levels in FMS patients but also strong increases after IL-6 injection, whereas no significant IL-6-induced norepinephrine increase was

observed in the healthy control group (Torpy, Papanicolaou, Lotsikas, Wilder, Chrousos & Pillemer, 2000).

Catecholamines exert distinguished effects on the immune system. Their administration in humans leads to a mobilization of lymphocytes within 30 minutes, followed by an increase of granulocytes and a drop of lymphocyte levels. The latter phase reaches its maximum 2-4 hours after catecholamine administration. It has been shown that especially natural killer (NK) cells and granulocytes are strongly affected by catecholamine infusion, whereas T and B cell numbers change to a lesser extent (Benschop et al., 1996). With regard to acute psychological stress, a transient increase in lymphocytes, particularly NK cell numbers, has been observed, with elevated catecholamine levels playing a documented role in these immune cell changes (see Elenkov et al., 2000). Interestingly, prolonged sympathetic activity might have opposite effects on NK cell numbers than acute catecholamine increases (Maisel et al., 1990b). Thus, while acute increases in SNS activity cause mobilization of NK cells from depots, long-term elevation of catecholamines leads to decreased NK cell numbers in the peripheral blood. This might be an important observation in the context of FMS studies, given the assumption that the SNS activity in FMS patients might be chronically elevated.

Only few studies have addressed the question of immune cell alterations in FMS patients, and the data are quite inconsistent. While Hernanz and colleagues found a reduced number of T cells with the activation markers CD25+ (interleukin (IL)-2 receptor) and CD69+ (activation inducer molecule marker) in FMS patients compared to healthy controls (Hernanz, Valenzuela, Quijada, Garcia, de la Iglesia, Gutierrez, Povedano et al., 1994), another study reported fewer lymphocyte counts but increased CD25+ T cell levels in the patient group (Russell et al., 1999). In a recent study, Landis and colleagues compared immune system markers in women with and without FMS. They found decreased NK cell numbers and a higher percentage of NK cells that expressed the IL-2 receptor in FMS patients than in controls, but the differences were no longer significant after Bonferroni correction (Landis et al., 2004). Thus, no clear direction in immune cell changes has so far arisen in FMS research. In addition, no study has integrated cortisol and catecholamine changes and alterations in immune system parameters after an applied psychosocial stressor in FMS patients.

The aim of the present study was to investigate endocrine, sympathetic, and immune activity in female FMS patients and healthy women before and after the the Trier Social Stress Test (TSST, Kirschbaum, Pirke & Hellhammer, 1993). The test combines public speaking and a cognitive task, which most reliably elicits substantial HPA responses, as confirmed by a recent meta analysis (Dickerson & Kemeny, 2004). We hypothesized that blunted HPA activity in FMS patients was accompanied by an exaggerated norepinephrine release which might be the result of a diminished inhibitory feedback activity of cortisol on catecholamine release and synthesis. Based on the above cited studies suggesting chronic basal SNS hyperactivity in FMS patients, we expected blunted NK cell levels in the patient group potentially associated with the inhibitory effects of chronic catecholamine exposure on NK cells.

3.3 Materials and methods

3.3.1 Study participants

The present study comprised 24 female FMS patients (mean age: 49.7 ± 9.2 SD) and 26 age-matched healthy women (mean age: 50.5 ± 8.4 SD). Patients were recruited via outpatient and specialist clinics, patient self-help groups, and newspaper advertisements. They were included into the study if they fulfilled the ACR 1990 Criteria for the Classification of Fibromyalgia (Wolfe et al., 1990) diagnosed by a rheumatologist, reported non-inflammatory origins of the pain, were free of medical diseases such as gastrointestinal, neurological or autoimmune disorders, had no psychotic or eating disorders, were not reporting presence of substance abuse or dependency, and discontinued potential antidepressant medication at least two weeks before study entry.

Healthy subjects were recruited via newspaper advertisements or were provided from the environment of participating FMS patients, respectively. They were included if they were free of antidepressant/antipsychotic drugs, medical diseases and mental disorders. Pregnancy, current breast feeding, hormonal medication (exception: oral contraceptives, hormone replacement therapy), and dietary weight loss of 5 kg or more within 6 weeks before study entry were regarded as further exclusion criteria for both the FMS patients and the healthy volunteers. The study subjects were evaluated with the German version of the Structured Clinical Interview for DSM-IV (Wittchen, Zaudig & Fydrich, 1997) and underwent a comprehensive medical

examination for past or current health problems. All persons participating in the study gave their written informed consent. The study protocol was approved by the Ethics Committee of the *Landesärztekammer Rheinland-Pfalz*.

3.3.2 Demographic and psychometric assessments

Demographic data including age, marital status, education, and job situation was assessed using a demographic questionnaire. The current health status of women with FMS compared to healthy subjects was measured by the *German version of the Fibromyalgia Impact Questionnaire* (FIQ-G, Offenbaecher, Waltz & Schoeps, 2000). The German version of the *Pain Disability Index* (PDI, Dillmann, Nilges, Saile & Gerbershagen, 1994) was used for the evaluation of the severity of pain symptoms. In addition, fatigue was assessed by the *Fatigue Scale (FS)* developed by Chalder and colleagues (Chalder, Berelowitz, Pawlikowska, Watts, Wessely, Wright & Wallace, 1993), sleep disturbances by the German version of the *Pittsburgh Sleep Quality Index* (Riemann & Backhaus, 1996). The degree of depressive symptoms was evaluated by the *Allgemeine Depressionsskala* (ADS, Hautzinger & Bailer, 1993). Finally, the German version of the *State-Trait-Anxiety-Inventory* (Laux, Glanzmann, Schaffner & Spielberger, 1981) was used to measure state (STAI-S) and trait (STAI-T) anxiety in FMS patients and healthy volunteers.

3.3.3 Experimental protocol

Study participants reported to the laboratory between 8.00 and 8.30 a.m. After the medical examination and the clinical interview, a catheter was inserted into the antecubital vein followed by a 45 min resting period. The first blood and saliva samples were taken 30 min before subjects were exposed to the Trier Social Stress Test (TSST, Kirschbaum et al., 1993), which consists of a three-minute speech preparation period, followed by a five-minute free speech task and a five-minute mental arithmetic task in front of an audience of one male and one female trained staff member. Further blood and saliva samples were taken 1, 10, 30, 60, and 120 min after the stress exposure, additional saliva samples 2 min before, and 20, 45, and 90 minutes after the TSST.

3.3.4 Laboratory assays

3.3.4.1 Salivary cortisol

All saliva samples were stored at -20°C until analysis. Salivary cortisol was measured with a time-resolved fluorescence immunoassay as described elsewhere (Dressendörfer, Kirschbaum, Rohde, Stahl & Strasburger, 1992). The intra-assay coefficient of variation is 4.0% to 6.7%, the inter-assay coefficient of variation 7.1% to 9.0%. All samples of one participant were analyzed in the same run in order to reduce error variance due to inter-assay imprecision.

3.3.4.2 Plasma cortisol, ACTH and norepinephrine

Blood samples were collected in ethylenediamine tetraacetic acid (EDTA) tubes (Sarstedt, Nümbrecht, Germany). After 15 min of centrifugation at 4°C and 1000g, plasma aliquots for the determination of cortisol were stored at -20°C, for the determination of ACTH and norepinephrine at -80°C until they were analyzed. Total cortisol was measured by a commercially available enzyme-linked immunosorbent assay (ELISA; Immuno-Biological Laboratories, IBL, Hamburg, Germany) with an intra-assay coefficient of variation between 5.6% and 8.1% and an inter-assay coefficient of variation between 6.5% and 7.7%. A chemiluminescent immunoassay (Nichols Institute Diagnostics, Bad Nauheim, Germany) was used for the analysis of ACTH. The assay has a lower and upper detection range of 0.5-1550 pg/ml, an intra-assay variation between 3.4% and 3.8%, and an inter-assay variation between 4.6% and 7.0%. Norepinephrine levels were detected by a standard high pressure liquid chromatography (HPLC, Waters GmbH, Eschborn, Germany). The required reagents for this assay were purchased from Recipe (Munich, Germany). A detailed description of the analysis of catecholamines by HPLC and their electrochemical detection has been provided by Kringe and colleagues (1982).

3.3.4.3 Lymphocyte subpopulations

Peripheral blood was collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) 30 min before as well as 1 min and 120 min after the TSST. Total numbers of leukocytes were determined by a cell counter in each sample (Coulter $A^{c}{\cdot}T$ diff^{TM}, Krefeld, Germany). Flow cytometry [fluorescence activated cell sorter (FACS), Becton

Dickinson, Heidelberg, Germany] using CellQuest Pro software version 3.4 was carried out for the analysis of the following circulating lymphocyte subsets: T cells (CD3+/CD19-), B cells (CD3-/CD19+), T helper cells (CD3+/CD4+), T suppressor/cytotoxic cells (CD3+/CD8+), and NK cells (CD3-/CD16,56+). For the staining protocol the kit Simultest^{TM} from Becton Dickinson was used according to the manufacturer's instructions.

3.3.5 Statistical analyses

Data was analyzed with SPSS 12.0. T-tests were used for normally distributed demographic variables, multivariate analyses of variance (MANOVAs) for psychometric data. In order to investigate differences in medication intake between the experimental groups, chi square tests were performed.

In case of skewed distributions, hormone raw data was normalized by logarithmic transformation for further analyses. For a better overview, however, raw data was retained unchanged in all figures in the results section. General linear models (GLMs) with repeated measures were performed to detect differences in the levels and the course of salivary cortisol (9 samples), plasma cortisol (5 samples), ACTH (6 samples), norepinephrine (6 samples) and lymphocyte subsets (3 samples each) between FMS patients and the healthy control group, with Greenhouse-Geisser or Huynh-Feldt corrections applied where appropriate. Due to a broad age range and the allowance of some medication intake, "age", "blood pressure medication", "estrogen intake" and "the intake of antidepressants (discontinued for at least two weeks)" were entered as covariates into all GLMs to control for the effects of these variables on the results.

In order to gain absolute cell numbers for the different lymphocyte subsets, the absolute number of leukocytes obtained by the cell count was multiplied by the percentage of lymphocytes received in the FACS analyses, thus yielding the total number of lymphocytes for each subject. Thereafter, the total number of lymphocytes was multiplied by the percentage of CD3+/CD19-, CD3-/CD19+, and CD3-/CD16,56+ cells to obtain the respective total numbers. The total number of T cells was multiplied by the percentage of CD3+/CD4+ and CD3+/CD8+ cells, respectively, in order to gain the total numbers of T cell subsets.

Area under the curve with respect to increase (AUCi) taking into account the time between the measurements as described by Pruessner and colleagues (Pruessner,

Kirschbaum, Meinlschmid & Hellhammer, 2003) was calculated for salivary cortisol levels using an algorithm derived from the trapezoid formula. Individual salivary cortisol levels up to 60 minutes after the TSST were aggregated relative to the individual level 2 minutes before the stress test. AUCi can attain both positive and negative values, the latter due to the diurnal decline in cortisol levels. For additional analyses, subgroups were built based on positive/negative AUCi values. It was suggested that subjects with a negative AUCi did not respond to the TSST in terms of cortisol changes, whereas a positive AUCi indicated a cortisol response to the stress test and thus a slowing down of the diurnal cortisol decline, which is more pronounced in the morning hours. With this procedure, four subgroups were built (AUCi positive: “FMS responders”, “controls responders”; AUCi negative: “FMS non-responders”, “controls non-responders”), which were further compared in terms of HPA axis responses to the TSST and data obtained from the questionnaires.

3.4 Results

3.4.1 Demographic and psychometric parameters

Twenty-four women with FMS and 26 female healthy volunteers participated in the study. All subjects were non-smokers. Demographic and psychometric characteristics of the patients and the healthy women are presented in Table 3.1. T-test comparisons between the FMS and the healthy control groups revealed no significant differences in age and body mass index (BMI). In addition, the number of women using blood pressure medication or oral contraceptives/ postmenopausal hormone substitution did not significantly differ between the groups. None of the healthy participants but ten of the FMS subjects took antidepressant medication, which they agreed to discontinue at least two weeks before entry into the study.

Results from MANOVAs indicated that FMS patients scored significantly higher on all psychometric scales included in the study. As expected, they reported higher pain, fatigue, and sleep difficulties, as well as more depressive symptoms and state and trait anxiety. The number of years since diagnosis ranged from 1 to 10 years, while symptoms of pain had been present for 1.5 up to 36 years.

Table 3.1: Demographic and psychometric characteristics of FMS patients and healthy volunteers

Variable	FMS patients (n=24)	Controls (n=26)	Statistics
Demographics			
Age, years *(mean, SD)*	49.7 (9.2)	50.5 (8.4)	t(48) = -0.349, p = 0.728
BMI *(mean, SD)*[a]	27.3 (6.0)	25.2 (4.2)	t(48) = 1.446, p = 0.155
Blood pressure medication *(n, %)*	5 (20.8%)	6 (23.1%)	Fisher's exact: p = 0.560
Intake of oral contraceptives, hormone replacement *(n, %)*	6 (25.0%)	10 (38.5%)	Fisher's exact: p = 0.372
Antidepressants *(n, %)*[b]	10 (41.7%)	-	
Psychometric parameters *(mean, SD[c])*			
FMS impact on functioning (FIQ-G)	45.3 (11.7)	5.4 (6.7)	$F_{1.43} = 207.30$, $p < 0.001$
Pain (PDI)	31.9 (11.3)	8.0 (11.9)	$F_{1.43} = 45.99$, $p < 0.001$
Fatigue (FS)	3.2 (0.6)	1.9 (0.3)	$F_{1.43} = 76.83$, $p < 0.001$
Sleep Disturbances (PSQI)	12.6 (4.7)	5.5 (2.8)	$F_{1.43} = 37.23$, $p < 0.001$
Depression (ADS)	23.3 (8.9)	7.7 (6.0)	$F_{1.48} = 53.47$, $p < 0.001$
Trait-Anxiety (STAI-T)	50.5 (10.6)	33.4 (7.2)	$F_{1.48} = 44.95$, $p < 0.001$
State-Anxiety (STAI-S)	42.5 (8.9)	33.9 (8.3)	$F_{1.48} = 12.56$, $p < 0.001$
FMS patient characteristics			
Number of years since diagnosed *(mean, range)*	4.3 (1-10)	-	
Number of years since first appearance of symptoms *(mean, range)*	14.9 (1.5-36)	-	

[a] *BMI: Body Mass Index (body weight [kg]/height [m]²)*
[b] *patients refrained from antidepressants - in most cases used as pain relievers - at least two weeks before study entry*
[c] *SD: Standard deviation*

3.4.2 Endocrine responses to the TSST

3.4.2.1 Salivary cortisol

GLMs with repeated measures were conducted for the investigation of differences in cortisol levels before and after the TSST between the FMS group and the healthy control group. As mentioned in Section 3.3.5, "age", "blood pressure medication", "estrogen intake" and "the intake of antidepressants (discontinued for at least two weeks)" were incorporated as covariates into all analyses due to their known effects

on HPA activity. One FMS patient suffered from a dry mouth and was unable to collect saliva samples 10 min and 30 min post-TSST. Therefore, her data on salivary cortisol was not included in the following analyses.

A Kolmogorov-Smirnov test indicated that some of the variables were not normally distributed. Consequently, logarithmized variables were entered into the GLM. Results revealed a significant time effect for the total group (*time*: $F_{3.3,142.4}$=7.30; p<0.001, η^2=0.15) and a significant interaction effect of the covariate age with time (*age*time interaction*: $F_{3.3,142.4}$=4.15; p<0.006, η^2=0.09). The additional covariates "blood pressure medication", "estrogen intake" and "the intake of antidepressants (discontinued for at least two weeks)" did not significantly affect the course of salivary cortisol in the TSST. Comparing the FMS group with the healthy control group, there was a trend towards a significant interaction effect group*time (*group*time interaction*: $F_{3.3,142.4}$=2.20; p=0.084, η^2=0.05), while the group effect was not significant (*group*: $F_{1,43}$=2.72; p=0.106, η^2=0.06). Covariate-adjusted salivary cortisol concentrations for the FMS group and the healthy control group are presented in Figure 3.1.

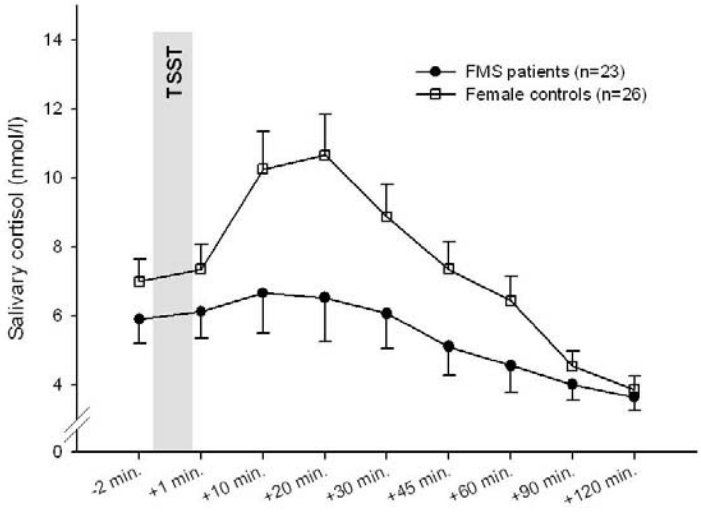

Figure 3.1: Salivary cortisol concentrations (mean ± SEM) before and after the TSST in women with FMS (n=23) and female controls (n=26).

To find out if salivary cortisol increases in response to the TSST were higher in the healthy control group compared to the FMS group, the AUCi was calculated. A one-way ANCOVA was conducted with the factor group (FMS, control) as independent variable, AUCi for salivary cortisol as dependent variable, and "age", "blood pressure medication", "estrogen intake" and "the intake of antidepressants (discontinued for at

least two weeks)" as covariates. Results revealed no significant group effect (*group*: $F_{1,43}$=2.26; p=0.14, η^2=0.05) but a significant effect for the covariate age (*age*: $F_{1,43}$=8.96; p=0.005, η^2=0.17). A t-test comparing subjects age 53 and older (n=20) with subjects younger than 53 (n=29) indicated lower AUCi for the elder group (t_{47}=-2.30, p=0.026). Figure 3.2 illustrates AUCi levels for the FMS group vs. the control group adjusted for the covariates. Figure 3.3 shows AUCi differences in the two age groups.

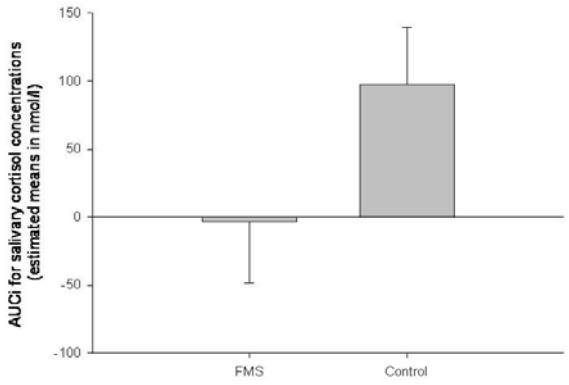

Figure 3.2: AUCi for salivary cortisol levels (mean ± SEM) for women with FMS (n=23) and female controls (n=26).
AUCi: area under the curve with respect to increase.

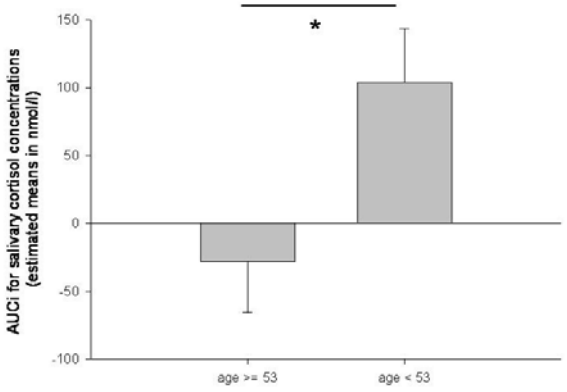

Figure 3.3: AUCi for salivary cortisol levels (mean ± SEM) for women ≥ 53 years of age (n=20) and women < 53 years (n=29).
Asterisk: p<0.05; AUCi: area under the curve with respect to increase.

For further analyses, the FMS group and the healthy control group were subdivided into TSST responders and TSST non-responders based on positive/negative AUCi values. It was suggested that subjects with negative AUCi levels did not respond to the TSST in terms of salivary cortisol increases (see Section 3.3.5). As shown in Table 3.2, 12 (52.2%) of the 23 patients were classified as cortisol responders due to positive AUCi values, whereas 11 (47.8%) were categorized as cortisol non-responders. Fifteen (57.7%) healthy women were responders, 11 (42.3%) non-responders. One-way ANOVAS with the factor group (FMS responders, FMS non-responders, controls responders, controls non-responders) as dependent variable and AUCi as independent variable indicated a significant group effect (*group*: $F_{3,45}$=23.71; p<0.001, η^2=0.61). Post-hoc analyses revealed that both responder groups differed significantly from the non-responder groups, whereas there was no statistically significant difference between FMS non-responders and healthy non-responders, or between FMS responders and healthy responders, respectively (Figure 3.4).

	Group		
	FMS Patients	Controls	n
Responders (AUCi > 0)	12	15	27
Non-Responders (AUCi ≤ 0)	11	11	22
n	23	26	49

Table 3.2: Classification of FMS patients and healthy subjects into TSST responders and TSST non-responders based on positive/ negative AUCi values.
AUCi: area under the curve with respect to increase.

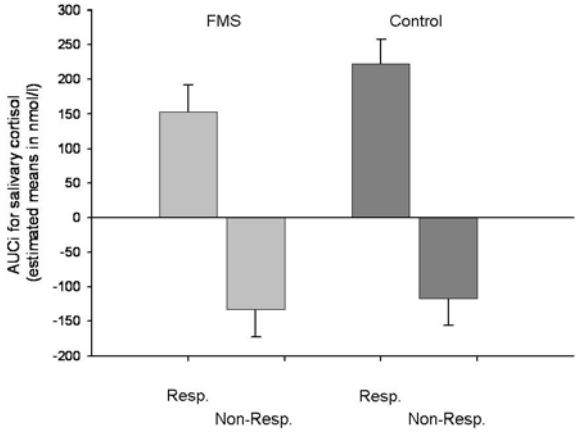

Figure 3.4: AUCi for salivary cortisol levels (mean ± SEM) for FMS responders (n=12), FMS non-responders (n=11), controls responders (n=15) and controls non-responders (n=11).
AUCi: area under the curve with respect to increase; Resp.: Responders, Non-Resp.: Non-Responders.

A GLM with repeated measures (9 saliva samples) was performed in order to investigate differences in cortisol levels before and after the TSST between the responder and non-responder groups. Again, “age”, “blood pressure medication”, “estrogen intake” and “the intake of antidepressants (discontinued for at least two weeks)” were entered as covariates into the model. Results for the log-transformed data revealed a significant time effect (*time*: $F_{4.0,163.9}$=3.67; p=0.007, η^2=0.08) and a significant group*time interaction effect (*group*time interaction*: $F_{12.0,163.9}$=5.42; p<0.001, η^2=0.28). In addition, there was a significant group effect (*group*: $F_{3,41}$=2.76; p=0.05, η^2=0.17). Post-hoc analyses demonstrated that healthy responders differed significantly from FMS responders and both non-responder groups. FMS responders did not differ from the non-responder groups but only from the healthy responder group. T-tests revealed that healthy responders had significantly higher salivary cortisol levels than FMS responders at the following measuring times (degrees of freedom for all t-tests: 25): *-2 min* (t = -2.13, p=0.043), *+10 min* (t = -2,13, p=0.043), *+20 min* (t = -2,42, p=0.023), *+30 min* (t = -2,24, p=0.034), *+45 min* (t = -2,57, p=0.017), *+60 min* (t = -2,01, p=0.055). None of the covariates significantly affected cortisol levels. Results for all four groups are shown in Figure 3.5A, for the two responder groups in Figure 3.5B.

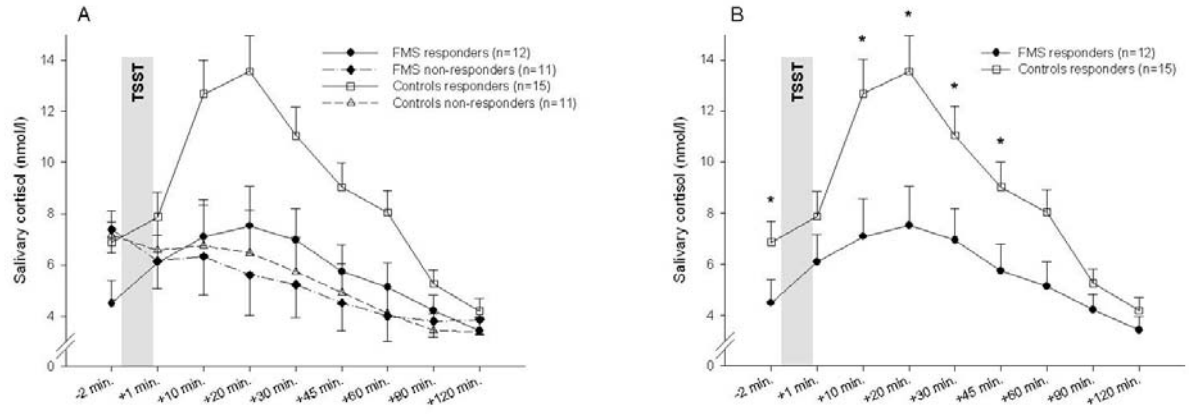

Figure 3.5: (A) Salivary cortisol concentrations (mean ± SEM) before and after the TSST in FMS responders (n=12), FMS non-responders (n=11), healthy responders (n=15), and healthy non-responders (n=11). (B) Salivary cortisol concentrations (mean ± SEM) before and after the TSST for the FMS responder group and the healthy responder group. Asterisk: $p<0.05$.

Differences in demographic and psychometric parameters between the four subgroups were investigated using one-way ANOVAs with the factor group (FMS non-/responders, controls non-/responders) as dependent variable and “age” and “BMI” as independent variables, and MANOVAs with the psychometric characteristics as independent variables. In addition, chi square tests were used to compare medication intake between the subgroups. Differences in time since diagnosis and years since first symptom appearance between FMS responders and non-responders were investigated by t-tests. As illustrated in Table 3.3, a trend towards a significant age effect was observed. However, post-hoc tests revealed no significant age differences between the four subgroups. In addition, BMI and medication intake did not differ between the groups. In terms of psychometric parameters, significant effects were found for all variables.

Subsequent post-hoc tests demonstrated that both healthy subgroups differed significantly from the two patient subgroups in all but one psychometric characteristics (exception: state anxiety). There were, however, no significant differences in any of the psychological variables between the two FMS subgroups, or between the two healthy subgroups, respectively. Time since diagnosis and years since first symptom appearance was similar for FMS responders and non-responders.

In sum, concerning data on salivary cortisol concentrations, the results indicated a trend towards lower levels in the FMS group. When observing only those subjects who responded to the TSST in terms of salivary cortisol increases (positive AUCi), it

was shown that FMS responders displayed significantly lower cortisol concentrations before and in response to the TSST than their healthy counterparts. While FMS patients differed significantly in all psychometric parameters from the control group, neither FMS responders did from FMS non-responders, nor healthy responders from healthy non-responders.

Table 3.3: Comparison of demographic and psychometric characteristics between FMS responders and non-responders and healthy responders and non-responders

Variables	FMS Resp. (n=12)	FMS Non-Resp. (n=11)	Controls Resp. (n=15)	Controls Non-Resp. (n=11)	Statistics
Demographics					
Age, years *(mean, SD)*	47.9 (9.8)	52.2 (8.8)	47.1 (6.9)	55.3 (8.1)	$F_{3,45}$=2.56, p=0.067
BMI *(mean, SD)*[a]	28.5 (7.7)	26.0 (3.8)	25.7 (4.0)	24.6 (4.6)	$F_{3,45}$=1.18, p=0.328
Blood pressure medication *(n, %)*	2 (16.7%)	3 (27.3%)	4 (26.7%)	2 (18.2%)	$X^2(3)$=0.886
Oral contra-ceptives, hormone replacement *(n, %)*	3 (25.0%)	3 (27.3%)	6 (40%)	4 (36.4%)	$X^2(3)$=0.825
Antidepressants *(n, %)*[b]	5 (41.7%)	5 (45.5%)	-	-	Fisher's exact: p = 0.59
Psychometric data *(mean, SD)*					
FMS impact on functioning (FIQ-G)	44.0 (12.0)	43.4 (11.1)	6.8 (8.3)	3.6 (3.5)	$F_{3,40}$=64.24, p<0.001
Pain (PDI)	33.8 (11.4)	32.0 (11.9)	5.8 (3.5)	5.2 (2.0)	$F_{3,40}$=15.88, p<0.001
Fatigue (FS)	3.2 (0.5)	3.0 (0.7)	1.9 (0.4)	1.9 (0.3)	$F_{3,40}$=24.15, p<0.001
Sleep Disturbance (PSQI)	12.7 (5.5)	11.8 (3.9)	5.8 (3.5)	5.2 (2.0)	$F_{3,40}$=11.09, p<0.001
Depression (ADS)	24.8 (9.5)	22.2 (8.8)	8.9 (6.7)	6.1 (4.7)	$F_{3,45}$=18.10, p<0.001
Trait-Anxiety (STAI-T)	52.4 (12.4)	47.9 (8.7)	33.7 (9.1)	33.0 (3.7)	$F_{3,45}$=14.38, p<0.001
State-Anxiety (STAI-S)	42.2 (9.2)	41.7 (8.2)	34.7 (9.8)	32.9 (6.0)	$F_{3,45}$=3.64, p=0.020
FMS patient characteristics					
Number of years since diagnosed *(mean, SD)*	4.46 (3.5)	4.10 (2.2)	-	-	t(20) = -0.283, p=0.780
Number of years since first symptom appearance *(mean, SD)*	13.78 (9.7)	16.32 (11.3)	-	-	t(18) = 0.533, p=0.601

[a] *BMI: Body Mass Index (body weight [kg]/height [m]²)*
[b] *patients refrained from antidepressants - in most cases used as pain relievers - at least two weeks before study entry*

3.4.2.2 Plasma Cortisol

On the TSST day, five blood samples were taken for the analysis of plasma cortisol (30 min before the TSST as well as 1 min, 10 min, 30 min, and 120 min after the TSST). Since plasma cortisol levels 120 min post-TSST were not normally distributed, data were logarithmized and then entered into a GLM with group (FMS patients, healthy volunteers) as independent variable and "age", "blood pressure medication", "estrogen intake" and "the intake of antidepressants (discontinued for at least two weeks)" as covariates. Two subjects, one FMS patient and one healthy woman, were excluded from the analyses because all their plasma cortisol samples were at least 2.6 standard deviations (range: 2.62 SD to 3.73 SD) above the mean of the respective values of the two groups.

Results demonstrated a significant time effect (*time*: $F_{3.5,138.5}$=4.76; p=0.002, η^2=0.11) and a significant age*time interaction (*age*time interaction*: $F_{3.4,134.2}$=2.63; p=0.046, η^2=0.06). Medication intake did not affect plasma cortisol levels. In terms of group differences, a significant group effect was observed (*group*: $F_{1,40}$=5.00; p=0.031, η^2=0.11). The group*time interaction was not significant (*group*time interaction*: $F_{3.4,138.5}$=0.99; p=0.407). T-test comparisons revealed significantly lower plasma cortisol levels in the FMS group *30 min pre-TSST* (t_{46} = -2.07, p=0.044), 10 min post-TSST (t_{44} = -1.97, p=0.055), and *30 min post-TSST* (t_{46} = -2.61, p=0.012). Covariate-adjusted results are shown in Figure 3.6.

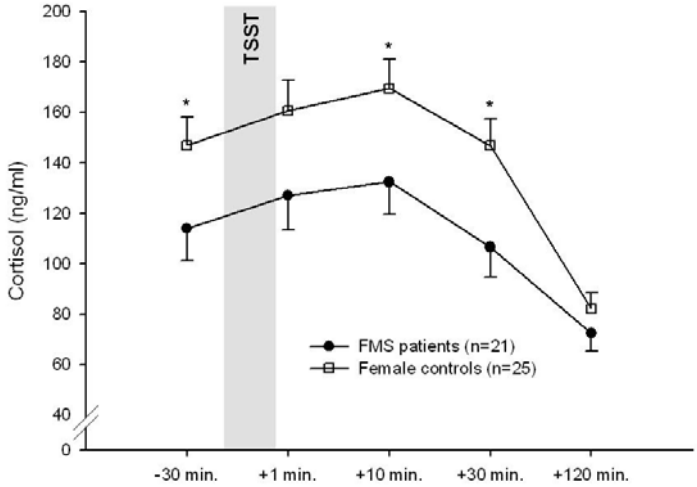

Figure 3.6: Plasma cortisol concentrations (mean ± SEM) before and after the TSST in women with FMS (n=21) and female controls (n=25). Asterisk: p<0.05

Plasma cortisol levels were then compared between the four subgroups that were built based on positive/negative AUCi for salivary cortisol (see Sections 3.3.5 and 3.4.2.1). The GLM with the independent factor group (FMS non-/responders, controls non-/responders), the log-transformed plasma cortisol levels as dependent variable, and the four covariates ("age", "blood pressure medication", "estrogen intake" and "the intake of antidepressants") revealed a significant interaction effect group*time (*group*time interaction*: $F_{7.7,97.6}$=4.53; p<0.001, η^2=0.26) but no significant main effect of the factor group on plasma cortisol levels (*group*: $F_{3,38}$=2.02; p=0.127). None of the covariates significantly influenced plasma cortisol levels or changes during the TSST, respectively. As illustrated in Figure 3.7A, the classification of subgroups based on salivary cortisol measures was also applicable to plasma cortisol changes in terms of decreasing levels in the non-responder groups and increasing levels in the responder groups after the TSST.

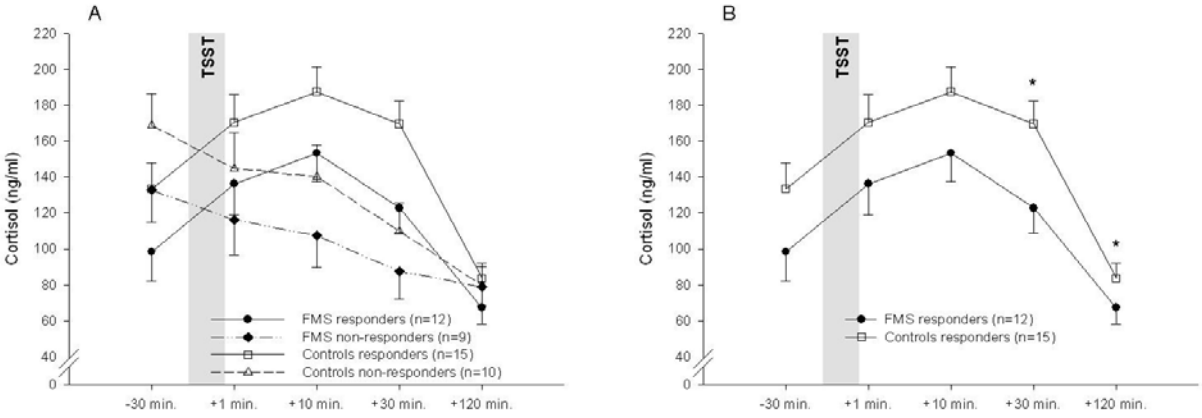

Figure 3.7: (A) Plasma cortisol concentrations (mean ± SEM) before and after the TSST in FMS responders (n=12), FMS non-responders (n=10), healthy responders (n=15), and healthy non-responders (n=11). (B) Plasma cortisol concentrations (mean ± SEM) before and after the TSST for the FMS responder group and the healthy responder group. Asterisk: p<0.05.

Figure 3.7B presents results of a GLM conducted with only the two responder groups. Since none of the covariates significantly influenced plasma cortisol levels when the four subgroups were compared, they were not included into the model. Results indicated a significant time effect (*time*: $F_{2.5,63.2}$=50.32; p<0.001, ω^2=0.67) as well as a significant group effect (*group*: $F_{1,25}$=5.75; p=0.024, ω^2=0.19). T-tests revealed marginally significant differences in plasma cortisol levels between FMS responders and healthy responders *30min pre-TSST* (t = -1.87, p=0.073) and *10min*

post-TSST (t = -1.84, p=0.077). In addition, at *30min post-TSST* (t = -2.48, p=0.020) and *120min post-TSST* (t = -2.09, p=0.047) cortisol levels were significantly lower in the FMS responder group compared to their healthy counterparts. All degrees of freedom were 25. A GLM conducted with only the two non-responder groups failed to show significant difference in plasma cortisol levels between the two groups (data not shown).

Thus, similar to the findings for free salivary cortisol, the results indicate lower total plasma cortisol levels in the FMS group, which was further confirmed when only FMS responders were compared with healthy volunteers.

3.4.2.3 ACTH

EDTA blood samples for the analysis of ACTH were collected 30 min before the TSST, as well as 1 min, 10 min, 30 min, 60 min, and 120 min after the TSST. Kolmogonov-Smirnov tests revealed that ACTH levels 120 min after the TSST were not normally distributed. Therefore, logarithmized data was entered into the statistical analyses. A GLM with group (FMS patients, healthy controls) as independent variable, ACTH levels as dependent variable, and “age”, “blood pressure medication”, “estrogen intake” and “the intake of antidepressants” as covariates yielded a significant time effect (*time*: $F_{2.3,95.6}$=18.20; p<0.001, η^2=0.30) and a significant age*time interaction (*age*time interaction*: $F_{2.3,95.6}$=12.21; p<0.001, η^2=0.23).

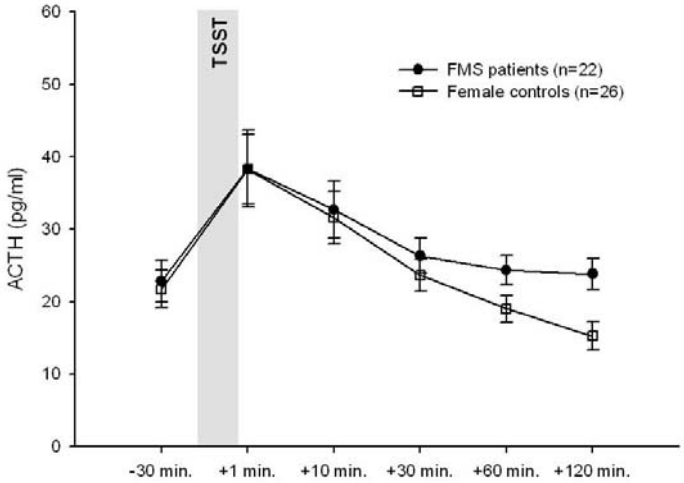

Figure 3.8: ACTH concentrations (mean ± SEM) before and after the TSST in women with FMS (n=22) and female controls (n=26).

Medication intake did not have any effect on ACTH levels. In terms of the influence of group affiliation on ACTH levels or ACTH changes during the TSST, no significant effect could be detected (*group*: $F_{1,42}$=1.46; p=0.234, *group*time interaction*: $F_{2.3,95.6}$=1.00; p=0.381; see Figure 3.8).

A comparison of ACTH levels between the four subgroups (FMS non-/responders, controls non-/responders in terms of negative/positive AUCi for salivary cortisol) revealed a significant time effect (*time*: $F_{2.5,99.8}$=7.93; p<0.001, η^2=0.17) and a significant age*time interaction (*age*time interaction*: $F_{2.5,99.8}$=4.73; p=0.007, η^2=0.11). In addition, a significant group*time interaction (*group*time interaction*: $F_{7.5,99.8}$=4.20; p<0.001, η^2=0.24) and a significant group effect (*group*: $F_{3,40}$=5.84; p=0.002, η^2=0.30) were observed. Post-hoc tests indicated that healthy non-responders differed significantly from the other three subgroups, whereas the patient non-responder group did not differ from the two responder groups (Figure 3.9A). As illustrated in Figure 3.9B, no significant group difference between the FMS responders and the healthy responders in terms of ACTH levels could be detected.

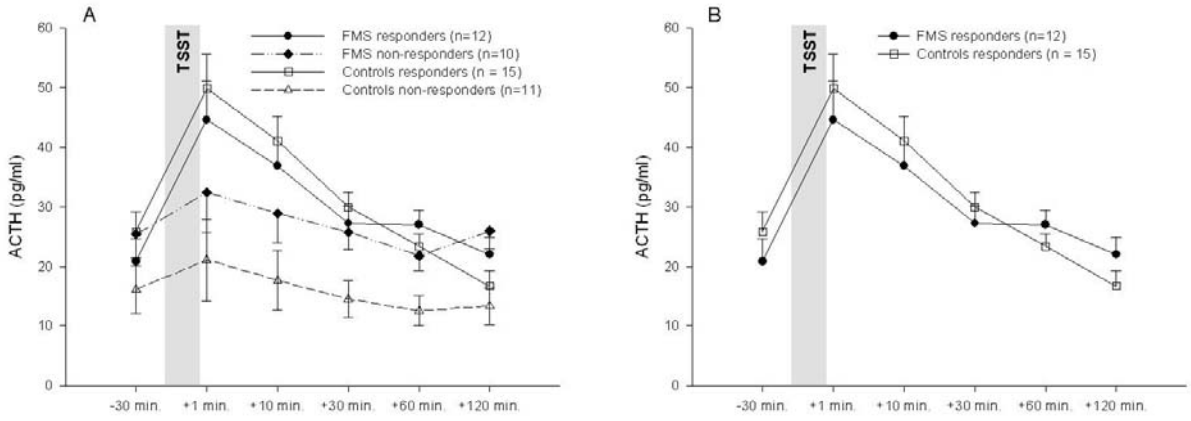

Figure 3.9: (A) ACTH levels (mean ± SEM) before and after the TSST in FMS responders (n=12), FMS non-responders (n=10), healthy responders (n=15), and healthy non-responders (n=11). (B) ACTH levels (mean ± SEM) before and after the TSST in the two responder groups.

To summarize, results on HPA axis activity in the TSST indicated a blunted adrenocortical activity in the FMS group compared to the healthy control group. In terms of salivary cortisol levels, this effect was only marginally pronounced when the patient group as a whole was compared to the group of healthy volunteers. However, a subdivision of the groups into TSST responders and non-responders based on the

AUCi for salivary cortisol allowed for the comparison of only those groups who showed a cortisol reaction to the TSST. This comparison revealed significantly lower salivary cortisol levels in the FMS responder group than in the healthy responder group.

With regard to plasma cortisol, hormone concentrations were significantly lower in the FMS group. This finding was supported by the observation that plasma cortisol levels were also significantly decreased in the FMS responder group than in the healthy responder group. No significant differences were found in ACTH responses between FMS patients and healthy volunteers with the exception that healthy non-responders had significantly lower ACTH levels then the other study participants.

3.4.2.4 Norepinephrine

It was hypothesized that hypocortisolemic FMS patients displayed a hyperactive SNS activity, which was supposed to be related to a diminished cortisol feedback on catecholamine synthesis and release. For the determination of norepinephrine, six blood samples were taken at 30 min pre-TSST as well as 1 min, 10 min, 30 min, 60 min, and 120 min post-TSST. A GLM was conducted with group (FMS patients, controls) as independent factor, norepinephrine levels as dependent factor and "age", "blood pressure medication", "estrogen intake" and "the intake of antidepressants" as covariates. As illustrated in Figure 3.10A, a significant group effect was seen (*group*: $F_{1,32}$=8.28; p=0.007, η^2=0.21). T-tests revealed significantly higher norepinephrine levels in the FMS group *30 min pre-TSST* ($t_{28.7}$ = 2.48, p=0.024), *1 min post-TSST* (t_{43} = 3.10, p=0.003), and *10 min post-TSST* (t_{41} = 2.30, p=0.027). Differences in norepinephrine levels 30 min and 60 min post-TSST were marginally significant (t_{43} = 1.84, p=0.072 and t_{43} = 1.80, p=0.079, respectively). In addition, results of the GLM indicated that norepinephrine changes in response to the TSST were significantly more pronounced in the FMS group than in the healthy control group (*group*time interaction*: $F_{3.1,99.9}$=2.75; p=0.044, η^2=0.08). This interaction effect was partly indicated by a trend towards a higher norepinephrine increase directly after the TSST ($t_{28.4}$ = 1.92, p=0.066) as demonstrated in Figure 3.10B. No significant effects of any of the covariates on norepinephrine levels were observed.

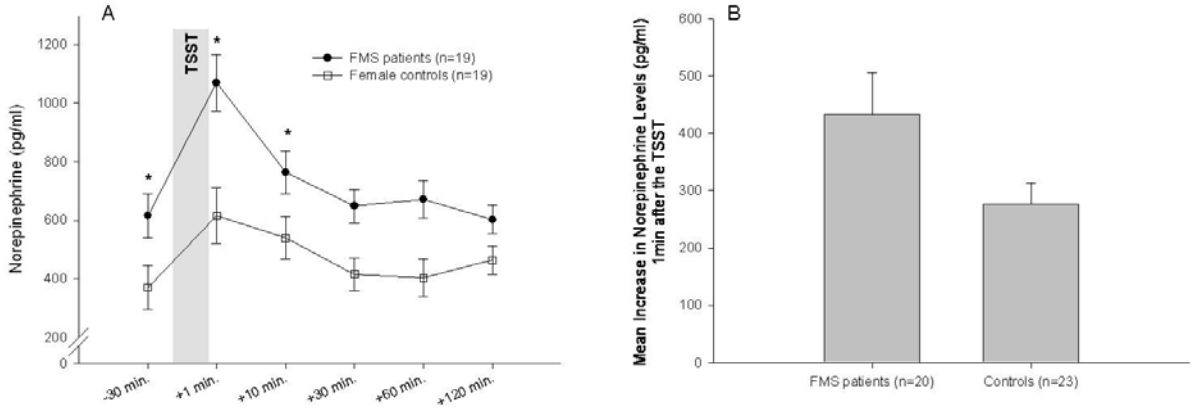

Figure 3.10: (A) Norepinephrine levels (mean ± SEM) before and after the TSST in FMS patients (n=19) and healthy volunteers (n=19). Asterisk: p<0.05. (B) Increase in norepinephrine concentrations (mean ± SEM) immediately after the TSST as compared to baseline levels 30 min before the TSST in FMS patients (n=20) and healthy controls (n=23).

3.4.2.5 Lymphocyte Subsets

Based on findings that NK cell levels are suppressed by chronic catecholamine elevations, we expected blunted NK cell concentrations in FMS patients. NK cells were analyzed in EDTA blood samples 30 min before the TSST, and 1 min and 120 min after the TSST. A GLM with group (FMS patients vs. controls) as independent factor, NK cell levels as dependent factor and the four covariates indicated a significant time effect (*time*: $F_{1.4,57.9}$=9.25; p=0.001 η^2=0.18) and lower NK cell concentrations in the FMS group (*group*: $F_{1,42}$=5.36; p=0.026 η^2=0.11). T tests revealed significantly higher NK cell levels in the control group 1 min after the TSST compared to the patient group (t_{46} = -2.47, p=0.017). No significant group*time interaction was observed (*group*time interaction*: $F_{1.4,57.9}$=2.07; p=0.150, see Figure 3.11).

Since neither age nor medication intake significantly added to the model, the same analysis was done without the covariates. In this case, the GLM revealed not only a considerable time effect (*time*: $F_{1.4,64.0}$=111.16; p<0.000 η^2=0.71) and a significant group effect (*group*: $F_{1,46}$=4.90; p=0.032 η^2=0.10) but also a significant group*time interaction (*group*time interaction*: $F_{1.4,64.0}$=4.62; p=0.024, η^2=0.09) indicating not only lower overall NK cell levels but also smaller increases in the FMS group than in the control group in response to the TSST.

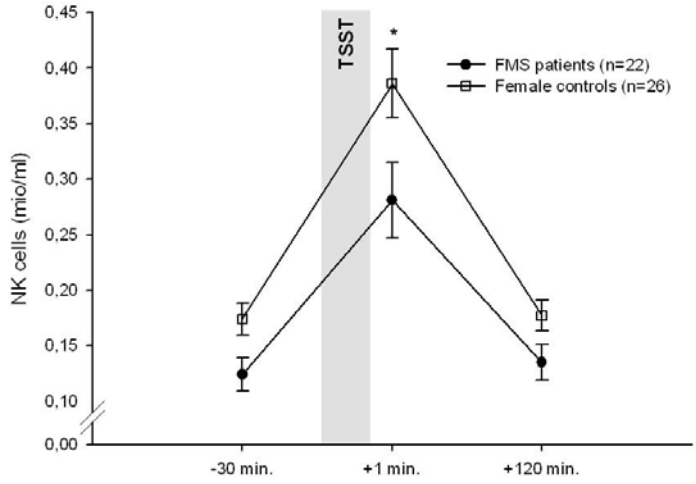

Figure 3.11: NK cell concentrations (mean ± SEM) before and after the TSST in women with FMS (n=22) and female controls (n=26). Asterisk: p<0.05.

With regard to lymphocyte, B cell, T cell, T helper cell, and T suppressor cell counts, no significant differences in the amount of cells and changes during the TSST were found between the FMS patients and the healthy volunteers (data not shown). It should be noted that “age” and “blood pressure medication” had a significant effect on T helper cell levels in terms of higher levels in elder subjects and lower levels in subjects taking blood pressure medication (*age*: $F_{1,42}$=5.91; p=0.019 η^2=0.12; *blood pressure medication*: $F_{1,42}$=5.28; p=0.027 η^2=0.11). The other immune cells were unaffected by the covariates.

3.5 Discussion

The present study shows blunted salivary and plasma cortisol levels in female FMS patients before and after the Trier Social Stress Test (TSST). These hormonal alterations were even more evident when only those patients and healthy volunteers were compared who responded to the TSST in terms of salivary cortisol increases (positive AUCi). In contrast, FMS patients did not differ from their healthy counterparts when ACTH levels were compared. These data confirm previous results from exercise and pharmacological studies that found blunted cortisol responses after an applied stress test or normal cortisol levels despite elevated ACTH responses, respectively (e.g., Calis et al., 2004; Crofford et al., 1996; Griep et al., 1993; Griep et al., 1998; Kirnap et al., 2001; Paiva et al., 2002).

Surprisingly, the non-responder rate in the TSST was unexpectedly high in the present study. Other studies have reported non-responder rates of below 30% (Kirschbaum et al., 1993; Schommer, Hellhammer & Kirschbaum, 2003). Since the non-responder rate in our study was above 40% even in the healthy volunteer group, we decided to build subgroups based on the AUCi for salivary cortisol. In addition to differences in hormonal responses to the TSST, we also compared demographic and psychometric parameters between the responder and non-responder groups. However, despite the expected findings that the FMS group as a whole had higher pain, fatigue, sleep disturbance, depression, and anxiety scores than the healthy volunteers, we were unable to find differences in psychometric variables between FMS responders and FMS non-responders, or between healthy responders and healthy non-responders, respectively. Therefore, none of the assessed parameters such as years since onset of the disorder (with regard to the FMS patients), amount of pain intensity, depression, or fatigue seemed to play a role in the differentiation between responders and non-responders. In accordance with results from a study investigating HPA and SNS reactivity in response to the TSST in healthy subjects (Schommer et al., 2003), no differences in the levels of norepinephrine were found between FMS responders and FMS non-responders, or between healthy responders and healthy non-responders, implying higher norepinephrine concentrations in both patient subgroups compared to the healthy subjects (data not shown).

Several reasons might apply to the higher non-responder rate found in our study. Firstly, the broad age range (34-67 yrs.) in the study sample must be taken into consideration. It is possible that elder subjects perceived the TSST less stressful than younger subjects because the first task consisting of a job interview did not apply as much to their life as to the life of younger subjects. In support of this assumption, we found lower AUCi in the elder subjects compared to the younger ones. Secondly, HPA axis activity follows a circadian rhythm characterized by highest hormone levels in the early morning hours and continuously decreasing levels over the day. In the present study, the TSST was conducted in the morning hours between 10.00 a.m. and 11.00 a.m. Since pre-stress cortisol levels were higher than in studies where the TSST is conducted in the afternoon hours, the stressor might have been not effective enough to elicit further cortisol increases. However, there are at least two reasons that contradict this assumption: Firstly, it was shown in a recent meta analysis that the TSST is most effective in eliciting HPA responses (Dickerson & Kemeny, 2004).

Since in our study the TSST was conducted by trained staff members, a reliable increase in HPA axis activity would have been expected in a higher percentage of study participants. Secondly, one study could show that ACTH, total plasma cortisol, and salivary cortisol stress responses to the TSST were comparable when the stress test was performed between 09.45 a.m. and 07.00 p.m. (Kudielka, Schommer, Hellhammer & Kirschbaum, 2004). Thus, it seems most unlikely that elevated pre-stress cortisol levels and/or an inappropriate choice of stressor were the reason for the observed high non-responder rate. We conclude that the factor “age” is most likely the main reason for the high non-responder rate in our study, which is confirmed by the trend towards age differences between the responder and non-responder groups as illustrated in Table 3.3.

In accordance with the observation that cortisol exerts negative feedback on catecholamine synthesis and release elevated norepinephrine levels were found in the FMS patients. This result might be explained by a reduced cortisol feedback capacity in the patients due to the observed mild hypocortisolism. To our knowledge, only three studies have looked at stress-induced changes in both cortisol and norepinephrine concentrations in FMS patients. In these studies, different stressors have been used such as exercise (van Denderen et al., 1992), hypoglycemia (Adler et al., 1999), and IL-6 administration (Torpy et al., 2000). While van Denderen and colleagues reported both lower cortisol and norepinephrine responses to exercise in FMS patients, Adler and coworkers found similar changes in cortisol and norepinephrine in FMS patients and healthy controls to hypoglycemia. In contrast, Torpy and colleagues observed no differences in plasma cortisol changes after IL-6 injection between the patient and the control group but strong norepinephrine increases in the patients, whereas catecholamine levels did not change significantly in the healthy control group. In the present study, a psychosocial stress test was applied, and the results confirm those of Torpy and colleagues in terms of exaggerated norepinephrine release. However, while we found at the same time blunted cortisol responses in the FMS patients, Torpy and colleagues reported no differences in cortisol levels between subjects with and without FMS. Interestingly, an exaggerated SNS activity after stressor exposure has been observed in other stress-related disorders with hypocortisolemic features such as PTSD. For example, in one study higher plasma catecholamine levels have been found in war veterans with PTSD during experimental exposure to combat sounds compared to veterans without

PTSD (Liberzon, Abelson, Flagel, Raz & Young, 1999). Another study reported higher levels of norepinephrine metabolites in police academy recruits with a history of childhood trauma after exposure to a video depicting officers confronted with highly stressful incidents (Otte, Neylan, Pole, Metzler, Best, Henn-Haase, Yehuda et al., 2005). Based on these results we conclude that further studies are needed in FMS patients to confirm our hypothesis that hypocortisolemic stress responses go along with increased SNS activity in this patient group.

While acute catecholamine increases lead to a quick and transient elevation of circulating NK cell levels, permanent SNS hyperactivity results in suppressed NK cell numbers (Maisel et al., 1990b). Even though we are aware that we cannot draw conclusions about the chronicity of SNS alterations from the present study results, we nevertheless found elevated norepinephrine levels at baseline as well as higher increases in response to the TSST in the FMS patients compared to the healthy controls. In accordance with the assumption of permanent basal SNS hyperactivity in FMS patients (Martinez-Lavin, 2004), increased norepinephrine levels in our study were accompanied by lower mean NK cell numbers in the FMS group, supporting our hypothesis of an association between SNS disturbances and immune system abnormalities. In CFS patients, a reduced number and cytotoxic activity of NK cells have also been observed in some studies (Masuda, Nozoe, Matsuyama & Tanaka, 1994; Masuda, Munemoto, Yamanaka, Takei & Tei, 2002; Ojo-Amaize, Conley & Peter, 1994; Zhang, Zhou, Denny, Ottenweller, Lange, LaManca, Lavietes et al., 1999). Given the high symptom overlap between FMS and CFS (Clauw & Chrousos, 1997) approved by high fatigue scores in the present patient group, our findings confirm reports on disturbed innate immune system activity in stress-related pain and fatigue syndromes. It should be noted that the assumption of changes in early immune responses are limited to NK cell numbers in our study because further immune cell counts were not affected in the FMS patients.

Another limitation of this study is the fact that we did not include male FMS patients into the study. About 90% of FMS patients are women making it difficult for us to assemble a group of affected men that was large enough for statistical analyses. Therefore, our findings might not generalize to male patients.

Finally, we allowed for the intake of several substances such as estrogens and blood pressure medication. In addition, some patients took antidepressants in the forefront of the study, which had in most cases been used as pain relievers, but had to be

discontinued at least two weeks before entry into the study. Due to very strict inclusion criteria including the absence of concomitant autoimmune disorders, we had to exclude at least 50 patients who were interested to participate in our study. In order to avoid further patient loss, we accepted subjects taking the above mentioned substances and entered them as covariates into all GLMs. Despite this conservative strategy, differences in HPA, SNS and immune system activities were still detected between the FMS and the control groups that were demonstrably independent from medication intake.

In sum, we have provided evidence for a blunted adrenal activity in FMS patients accompanied by exaggerated SNS activity and reduced NK cell activity. The present study is the first one in FMS patients investigating changes in three different but strongly interacting bodily systems. Interestingly, based on basic principles of the interplay between the HPA axis, the SNS and the immune system, we have found changes in one system that seem to be associated to changes in other systems such as disinhibited catecholamine release that might be due to a lack of cortisol feedback, and blunted NK cell levels potentially related to chronic SNS input. However, one should be aware that this suggestion should be interpreted with caution because our study results do not allow for the setting up of causalities but only for the note that at least three biological systems are simultaneously affected in FMS patients. Future studies are needed that concentrate on altered biological interactions in order to gain a more consistent insight into the mechanisms that underlie pain and fatigue symptoms such as fibromyalgia.

CHAPTER 4

4 Macrophage Migration Inhibitory Factor in the TSST: Comparison between Fibromyalgia Patients and Healthy Subjects

4.1 Abstract

Background: Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine with distinctive features. Unlike other pro-inflammatory cytokines, MIF acts as a potent physiological counter-regulator of the anti-inflammatory effects of glucocorticoids and is enhanced rather than suppressed by physiological glucocorticoid dosages. MIF is considered a critical mediator of a number of diseases including septic shock, cancer, atopic dermatitis, atherosclerosis, rheumatoid arthritis, and obesity. So far, no study has looked at MIF levels in stress-related disorders that are characterized by blunted cortisol levels, such as fibromyalgia, chronic fatigue syndrome, and post-traumatic stress disorder. The aim of the present study was to investigate MIF responses to a psychosocial stress test in fibromyalgia patients and healthy subjects and to find out if blunted cortisol levels as repeatedly observed in these patients are accompanied by changes in the cortisol/MIF ratio in terms of a comparatively higher inflammatory activity.

Methods: The study sample consisted of 24 female fibromyalgia patients and 26 healthy, age-matched women. For the determination of cortisol and MIF, blood was taken 30 minutes before, as well as 1 min and 30 min after a psychosocial stressor, the Trier Social Stress Test (TSST). Demographic characteristics and body mass index (BMI) were assessed by a questionnaire and during a comprehensive medical examination.

Results: The TSST caused significant increases in MIF levels in the whole study sample. As expected, MIF levels were higher in overweight/obese subjects than in normal-weight subjects. A comparison between FMS patients and healthy controls revealed blunted cortisol levels in the patient group supporting the assumption of a mild hypocortisolism in this disorder. While no group differences could be detected in total MIF levels, MIF increases after the stress test as compared to baseline were significantly higher in the patients. In addition, the cortisol/MIF ratio tended to be decreased in the FMS group, which was especially apparent post-TSST.

Conclusion: The TSST successfully elicited a MIF response in our study subjects. Higher stress-induced increases of MIF in relation to baseline levels and a slightly decreased cortisol/MIF ratio in the patient group especially after the TSST indicate a higher stress-induced inflammatory activity in FMS patients, which might result in worsening of symptoms and the induction of sickness behavior via activation of other

pro-inflammatory cytokines. Further studies are needed that extend the post-stress monitoring period in order to be able to confirm this assumption.

4.2 Introduction

Stress-related disorders such as the fibromyalgia syndrome (FMS), chronic fatigue syndrome (CFS), and post-traumatic stress disorder (PTSD) have been associated with disturbed hypothalamus-pituitary-adrenal (HPA) axis responses to stress. These disorders have in common that they often start in the aftermath of a prolonged period of stress and are triggered by injuries, infection, or traumatic experiences. It has been suggested that the onset of the disease might be facilitated by a shift from chronic hyperfunction to hypofunction of the HPA axis, which might result in an inability to mount an adequate stress response and, eventually, might cause long-term alterations in stress-regulating and immune mechanisms (Fries et al., 2005; Heim et al., 2000; Van Houdenhove & Egle, 2004). Since cortisol is a well-known inhibitor of pro-inflammatory cytokine production, several studies have been conducted that investigated cytokine alterations in stress-related disorders. In accordance with the finding of blunted cortisol activity, some studies have reported elevated pro-inflammatory cytokine levels in FMS, CFS and PTSD patients (Gupta, Aggarwal, See & Starr, 1997; Hader et al., 1991; Maes, Lin, Delmeire, Van Gastel, Kenis, De Jongh & Bosmans, 1999; Moss, Mercandetti & Vojdani, 1999; Rohleder et al., 2004; Wallace et al., 2001), whereas other studies have been unable to find cytokine alterations in these patients when compared to healthy subjects (Amel Kashipaz et al., 2003; Lloyd, Gandevia, Brockman, Hales & Wakefield, 1994).

So far, one pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF), that has very distinctive features and differs from other pro-inflammatory cytokines in several aspects has found little to no consideration in the context of endocrine-immune interactions in stress-related disorders. MIF is one of the first identified pro-inflammatory cytokines. It was originally discovered as a protein factor produced by sensitized lymphocytes, preventing the random migration of macrophages and collecting them at inflammatory sites (Bloom & Bennett, 1966; David, 1966). Beside its role in the regulation of macrophage function, MIF is able to induce the production of several pro-inflammatory molecules including nitric oxide, cyclooxygenase 2 (COX2), prostaglandin E_2 (PGE_2), and the release of other pro-inflammatory

cytokines such as tumor necrosis factor (TNF)-α, which, in terms of a positive feedback loop, is itself able to induce MIF secretion (Bernhagen, Mitchell, Calandra, Voelter, Cerami & Bucala, 1994; Calandra, Bernhagen, Mitchell & Bucala, 1994; Calandra & Roger, 2003). Another important function of MIF is the upregulation of Toll-like receptor (TLR-) 4 thus facilitating the detection of endotoxin-containing bacteria and enabling cells of the innate immune system to rapidly respond to invasive gram-negative bacteria (Roger, David, Glauser & Calandra, 2001; Roger, Froidevaux, Martin & Calandra, 2003).

For almost 30 years, MIF was thought to be a T cell cytokine released upon lymphocyte-specific stimulus. Nowadays, additional immune cells have been identified that express MIF such as monocytes, macrophages, blood dendritic cells, B cells, mast cells, and granulocytes (reviewed by Calandra & Roger, 2003). In addition, MIF is widely expressed by several body tissues including the lungs, the skin, the gastrointestinal and genitourinary tract, the eye, the liver, the kidney, the bone, the joints, and the brain (Calandra & Roger, 2003). Importantly, MIF can also be found in several tissues of the endocrine system, especially in organs that are involved in stress responses such as the hypothalamus, pituitary and adrenal glands (Bacher, Meinhardt, Lan, Mu, Metz, Chesney, Calandra et al., 1997; Bacher, Meinhardt, Lan, Dhabhar, Mu, Metz, Chesney et al., 1998; Bernhagen, Calandra, Mitchell, Martin, Tracey, Voelter, Manogue et al., 1993; Fingerle-Rowson, Koch, Bikoff, Lin, Metz, Dhabhar, Meinhardt et al., 2003). For example, Bernhagen and colleagues observed in mice that MIF was released, similar to a hormone, by cells of the anterior pituitary gland after exposure to lipopolysaccharide (LPS) (Bernhagen et al., 1993). Interestingly, MIF seems to co-localize in the same population of secretory granules as adrenocorticotrophic hormone (ACTH). Stimulation of murine corticotrophic cells with corticotrophin releasing hormone (CRH) results in the release of MIF at concentrations lower than that required for ACTH secretion (Nishino, Bernhagen, Shiiki, Calandra, Dohi & Bucala, 1995). These findings indicate that MIF might play a role in the stress response to infection and to other stimuli activating the HPA axis and that the cytokine could be an important mediator linking the endocrine and the immune systems.

In support of this assumption, MIF has been shown to act as a potent physiological counter-regulator of the anti-inflammatory and immunosuppressive effects of glucocorticoids (Bucala, 1996), and MIF levels just as glucocorticoid levels are

increased during inflammation, infection, and stress (Beishuizen, Thijs, Haanen & Vermes, 2001; Calandra, Bernhagen, Metz, Spiegel, Bacher, Donnelly, Cerami et al., 1995). Calandra and colleagues (1995) demonstrated that low levels of glucocorticoids were able to induce MIF release from macrophages. MIF in turn counterbalanced the immunosuppressive effects of glucocorticoids by overriding glucocorticoid-mediated inhibition of cytokine secretion by LPS-stimulated monocytes.

Recent studies have provided evidence that MIF is able to counterbalance glucocorticoid effects by affecting both transcriptional and post-transcriptional regulation of cytokine gene expression. On the transcriptional level, Daun and Cannon (2000) demonstrated that MIF exerts its overriding effects on anti-inflammatory glucocorticoid activity by inhibiting glucocorticoid-induced up-regulation of IκBα expression. As a result, the glucocorticoid-induced suppression of nuclear factor (NF)-κB translocation to the nucleus and activation of transcription of inflammatory markers is prevented. With regard to the post-transcriptional regulation of cytokine production, Roger and colleagues (2005) have lately identified mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) as a major target of MIF-glucocorticoid crosstalk. MKP-1 is able to dephosphorylate and hence inactivate members of the MAPK family that are involved in the biosynthesis of pro-inflammatory mediators (English, Pearson, Wilsbacher, Swantek, Karandikar, Xu & Cobb, 1999). It has been shown that MKP-1 is induced by glucocorticoids, consequently resulting in negative regulation of the MAPK signal transduction pathways (Clark, 2003). Roger and colleagues (2005) demonstrated that MIF is able to override the glucocorticoid-induced MKP-1 up-regulation in a dose-dependent manner thus counter-regulating the anti-inflammatory effects of glucocorticoids at post-transcriptional level.

MIF differs from other pro-inflammatory cytokines in several aspects. For example, MIF is constitutively expressed by endocrine and immune cells, implying that *de novo* protein synthesis is not required before secretion (Calandra & Roger, 2003). Mean circulating MIF concentrations usually range between 2 and 6 ng/ml, whereas the concentration of most other cytokines in the blood is 500- to 1000-fold lower. Interestingly, MIF also differs from other pro-inflammatory cytokines in the circadian rhythmicity. Studies using LPS-stimulated whole blood have shown that cytokines such as interferon (IFN)-γ, TNF-α, interleukin (IL)-1, and IL-12 exhibit diurnal rhythms

with peaks between 9 pm and 3 am, the time of the nadir of plasma cortisol, and lowest levels in the morning hours when plasma cortisol levels are highest, indicating an inverse relationship between the circadian rhythms of these pro-inflammatory cytokines and cortisol (Petrovsky & Harrison, 1998; Petrovsky, McNair & Harrison, 1998). In contrast to these findings, Petrovsky and colleagues showed in another study that mean plasma MIF levels peaked at 8 am and reached a nadir at 3 am pointing toward a close temporal relationship between the rhythms of MIF and cortisol (Petrovsky, Socha, Silva, Grossman, Metz & Bucala, 2003). In the same study, an oral dose of 25 mg cortisone acetate resulted in a significant suppression of LPS-stimulated whole blood IFN-γ, TNF-α, and IL-12 production, whereas plasma MIF levels increased in response to the low-dose glucocorticoid stimulation. Interestingly, dexamethasone in pharmacological dosages resulted in a reduction of plasma MIF levels suggesting a bimodal sensitivity of MIF to glucocorticoids depending whether they are at physiological or pharmacological dosages (Petrovsky et al., 2003).

MIF has been identified as a critical mediator of a number of diseases such as septic shock, delayed-type hypersensitivity, inflammatory lung diseases, atopic dermatitis, cancer, diabetes, ulcerative colitis, Crohn's disease, atherosclerosis, multiple sclerosis, and rheumatoid arthritis (for comprehensive reviews see Calandra & Roger, 2003; Lue et al., 2002; Ohkawara et al., 2005). Elevated MIF concentrations have also been reported in obese subjects in terms of plasma protein and mRNA levels (Dandona, Aljada, Ghanim, Mohanty, Tripathy, Hofmeyer & Chaudhuri, 2004). A recent study reported that human adipocytes were able to produce MIF indicating that MIF might be an obesity-dependent mediator of macrophage infiltration of adipose tissue (Skurk, Herder, Kraft, Muller-Scholze, Hauner & Kolb, 2005). Interestingly, participation of obese subjects displaying elevated MIF levels in physical activity and a weight-loss program resulted in a significant reduction of MIF levels (Church, Willis, Priest, Lamonte, Earnest, Wilkinson, Wilson et al., 2005).

To our knowledge, no study has looked at MIF levels in stress-related disorders with hypocortisolemic features, such as FMS, CFS, and PTSD. FMS is a distinct rheumatologic disorder characterized by chronic wide-spread pain for at least three months duration and pain at 11 of 18 defined tender points (Wolfe et al., 1990). FMS patients frequently suffer from additional symptoms such as sleep disturbances, fatigue, depression, headaches, concentration difficulties, irritable bowel, and obesity

(Thompson et al., 2003). As described in more detail in Chapter 3 (Section 3.2), some studies have reported blunted basal and stress-induced HPA activity in FMS patients primarily on adrenal level as indicated by reduced cortisol concentrations (e.g., Calis et al., 2004; Griep et al., 1998; Gur et al., 2004a; Gur et al., 2004b; Kirnap et al., 2001; Lentjes et al., 1997; Paiva et al., 2002).

In the present study we investigated whether differences in cortisol levels between female FMS patients and a group of healthy, age-matched women in the Trier Social Stress Test (TSST, Kirschbaum et al., 1993) were reflected on immunological level in terms of an altered MIF reactivity. We hypothesized that the cortisol/MIF ratio was decreased in the FMS group, which might serve as a new indicator for increased pro-inflammatory activity in stress-related disorders.

4.3 Materials and methods

4.3.1 Study participants

Twenty-four female patients (mean age: 49.7 ± 9.2 years) who fulfilled the ACR 1990 Criteria for the Classification of Fibromyalgia (Wolfe et al., 1990) - as diagnosed by a rheumatologist – were recruited via outpatient and specialist clinics, patient self-help groups, and newspaper advertisements. Patients were included into the study if they reported non-inflammatory origins of the pain, were free of medical diseases such as gastrointestinal, neurological or autoimmune disorders, as well as free of psychotic or eating disorders, were not reporting presence of substance abuse or dependency, and refrained from potential antidepressant medication at least two weeks before study entry.

The healthy control group comprised 26 healthy, age-matched women (50.5 ± 8.4 years) who were recruited via newspaper advertisements or were provided from the environment of participating FMS patients. They were allowed participation if they were free of antidepressant/antipsychotic drugs, medical diseases, and mental disorders. Pregnancy, current breast feeding, hormonal medication (exception: oral contraceptives, hormone replacement therapy) and dietary weight loss of 5 kg or more within 6 weeks before study entry were regarded as further exclusion criteria for both patients and healthy volunteers. All participants were evaluated with the German Version of the Structured Clinical Interview for DSM-IV (Wittchen et al., 1997) and underwent a comprehensive medical examination for past and current health

problems. Demographic characteristics such as age, marital status, and job situation were assessed by a demographic questionnaire. All subjects gave written informed consent after they were given a detailed description of the study. The study protocol was approved by the Local Ethics Committee.

4.3.2 Experimental protocol

Laboratory sessions were carried out in the morning and began between 8.00 and 8.30 am with the medical examination and the clinical interview. A catheter was then inserted into the antecubital vein. After a 45-min resting period, blood was taken for the determination of baseline plasma cortisol and serum MIF levels. Thirty minutes later, the subjects were exposed to the Trier Social Stress Test (TSST, Kirschbaum et al., 1993). In brief, the participant was guided into a room where she faced a committee consisting of one male and one female staff member dressed in white coats. The participant was told to imagine that she had to apply for a job she most desired. The committee was introduced as the personnel board having to decide on the employment of the participant and being trained in behavioral analysis. The participant was given a 3-minute preparation period for the job interview at a table in the same room, then she was asked to approach a microphone in front of the committee and deliver a 5-minute free speech on personality traits that would qualify her for the job. In case of a break of more than 20 seconds during that task, one member of the committee encouraged the participant to continue or asked standardized questions. Then a second task was introduced consisting of 5 minutes of serial subtraction in steps of 17 beginning with the number 2023. The participant was told to calculate as correctly and quickly as possible. With every mistake made, the participant had to start again at number 2023. During both tasks, a video camera and a tape recorder were running, and the committee took notes, all of which the participant was made believe was analyzed after the test. However, during a comprehensive debriefing at the end of the experiment, it was clarified that no written or recorded information would be reviewed.

Additional plasma and serum blood samples for the determination of cortisol and MIF levels were taken 1 min and 30 min after the TSST.

4.3.3 Laboratory assays

Serum blood for the determination of MIF and plasma for the determination of cortisol were drawn 30 min pre-TSST as well as 1 min and 30 min post-TSST. Total cortisol was assayed by using a commercially available enzyme-linked immunosorbent assay (ELISA; Immuno-Biological Laboratories, IBL, Hamburg, Germany) with an intra-assay coefficient of variation between 5.6% and 8.1% and an inter-assay coefficient of variation between 6.5% and 7.7%.

MIF was measured using an enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Wiesbaden, Germany). The limit of detection of this assay was 0.017 ng/ml; intra- and inter-assay coefficients of variation ranged between 3.8% and 6.6% and between 5.0% and 9.1%, respectively.

4.3.4 Statistical analyses

The FMS and the healthy control groups were compared on demographic characteristics using t tests and chi square tests as appropriate. For the investigation of changes in the course of cortisol levels, MIF concentrations and the cortisol/MIF ratio, general linear models (GLMs) with repeated measures were conducted, with Greenhouse-Geisser or Huynh-Feldt corrections of degrees of freedom appropriately applied where the sphericity assumption was violated. Cortisol and MIF data was available at the following time points: baseline (30 min pre-stress), 1 min post-stress and 30 min post-stress. BMI was entered as a covariate into the GLMs due to the reported positive relationship between MIF levels and BMI. In addition, changes in MIF between baseline and 1 min and 30 min post-TSST were calculated and compared between the FMS and healthy control groups by performing another GLM with repeated measures. All analyses were carried out using SPSS V 13.0.

4.4 Results

4.4.1 Participant information

A total of 24 female FMS patients (mean age: 49.7 ± 9.2 years) and 26 healthy women (50.5 ± 8.4 years) participated in the study. As shown in Table 4.1, there were no significant differences in the number of women using blood pressure medication or oral contraceptives/postmenopausal hormone substitution between the

two groups. Ten of the FMS subjects were on antidepressants which they agreed to discontinue at least two weeks before study entry. The number of years since diagnosis varied between 1 and 10 years in the FMS group, the presence of pain symptoms between 1.5 and 36 years. All subjects were non-smokers.

Table 4.1: Demographic characteristics of FMS patients and healthy volunteers

Variable	FMS patients (n=24)	Controls (n=26)	Statistics
Demographics			
Age, years *(mean, SD)*	49.7 (9.2)	50.5 (8.4)	t(48) = -0.349, p = 0.728
Married *(n, %)*	15 (62.5%)	20 (76.9%)	Fisher's exact: p = 0.358
Full- or part-time job *(n, %)*	12 (50.0%)	16 (61.5%)	Fisher's exact: p = 0.569
BMI *(mean, SD)*[a]	27.3 (6.0)	25.2 (4.2)	t(48) = 1.446, p = 0.155
Blood pressure medication *(n, %)*	5 (20.8%)	6 (23.1%)	Fisher's exact: p = 0.560
Intake of oral contraceptives, hormone replacement *(n, %)*	6 (25.0%)	10 (38.5%)	Fisher's exact: p = 0.372
FMS patient characteristics			
Antidepressants *(n, %)*[b]	10 (41.7%)	-	
Number of years since diagnosed *(mean, range)*	4.3 (1-10)	-	
Number of years since first appearance of symptoms *(mean, range)*	14.9 (1.5-36)	-	

[a] *BMI: Body Mass Index (body weight [kg]/height [m]²)*
[b] *patients refrained from antidepressants - in most cases used as pain relievers - at least two weeks before study entry*

4.4.2 MIF responses to the TSST in the whole study group and its relation to BMI

While the TSST has been shown to most reliably induce HPA axis responses (Dickerson & Kemeny, 2004), no study has been conducted investigating changes in MIF concentrations in response to the TSST. Therefore, we first analysed MIF levels 30 min before and 1 min and 30 min after the TSST in the whole study group in order to find out if the psychosocial stress test was efficient in inducing changes in the level of the pro-inflammatory cytokine. A GLM with repeated measures (3 samples)

revealed a significant time effect (*time:* $F_{2,98}$=22.66, p<.001, η^2=0.32) indicating a pronounced increase of MIF levels after the TSST (Figure 4.1). When looked at the FMS group and at the healthy control group separately, this effect was significant in both groups (data not shown).

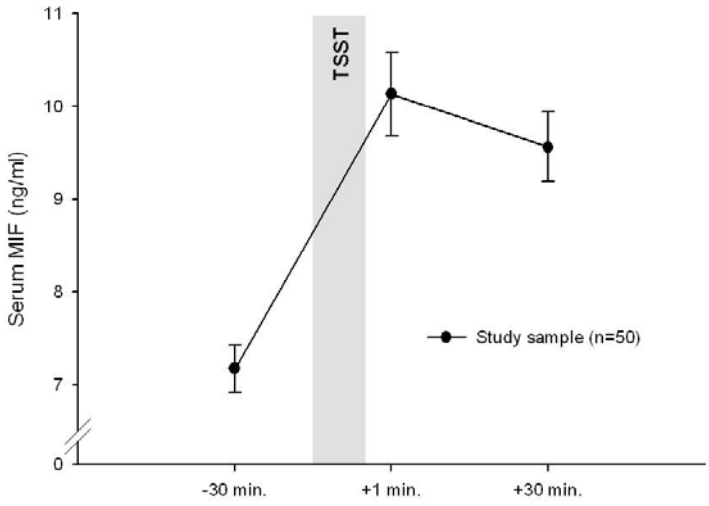

Figure 4.1: Mean serum MIF concentrations 30 min before, and 1 min and 30 min after the TSST in the study sample (n=50). A significant time effect was found (p<.001) indicating a substantial increase in MIF levels in response to the TSST. Bars indicate standard errors.

Since MIF has been reported to be elevated in obese subjects, study participants were categorized according to their BMI: 24 subjects were classified as normal-weight (BMI < 25), 20 were overweight (BMI 25-30), 6 were obese (BMI > 30). In order to avoid unequal group sizes, the overweight and obese subjects were combined in one group. Another GLM was then conducted with group (normal weight group, overweight/obese group) as independent variable. Again, a significant time effect could be observed (*time:* $F_{2,96}$=22.11, p<.001, η^2=0.32). In addition, as illustrated in Figure 4.2, a significant group effect was found demonstrating higher MIF levels in the overweight/obese group (*group:* $F_{1,48}$=7.70, p=.008, η^2=0.14), while no significant group*time interaction was detected (*group*time:* $F_{2,96}$=0.03, p=.97). These findings support previous studies that have reported a positive correlation between MIF concentrations and BMI. In all further analyses, BMI was therefore entered as a covariate into the model.

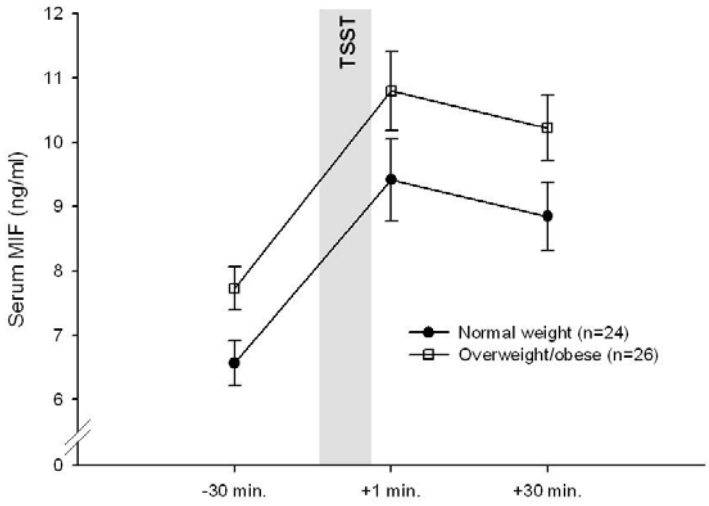

Figure 4.2: Comparison of MIF levels before and after the TSST between normal weight (n=24) and overweight/obese subjects (n=26). Higher MIF concentrations were found in the overweight/obese group (p<.01). Bars indicate standard errors.

4.4.3 Differences in MIF and cortisol responses between the FMS and the healthy control groups

Serum and plasma samples for the determination of MIF and cortisol, respectively, were taken from the participants 30 min before as well as 1 min and 30 min after the TSST. A repeated-measures GLM with MIF levels as dependent variable failed to reveal significant differences in MIF concentrations or in TSST-induced changes of the pro-inflammatory cytokine between FMS patients and healthy controls (*group:* $F_{1,47}$=0.81, p=.37; *group*time:* $F_{2,94}$=2.13, p=.12). Similar to the above reported results, MIF levels were influenced by BMI scores indicating higher MIF concentrations in overweight and obese subjects (*BMI:* $F_{1,47}$=5.37, p=.03, η^2=0.10). The BMI-adjusted course of MIF in the patient and the control groups during the TSST is shown in Figure 4.3A.

Since we were interested not only in MIF levels and changes in the TSST but also in the amount of MIF increases after the stress test, we calculated net increases of MIF 1 min and 30 min post-TSST from baseline. An additional GLM with repeated measures was then conducted analyzing the MIF increase in the two study groups for each of the two time points after the TSST compared to baseline. Results revealed a significant group effect (*group:* $F_{1,47}$=4.97, p=.03, η^2=0.10) that was due to a significantly higher net increase in the FMS group *1 min after the TSST* ($t_{34.4}$ = 2.12, p=0.04) and a trend towards elevated levels *30 min after the TSST* (t_{48} = 1.92,

p=0.06, Figure 4.3B). BMI did not significantly affect net increases of MIF after the TSST compared to baseline (*BMI:* $F_{1,47}$=0.56, p=.46).

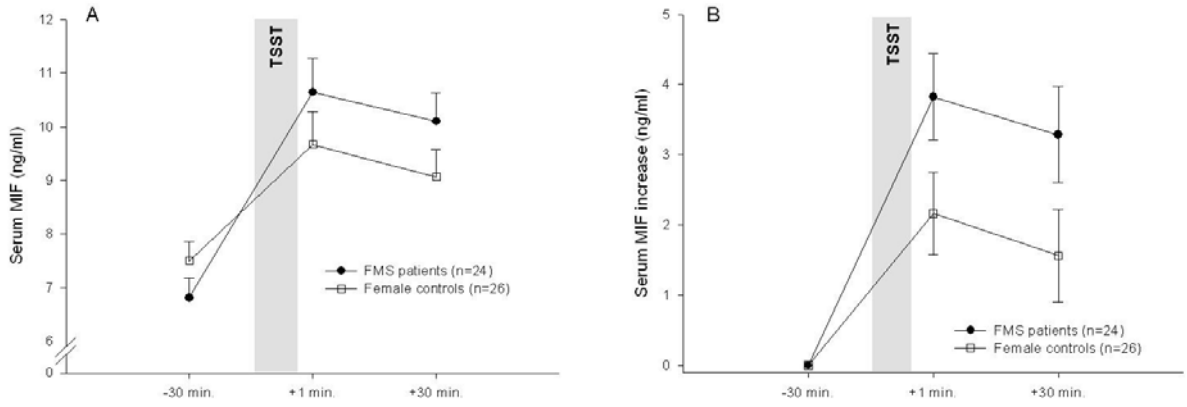

Figure 4.3: Comparison of serum MIF levels (A) and serum MIF increases (B) in the TSST between female FMS patients (n=24) and healthy women (n=26). While no statistically significant differences were found in serum MIF levels between the FMS group and the healthy control group (A), MIF increases after the TSST as compared to baseline levels were significantly higher in the patient group (p<.05) (B). Bars indicate standard errors.

With regard to cortisol analyses, data was available from 21 FMS patients and 25 healthy subjects. One patient and one healthy subject were excluded because their cortisol levels at all time points were at least 2.6 standard deviations (range: 2.62 SD to 3.73 SD) above the mean of the respective values of the two groups. In addition, two plasma samples of two patients were missing in each case resulting in their exclusion from cortisol analyses. A repeated-measures GLM with group (FMS patients, healthy volunteers) as dependent variable and BMI as covariate indicated significant differences in cortisol levels between the FMS group and the healthy control group (*group:* $F_{1,43}$=3.97, p=.05, η^2=0.09). T-test comparisons revealed significantly lower plasma cortisol concentrations in FMS patients *30 min pre-TSST* (t_{46} = -2.067, p=0.04) and *30 min post-TSST* (t_{46} = -2.61, p=0.012). BMI scores only marginally affected cortisol levels in the stress test (*BMI:* $F_{1,43}$=3.10, p=.09, η^2=0.07). No interaction effect between group and time or between BMI and time could be observed. Covariate-adjusted results are presented in Figure 4.4.

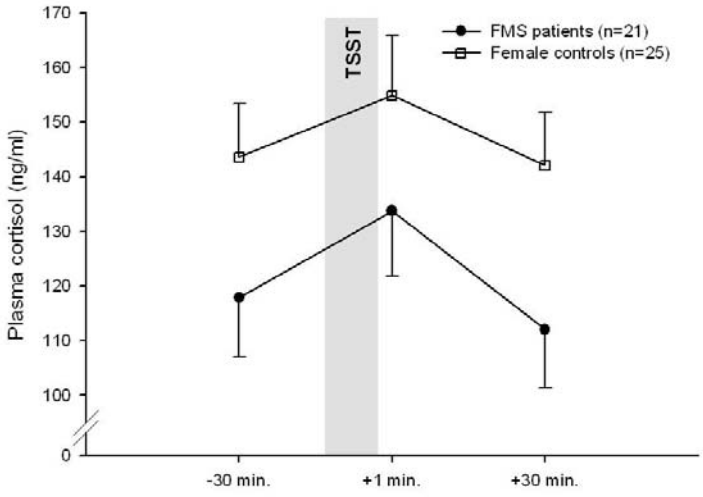

Figure 4.4: Plasma cortisol concentrations before and after the TSST in women with FMS (n=21) and female controls (n=25). Women with FMS had significantly lower cortisol levels than healthy women (p=.05). Bars indicate standard errors.

4.4.4 Cortisol/MIF ratio

It is well-known that MIF is a strong counter-regulator of cortisol activity. However, little is known about potential changes in the cortisol/MIF ratio during an applied psychosocial stressor. We therefore calculated the ratio of cortisol and MIF 30 min pre-TSST as well as 1 min and 30 min post-TSST and investigated its course during the stress test. A GLM with repeated measures and group (FMS patients, healthy volunteers) as independent variable resulted in a significant time effect (*time:* $F_{1.8,79.4}$=9.14, p<.001, η^2=0.17) indicating decreasing cortisol/MIF ratios after the TSST. Both the 1 min and 30 min post-TSST cortisol/MIF ratio were significantly lower than the cortisol/MIF ratio at baseline, as shown in post-hoc pair-wise comparisons. In addition, FMS patients had significantly lower cortisol/MIF ratios than their healthy counterparts (*group:* $F_{1,44}$=4.39, p=.04, η^2=0.09). T-tests comparing the cortisol/MIF ratio at baseline and the mean ratio after the stress test revealed a trend towards a significant difference between the FMS patients and healthy controls at baseline ($t_{40.5}$ = -1.7, p=0.097) and a significantly lower mean cortisol/MIF ratio after the TSST (t_{44} = -2.0, p=0.05). Results are presented in Figures 4.5A and 4.5B. When BMI was entered as a covariate into the GLM, it significantly affected cortisol/MIF ratios (*BMI:* $F_{1,43}$=4.05, p=.05, η^2=0.09), while the group effect remained marginally significant (*group:* $F_{1,43}$=3.18, p=.08, η^2=0.07).

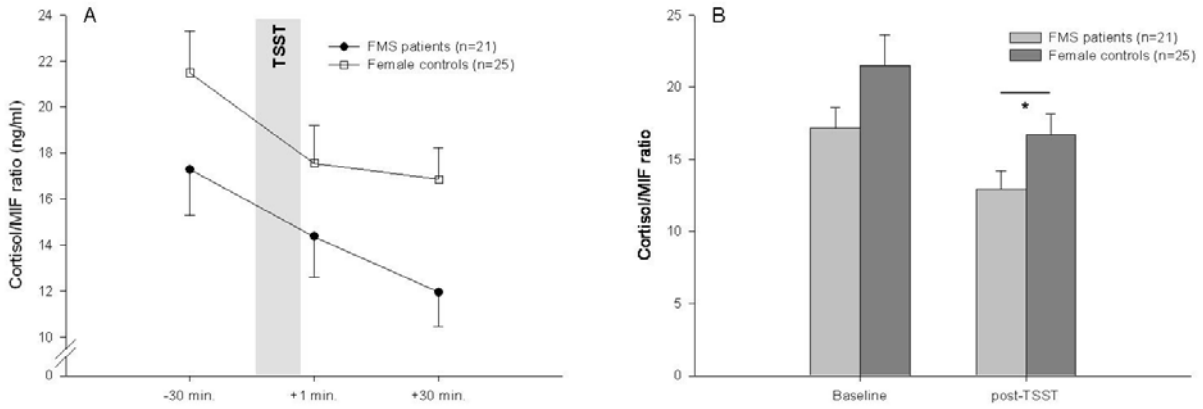

Figure 4.5: Cortisol/MIF ratio in the TSST. (A) Comparison of the course of the cortisol/MIF ratio during the TSST in female FMS patients (n=21) and healthy women (n=25). The cortisol/MIF ratio was significantly lower in the FMS group ($p<.05$); however, when BMI was entered as a covariate, the group difference was only marginally significant ($p=.08$). (B) Comparison of the cortisol/MIF ratio before and after the TSST, respectively, between 21 female FMS patients and 25 healthy women. There was a trend towards a lower cortisol/MIF ratio at baseline ($p=.097$) and a significant difference in the mean cortisol/MIF ratio between the FMS group and the healthy control group after the TSST ($p=.05$). Bars indicate standard errors. Asterisk: $p<.05$.

4.5 Discussion

The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has been identified as a potent counter-regulator of the immunosuppressive effects of glucocorticoids (Bucala, 1996; Calandra et al., 1995). However, little is known about MIF reactivity and changes in the cortisol/MIF ratio in response to a psychosocial stress test. We therefore assessed MIF and cortisol levels 30 min before, as well as 1 min and 30 min after the TSST in order to investigate if changes in MIF levels and in the cortisol/MIF ratio can be seen after psychosocial stress.

The study population consisted of 26 healthy women and 24 female FMS patients. FMS shares some characteristic symptoms with rheumatoid arthritis. Increased rates of obesity have also been reported in FMS patients (Thompson et al., 2003). Based on studies reporting elevated MIF levels in patients with rheumatoid arthritis and obesity, we therefore expected altered MIF concentrations in the FMS group. In addition, we hypothesized that the cortisol/MIF ratio was decreased in the patients given the assumption that FMS is a stress-related disorder with hypocortisolemic features (Fries et al., 2005; Heim et al., 2000).

The TSST led to a significant increase in MIF levels in the whole study group, indicating that the psychosocial stress test was appropriate to induce changes in the

level of this pro-inflammatory cytokine. Consistent with other studies (Dandona et al., 2004; Skurk et al., 2005), we observed higher MIF concentrations in overweight/obese subjects. Importantly, FMS patients did not differ significantly in their BMI from healthy volunteers in our study indicating that the BMI effect occurred independently of group affiliation.

As described in more detail in Chapter 3 (Section 3.4.2.2) FMS patients displayed lower total cortisol levels than their healthy counterparts. This observation supports other studies that have reported blunted plasma and serum cortisol levels in FMS patients compared to healthy subjects (Griep et al., 1998; Gur et al., 2004a; Gur et al., 2004b; Lentjes et al., 1997). Interestingly, similar results have been found in rheumatoid arthritis patients. For example, a reduced cortisol production, especially in the early morning hours, has been observed in these patients (Cutolo, Villaggio, Otsa, Aakre, Sulli & Seriolo, 2005). In addition, blunted cortisol levels in response to pharmacological stress tests (insulin tolerance test, CRH test) have been found despite normal ACTH levels (Eijsbouts, van den Hoogen, Laan, Hermus, Sweep & van de Putte, 2005; Gudbjornsson, Skogseid, Oberg, Wide & Hallgren, 1996). The latter results are in accordance with studies in FMS patients reporting diminished cortisol increases in response to 1µg and 250µg ACTH (Calis et al., 2004; Kirnap et al., 2001). Based on these findings, a mild, subclinical glucocorticoid insufficiency on adrenal level has been suggested in both disorders. As demonstrated in Chapter 3 (Section 3.4), our results support this assumption in terms of normal ACTH levels accompanied by blunted cortisol levels in the FMS patients during the TSST.

Unlike studies with rheumatoid arthritis patients, the total level of MIF was similar in our study between the FMS and the healthy control groups. As shown by Calandra and colleagues (1995), glucocorticoids are able to provoke MIF secretion from macrophages. Thus, if the main source of circulating MIF in our study sample were phagocytes, a lower concentration of cortisol might have been sufficient in the FMS patients to induce MIF release. In this context, glucocorticoid receptor upregulation or a higher binding affinity of cortisol to its receptor in consequence of a mild hypocortisolism could explain an unchanged MIF release despite low cortisol levels in the FMS patients. However, the only study that has investigated this issue has reported similar glucocorticoid receptor numbers in FMS patients and healthy controls. Moreover, contrary to our expectations, the patients in that study showed lower affinity for the binding of glucocorticoids to the receptor (Lentjes et al., 1997).

Hence, the interplay between cortisol and MIF in disorders characterized by a mild hypocortisolism needs further elucidation. In addition, given the similarities in endocrine stress responses between rheumatoid arthritis and FMS patients, it would be interesting to compare stress-induced MIF reactivity in the two patient groups. To our knowledge, no study has investigated MIF responses to a standardized laboratory stressor in rheumatoid arthritis patients so far.

When we looked at differences in MIF levels after the stress test as compared to baseline, the net increase of MIF was significantly higher in FMS patients than in healthy controls. This might imply that the TSST elicited a relatively stronger immune reaction in the patient group than in the healthy volunteer group. One possible consequence of the stress-induced enhanced MIF activity in the patients is that it might trigger the proliferation and secretion of further inflammatory markers such as the pro-inflammatory cytokines IL-1, TNF-α and IL-6 (Calandra et al., 1994; Calandra & Roger, 2003). This could result in a worsening of symptoms and feelings of sickness (Dantzer, 2001; Kelley et al., 2003) and might partly explain a higher symptom burden in many pain and fatigue patients in response to stress. In this context, it might be of special importance that cortisol levels were blunted in the patients indicating that the hormone as one important modulator of the immune system might exert diminished inhibitory effects on inflammatory activity in the patient group. However, in order to be able to investigate this train of thought in future studies, post-stress monitoring should be further extended to enable the detection of cytokine stress responses other than MIF. For example, Steptoe and colleagues have shown that IL-6 increases after acute mental stress in humans were most apparent two hours post-stress (Steptoe, Willemsen, Owen, Flower & Mohamed-Ali, 2001).

Given the role of MIF as a potent counter-regulator of cortisol activity, we were also interested in the ratio of the two substances to investigate potential changes in the TSST and to find out if there were differences between FMS patients and healthy subjects. In both groups, there was a significant decrease in the cortisol/MIF ratio after the TSST indicating a higher MIF increase relative to baseline compared to the relative cortisol increase in response to the stress test. When the two study groups were compared, a significant group difference was observed that remained marginally significant when BMI was included in the analyses, revealing a lower cortisol/MIF ratio in the patient group. Further t-test comparisons taking into

consideration the baseline cortisol/MIF ratio and the mean ratio after the TSST, respectively, indicated that especially after the stress test cortisol/MIF ratios were significantly lower in FMS patients than in healthy controls. These results suggest that the hypocortisolemic features as observed in our patient sample were accompanied by a relatively higher inflammatory activity and support our findings of a stronger MIF increase in the patient group after the TSST relative to baseline.

Taken together, our results indicate that the TSST is a useful tool in eliciting elevations in the level of the pro-inflammatory cytokine MIF. As expected, MIF concentrations were higher in overweight/obese subjects, which was consistent with previous findings. A comparison between FMS patients and healthy volunteers revealed that despite lower plasma cortisol concentrations, the patients displayed normal MIF levels and higher net increases of MIF after the TSST compared to baseline. This observation was underlined by a blunted cortisol/MIF ratio in the patient group, which was especially apparent after the stress test.

We conclude that a blunted cortisol activity in stress-related disorders such as FMS might be associated with an increased inflammatory activity as reflected by higher net increases of the pro-inflammatory cytokine MIF and an altered cortisol/MIF ratio after an applied psychosocial stressor. This is the first study investigating MIF levels in a stress-related disorder that is linked to HPA abnormalities with regard to a hypocortisolemic activity. It should be noted that we included only women in our study; therefore the findings might not generalize to male FMS patients. Thus, further studies are needed that support our results in terms of an increased MIF sensitivity to stress and a blunted cortisol/MIF ratio in both male and female patients with stress-related disorders such as FMS, CFS, and PTSD.

CHAPTER 5

5 The Role of Th1/Th2 Cytokines in Fibromyalgia and Their Association with Cortisol Responses to a Psychosocial Stress Test

5.1 Abstract

Background: Cytokines play a pivotal role in the initiation and maintenance of the sickness response during infection and inflammation. The fibromyalgia syndrome (FMS) is a chronic pain syndrome of unexplained medical origin whose symptoms show a striking resemblance with those of the sickness response. However, only few studies have investigated cytokine levels in FMS patients revealing rather ambiguous results. In addition, there is a lack of studies combining altered adrenal activity in terms of reduced cortisol levels with potential changes in cytokine production in FMS patients.

The aim of the present study was to investigate cytokine production by lymphocytes before and after a psychosocial stress test in female FMS patients and healthy women. Within the FMS group, the impact of sickness-response-like symptoms on cytokine production was examined in order to find out if it was more pronounced in patients with highest symptom burden. Finally, subgroups of patients and healthy controls according to cortisol responses in the stress test were compared with regard to post-stress cytokine production.

Methods: The study sample comprised 24 female fibromyalgia patients and 26 healthy, age-matched women. A cytokine biochip array was used for the simultaneous quantification of different pro- and anti-inflammatory cytokines in Phytohaemagglutinin- (PHA-) stimulated peripheral blood mononuclear cells (PBMC). Blood samples were taken 30 min before and 1 min and 120 min after a psychosocial stressor, the Trier Social Stress Test (TSST). In addition, saliva samples were taken before and after the stress test for the determination of cortisol levels. Demographic characteristics, medical data, the impact of FMS symptoms on daily life, and sleep disturbances were assessed by questionnaires and during a comprehensive medical examination.

Results: FMS patients displayed a significantly elevated IL-2 production and a trend towards a decreased IL-4 production that was reflected by an increased IL-2/IL-4 ratio. Higher symptom burden and more pronounced sleep disturbances within the patient group were accompanied by an increased pro-inflammatory activity. A subgroup of FMS patients who responded to the TSST with increases in cortisol levels that were significantly lower than the cortisol levels of healthy responders, showed an elevated IL-2/IL-4 ratio 120 min after the stress test.

Conclusion: The study results suggest that FMS is linked to enhanced pro-inflammatory activity reflected by an increased IL-2 production and an elevated IL-2/IL-4 ratio, which was especially pronounced in patients with highest scores on sickness-response-like symptoms. In addition, endocrine-immune interactions might play a role in disturbances of the Th1/Th2 balance occurring with some delay after stress cessation.

5.2 Introduction

The fibromyalgia syndrome (FMS) is a disorder of poorly understood aetiology, which is characterized by diffuse, widespread pain and tenderness on palpation at 11 or more of 18 defined tender points (Wolfe et al., 1990). The disorder typically goes along with several additional symptoms such as fatigue, sleep disturbances, irritable bowel and bladder syndrome, chronic headaches, paresthesia, concentration difficulties, depression, and increased stress sensitivity (Bruckle & Zeidler, 2004; Thompson et al., 2003). There is a striking resemblance of FMS symptoms with symptoms of the sickness response that usually occur during inflammation and infection in humans and animals. Typical behavioral features of the sickness response include weakness, fatigue, increased pain sensitivity and reactivity, changes in sleep patterns, malaise, listlessness, inability to concentrate, depressed and lethargic mood, and reduced social behavior (Dantzer, 2004; Kelley et al., 2003; Maier & Watkins, 1998). In the 1980's, Hart was amongst the first who suggested that the behavioral part of sickness, together with the fever response, constitutes a highly organized and adaptive strategy of the organism to fight infection (Hart, 1988).

Interestingly, several studies have proposed a link between pain and fatigue syndromes and underlying infections. For example, about 15-16% of patients with chronic hepatitis C virus (HCV) infection have been co-diagnosed with FMS - a rate significantly higher than the usually reported 1-4% in the general population (Buskila et al., 1998; Rivera et al., 1997; Thompson & Barkhuizen, 2003). It should be noted, however, that a recent study comparing the prevalence of HCV in 115 FMS patients with the prevalence in the general population has failed to confirm an association between FMS and HCV (Narvaez et al., 2005). The chronic fatigue syndrome (CFS) represents another idiopathic syndrome that shares many common features with FMS (see Clauw & Chrousos, 1997; Clauw & Crofford, 2003). In both FMS and CFS

patients, a higher prevalence of mycoplasmal infections has been observed (Nasralla et al., 1999; Nijs, Nicolson, De Becker, Coomans & De Meirleir, 2002).

In this context, Van Hoof and colleagues have recently hypothesized that atypical depression as one of the most common affective disorders in CFS patients constitutes sickness behavior rather than an affective disorder (Van Hoof, Cluydts & De Meirleir, 2003). The authors argue that a lot of the observed behavioral, affective, and cognitive phenomena in CFS patients might be driven by events in the immune system. They hypothesized that the state of atypical depression in CFS patients is characterized by decreased energy consumption during periods of sickness to promote recuperation, thus serving an important function for survival. Similarly, Van Houdenhove and Egle have suggested that typical FMS symptoms may be linked to immune activation thus comprising symptoms of the sickness response (Van Houdenhove & Egle, 2004).

Numerous studies have demonstrated that the sickness response during infections and inflammation is primarily cytokine-mediated. Thus, responses characteristic of sickness can be elicited by subcutaneous, intravenous, and intraperitoneal administration of pro-inflammatory cytokines including interleukin (IL)-1, IL-2, tumor necrosis factor (TNF)-α, and interferon (IFN)-α. In addition, symptoms induced by lipopolysaccharide (LPS) can be attenuated by antagonists of the IL-1 and TNF-α receptors (Bluthe et al., 1992; Cunha et al., 2000; Dantzer, 2001; Konsman et al., 2002; Larson & Dunn, 2001; Lee et al., 2004; Watkins, Maier & Goehler, 1995; Watkins & Maier, 2000).

The role of cytokines in syndromes with idiopathic chronic fatigue and pain symptoms such as CFS and FMS is more ambiguous. As reviewed by Patarca-Montero and colleagues (2001) and by Lyall and colleagues (2003), no clear picture has emerged from the multitude of studies investigating cytokine abnormalities in CFS patients. While some studies have reported elevated pro-inflammatory cytokine levels or increased cytokine production after lymphocyte stimulation, as observed for IFN-γ, TNF-α, and IL-2, other studies have been unable to replicate the results or reported opposite findings in terms of diminished cytokine levels in CFS patients (Lyall, Peakman & Wessely, 2003; Patarca-Montero et al., 2001). Cytokine alterations in the context of FMS have been investigated to a lesser extend, however, with similarly inconclusive results. In three studies, elevated serum IL-8 levels have been found (Gur et al., 2002a; Gur et al., 2002b; Wallace et al., 2001). After stimulation of

peripheral blood mononuclear cells (PBMC) with phorbol myristate acetate (PMA), Wallace and colleagues reported not only increased IL-8 concentrations but also elevated IL-6 levels in FMS patients compared to healthy controls (Wallace et al., 2001). In addition, higher IL-2 levels have been observed in serum and after lymphocyte stimulation in FMS patients (Wallace et al., 1989). In contrast, Hader and colleagues (1991) found significantly reduced IL-2 levels 24h and 48h after stimulation of T lymphocytes with 4 µg/ml or 10 µg/ml concanavalin A (Con A). In the same study, however, IL-2 levels were significantly higher in FMS patients than in healthy subjects after 48 and 72 hours of culture when the dosage was increased to 25 µg/ml Con A (Hader et al., 1991). A recent study investigated cytokine profiles in 40 patients with chronic widespread pain of whom 26 were diagnosed with FMS. Compared to a healthy control group, IL-4 and IL-10 mRNA expression and serum protein levels were significantly lower in the patient group, while no differences were found with regard to pro-inflammatory cytokine mRNA and protein levels (Uceyler et al., 2006). Cytokine expression in skin biopsies of FMS patients compared to healthy controls revealed that IL-1β was detectable in skin tissues from 38% of the patients, IL-6 in 27%, and TNF-α in 32%, whereas none of the cytokines could be detected in skin tissues of healthy subjects (Salemi et al., 2003). Other studies could not find differences in cytokine levels between FMS patients and healthy volunteers (Amel Kashipaz et al., 2003; Pay et al., 2000).

It has been shown that glucocorticoids belong to the most potent modulators of inflammatory activity in the body by both suppressing and stimulating pro- and anti-inflammatory mediators. For example, glucocorticoids promote Th2 development by enhancing the expression of cytokines such as IL-4, IL-10 and IL-13, while they inhibit inflammatory responses by suppressing the synthesis and release of pro-inflammatory cytokines including IL-1, IL-2, IL-6, IL-12, IFN-γ, and TNF-α (Franchimont et al., 2003; Kovalovsky, Refojo, Holsboer & Arzt, 2000; Petrovsky et al., 1998; Ramierz et al., 1996). Consequently, low levels of glucocorticoids increase susceptibility to and the severity of inflammatory activity, which becomes most obvious in patients with Addison's disease who suffer from severe glucocorticoid deficiency. In these patients, supplemental glucocorticoids are required during infection and inflammation to prevent damaging effects of pro-inflammatory cytokines on the organism (Kapcala et al., 1995).

Interestingly, a mild hypocortisolism has been observed in different studies investigating the activity of the hypothalamus-pituitary-adrenal (HPA) axis in FMS patients. As described in more detail in Chapter 3 (Section 3.2), lower cortisol levels have been reported in FMS patients compared to healthy volunteers both under basal conditions (Crofford et al., 1994; Griep et al., 1998; Gur et al., 2004a; Gur et al., 2004b; Lentjes et al., 1997), in exercise tests (Paiva et al., 2002; van Denderen et al., 1992) and after pharmacological stimulation (Calis et al., 2004; Kirnap et al., 2001). In Chapter 3, we described lower cortisol levels before and after a psychosocial stress test (see Section 3.4.2). Consequently, a mild immune activation as reported in some but not all studies with FMS patients might be due to a relative inability of FMS patients to mount adequate levels of cortisol. There is, however, a lack of studies investigating endocrine-immune interactions in FMS patients.

The aim of the present study was to examine the level of different cytokines after lymphocyte stimulation before and after a psychosocial stress test in female FMS patients and healthy women. Based on the assumption that FMS reflects sickness behavior, we hypothesized that the production of pro-inflammatory cytokines was higher in FMS patients than in healthy subjects, while the synthesis of anti-inflammatory cytokines was diminished in the patient group. This shift to Th1 immunity would also be reflected by an elevated Th1/Th2 ratio.

We next addressed the question of whether the subjective impact of fibromyalgia symptoms and sleep disturbances were associated with cytokine levels in the FMS group. As mentioned above, typical FMS symptoms such as pain, tiredness, and stiffness comply with those of sickness response and might be cytokine-induced. In addition, sleep disturbances that frequently occur in FMS patients have been associated with elevations in pro-inflammatory cytokine levels (Mullington, Hinze-Selch & Pollmacher, 2001; Vgontzas, Zoumakis, Bixler, Lin, Prolo, Vela-Bueno, Kales et al., 2003; Vgontzas, Zoumakis, Bixler, Lin, Follett, Kales & Chrousos, 2004). We therefore hypothesized that FMS patients with highest symptom burden and worst sleep disturbances had significantly higher pro-inflammatory cytokine levels and an elevated Th1/Th2 ratio than patients with lower symptom burden and less sleep disturbances.

In Chapter 3, the FMS and healthy volunteer groups were each subdivided into responders and non-responders based on salivary cortisol responses to the psychosocial stress test. In the present chapter, we expected group differences in

cytokine levels and the Th1/Th2 ratio especially between FMS responders and controls responders according to our finding that FMS responders had significantly lower cortisol levels than their healthy counterparts. By contrast, the non-responder groups did not differ from each other with regard to cortisol concentrations (see Chapter 3, Section 3.4.2.1). Taking into consideration that stress-induced increases in plasma cytokine levels are most apparent after some delay, e.g. in the case of IL-6 two hours following a mental stress test (Steptoe et al., 2001), post-stress cytokine monitoring was extended to 120 min in the present study.

5.3 Materials and methods

5.3.1 Study participants

The study sample consisted of 24 female FMS patients (mean age: 49.7 ± 9.2 SD) and a control group of 26 healthy, age-matched women (mean age: 50.5 ± 8.4 SD). Patients were made aware of the study by information sheets in outpatient and specialist clinics, by contacting patient self-help groups and by newspaper advertisements. Healthy volunteers were recruited via newspaper advertisements or were provided from the environment of participating FMS patients. Patients were included into the study if they fulfilled the ACR 1990 Criteria for the Classification of Fibromyalgia (Wolfe et al., 1990) as diagnosed by a rheumatologist, reported non-inflammatory origins of the disease symptoms, were free of chronic medical diseases including gastrointestinal, neurological and autoimmune disorders, as well as free of psychotic or eating disorders and of substance abuse or dependency, and refrained from potential antidepressant medication at least two weeks before study entry.

Healthy women had to be free of antidepressant/antipsychotic drugs, medical diseases and mental disorders in order to be allowed to participate in the study. Further exclusion criteria for both FMS patients and healthy controls included pregnancy, current breast feeding, dietary weight loss of 5 kg or more within 6 weeks before study entry, hormonal medication with the exception of oral contraceptives and hormone replacement therapy, and infections during the preceding two weeks before study entry. In the latter case, appointments were postponed for at least two weeks.

The participants were evaluated with the German version of the Structured Clinical Interview for DMS-IV (Wittchen et al., 1997) and underwent a comprehensive medical examination for past and current health problems. Written informed consent was provided by all subjects after they were given a detailed description of the study. The study protocol was approved by the Landesärztekammer Rheinland-Pfalz.

5.3.2 Demographic and psychosocial assessments

A demographic questionnaire was used for the assessment of demographic data such as age, marital status, and job situation. During the medical examination, parameters such as weight, height, waist and hip circumference were measured. The current health status of the FMS patients as compared to the healthy volunteers was assessed with the *German version* of the *Fibromyalgia Impact Questionnaire* (FIQ-G, Offenbaecher et al., 2000). The FIQ-G consists of 10 items, with the first item containing 10 sub-items that focus on the patients' ability to perform daily tasks involving large muscles such as cooking, cleaning and walking. The remaining items refer to the number of days felt good, number of days missed work, ability to do one's job, pain, fatigue, morning tiredness, stiffness, anxiety, and depression. In the evaluation study by Offenbaecher and colleagues (2000), test-retest reliability for the total FIQ-G score was r = 0.85, internal consistency alpha = 0.92, and construct validity ranged from 0.48 to 0.67.

Sleep disturbances were evaluated with the German version of the *Pittsburgh Sleep Quality Index* (PSQI, Riemann & Backhaus, 1996). The PSQI is a 19-item questionnaire assessing sleep quality and disturbances over a 1-month period. The 19 items generate seven component scores: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. According to the original version by Buysse and colleagues (1989), the sum of scores for these seven components yields one global PSQI score that – at a cut-off score of 5 - distinguished good and poor sleepers with a diagnostic sensitivity of 89.6% and specificity of 86.5% (Buysse, Reynolds, Monk, Berman & Kupfer, 1989).

5.3.3 Experimental protocol

The study took place at the Department of Psychobiology, University of Trier, Germany. Laboratory sessions started between 8.00 and 8.30 a.m. with the medical examination and the clinical interview. Afterwards, a catheter was inserted into the antecubital vein, followed by a 45-min resting period. Next, saliva and blood samples were taken for the determination of basal endocrine and immune markers. Thirty minutes later, the participant was asked to collect a second saliva sample for the determination of cortisol and was then guided into another room where she was exposed to the Trier Social Stress Test (TSST, Kirschbaum et al., 1993). In brief, the participant faced a committee of one male and one female trained staff member who were dressed in white coats. The participant was told to imagine that she had to apply for a job she most desired. After a three-minute speech preparation period in the same room, the participant had to deliver a five-minute free speech on personality traits that would qualify her for the job in front of the committee. The job interview was followed by a five-minute mental arithmetic task. Further blood samples were taken 1 min and 120 min after stress exposure, saliva was collected 1, 10, 20, 30, 45, 60, 90, and 120 min post-TSST.

5.3.4 Laboratory assays

5.3.4.1 Cytokines

Cytokines were quantified in Phytohaemagglutinin- (PHA-) stimulated peripheral blood mononuclear cells (PBMC). Blood samples were collected in syringes prepared with heparin (500IE/ml) 30 min before and 1 min and 120 min after the TSST. PBMCs were isolated according to Boyum (1968) using Ficoll-Paque® (Pharmacia Biotech Europe GmbH, Freiburg, Germany) for separation. Cell numbers were counted in each sample using a cell counter (Coulter $A^c{\cdot}T$ diff^{TM}, Krefeld, Germany), and were adjusted to 10^6 cells/ml by adding according amounts of the cell culture medium (Roswell Park Memorial Institute, RPMI), which was supplemented with 10% fetal calf serum, 2nM L-Glutamine, and 100U/ml Penicillin/Streptomycin. 1.5ml cell suspension was stimulated with 15µl of the T-cell stimulant PHA (0.5mg/ml) and incubated for 48 hours under 37°C and 5% CO_2. Finally, the samples were spun down and the supernatants were stored at -80°C until they were analyzed.

For the determination of different cytokine levels, a cytokine panel (evidence® biochip array technology, Randox Laboratories, Crumlin, UK) was used that is designed for the simultaneous quantitative detection of multiple related cytokine immunoassays from a single subject sample run on an automated biochip array analyser. The biochip contains an array of discrete test regions including immobilized antibodies specific to different cytokines. A sandwich chemiluminescent immunoassay was employed for the cytokine assays. The light signal generated from each of the test regions on the biochip was detected using digital imaging technology. The signal was compared to that from a stored calibration curve thus enabling the calculation of the concentration of analyte present in the sample.

The following simultaneously measured cytokines were included in the present study: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. Different dilution factors had been employed for the determination of cytokines (neat, 1:10, 1:200) that were taken into consideration in the analyses. Assay sensitivities as well as intra- and inter-assay coefficients of variance for the respective cytokine assays are presented in Table 5.1.

Table 5.1: Assay sensitivities and intra- and inter-assay coefficients of variance for the cytokine assays

Cytokines	Assay sensitivities (pg/ml)	Intra-assay coefficient of variance (%)	Intra-assay coefficient of variance (%)
IL[a]-1α	3.6	3.9 - 5.3	5.8 - 7.5
IL-1β	1.8	4.3 - 8.2	7.9 - 10.5
IL-2	5.1	5.5 - 10.3	5.3 - 9.6
IL-4	5.3	4.9 - 6.8	9.0 - 11.9
IL-6	1.1	5.8 - 7.8	6.2 - 7.3
IL-8	8.9	6.3 - 11.0	6.8 - 10.6
IL-10	1.8	4.4 - 6.5	6.4 - 6.9
IFN[b]-γ	18.0	3.7 - 6.8	8.1 - 11.8
TNF[c]-α	7.7	4.8 - 7.3	7.9 - 12.0

[a] *IL: interleukin,* [b] *IFN: interferon,* [c] *TNF: tumor necrosis factor*

5.3.4.2 CD3 T cells

Peripheral blood was collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) 30 min before as well as 1 min and 120 min after the TSST. Total numbers of leukocytes were determined by a cell counter in each sample (Coulter A[c]·T diff[TM], Krefeld, Germany). For the analysis of circulating T cells (CD3+/CD19-), flow cytometry

[fluorescence activated cell sorter (FACS), Becton Dickinson, Heidelberg, Germany] using CellQuest Pro software version 3.4 was carried out. The kit Simultest^{TM} from Becton Dickinson was used according to the manufacturer's instructions for the staining protocol.

5.3.4.3 Salivary cortisol

Saliva samples were stored at -20°C until they were analyzed. For the determination of salivary cortisol, a time-resolved fluorescence immunoassay was used that is described in detail by Dressendörfer and colleagues (Dressendörfer et al., 1992). The assay has an intra-assay variation between 4.0% and 6.7%, and an inter-assay variation between 7.1% and 9.0%. Error variance due to inter-assay imprecision was reduced by analyzing all samples of one participant in the same run.

5.3.5 Statistical analyses

T-tests and X^2-tests were used as appropriate for the comparison of demographic characteristics between FMS patients and healthy volunteers. Differences in FIQ-G and PSQI scores between the two groups were analyzed with a multivariate analysis of variance (MANOVA).

General linear models (GLMs) with repeated measures were performed in order to compare PHA-stimulated cytokine levels and their changes in the TSST between the FMS and healthy control groups. Greenhouse-Geisser or Huynh-Feldt corrections were applied where appropriate. Cytokine and levels that diverged three or more standard deviations from the mean of the respective group led to the exclusion of the participants' values in the relevant analyses.

Since PHA is a potent T cell stimulator, the role of T cell levels on cytokine concentrations was investigated for those cytokines that differed significantly between the patient and the control group. Univariate analyses of covariance (ANCOVAs) with the factor group (FMS, control) as independent variable and T cell levels as covariates were used to investigate if group differences in PHA-stimulated cytokine levels remained significant when T cell concentrations were taken into consideration. In order to avoid accumulation of the type 1 error due to multiple comparisons, Bonferroni adjustments of p values were performed. Thereafter, $p < 0.017$ was considered significant.

Absolute T cell numbers were gained by multiplying the absolute number of leukocytes by the percentage of lymphocytes received in the FACS analyses, thus yielding the total number of lymphocytes for each subject. The total lymphocyte number was then divided by the percentage of CD3+/CD19- cells to obtain the total number of T cells. T cell, cytokine counts were available 30 min before as well as 1 min and 120 min after the stress test.

The impact of the current health status (FIQ-G) and sleep disturbances (PSQI) on cytokine levels in the FMS group was investigated by building tertiles for the scores of the two questionnaires. Cytokine levels of FMS patients in the highest tertile were compared with cytokine levels of the remaining patients in order to find out if differences in subjective symptom burden were reflected in immunological parameters. Repeated-measures GLMs with group (high FMS impact vs. low to medium FMS impact; high sleep disturbance vs. low to medium sleep disturbance, respectively) as independent variable and cytokine levels before and after the TSST as dependent variable were conducted to reveal potential differences in cytokine levels in the patient subgroups.

In Chapter 3, subgroups were built within the patient and the control groups based on positive/negative values of the area under the curve with respect to increase (AUCi) for salivary cortisol levels. The following four subgroups were compared in terms of endocrine and psychosocial data in Chapter 3: AUCi positive – “FMS responders”, “controls responders”; AUCi negative – “FMS non-responders”, “controls non-responders” (see Sections 3.3.5 and 3.4.2.1). In the present chapter, the four subgroups were compared with respect to cytokine levels that differed significantly between the FMS group and the healthy control group in order to investigate the potential impact of free cortisol responses in the stress test on post-TSST cytokine concentrations. MANOVAs were conducted with the respective cytokine levels as dependent variables and the factor group (FMS non-/responders, controls non-/responders) as independent variable. All data was analyzed with SPSS 12.0.

5.4 Results

5.4.1 Study participants

The study sample consisted of 24 female FMS patients and 26 healthy, age-matched women. All study participants were non-smokers. As shown in Table 5.2, there were no significant differences in demographic characteristics between the patient and the control groups. Twelve (50%) patients and 16 (61.5%) controls were employed full- or part-time, while 2 patients were unemployed, 3 patients and 7 controls were housewives, and 7 patients and 3 controls were retired. Ten of the FMS patients took antidepressants, which they agreed to discontinue at least two weeks before their TSST appointment. As expected, FMS patients had significantly higher FIQ-G and PSQI scores than their healthy counterparts, indicating a worse health status and more sleep disturbances in the patient group.

Table 5.2: Demographic and psychometric characteristics of FMS patients and healthy volunteers

Variable	FMS patients (n=24)	Controls (n=26)	Statistics
Demographics			
Age, years *(mean, SD)*	49.7 (9.2)	50.5 (8.4)	t(48) = -0.349, p = 0.728
Married *(n, %)*	15 (62.5%)	20 (76.9%)	Fisher's exact: p = 0.358
Full- or part-time job *(n, %)*	12 (50.0%)	16 (61.5%)	Fisher's exact: p = 0.569
BMI *(mean, SD)*[a]	27.3 (6.0)	25.2 (4.2)	t(48) = 1.446, p = 0.155
WHR *(mean, SD)* [b]	0.84 (0.06)	0.83 (0.05)	t(48) = 0.154, p = 0.878
Intake of oral contraceptives, hormone replacement *(n, %)*	6 (25.0%)	10 (38.5%)	Fisher's exact: p = 0.372
Antidepressants *(n, %)*[c]	10 (41.7%)	-	
Psychometric parameters *(mean, SD)*			
FMS impact on functioning (FIQ-G)	45.5 (11.9)	5.4 (6.7)	$F_{1.43}$ = 211.59, **p < 0.001**
Sleep Disturbances (PSQI)	12.6 (4.7)	5.5 (2.9)	$F_{1.43}$ = 40.26, **p < 0.001**

[a] *BMI: Body Mass Index (body weight [kg]/height [m]²)*
[b] *WHR: waist-to-hip ratio*
[c] *patients refrained from antidepressants - in most cases used as pain relievers - at least two weeks before study entry*

5.4.2 Changes of cytokine levels in the TSST

Figure 5.1 presents the levels of all included cytokines at baseline and in response to the TSST for the FMS and healthy control groups. As illustrated in Table 5.3, significant changes over time occurred for the following cytokines: IL-1α, IL-1β, IL-2, IL-4, IFN-γ, and TNF-α, whereas IL-6, IL-8, and IL-10 levels remained relatively unaffected by the stress test. In terms of IFN-γ changes in the TSST, we found a significant group*time interaction effect (*group*time*: $F_{2,78}$=6.17; p=0.003; η^2=0.14) indicating a stronger IFN-γ reactivity in the healthy control group. Two separate GLMs for the control and the FMS groups revealed that the significant time effect on IFN-γ levels was due to changes in the course of IFN-γ in the control group (*time*: $F_{2,40}$=18.40; p=0.000; η^2=0.48), while IFN-γ levels changed only marginally in the FMS group (*time*: $F_{2,38}$=2.36; p=0.108).

Table 5.3: Time effect on PHA-stimulated cytokine levels in the TSST

	Time Effect				
	df effect	**df error**	**F**	**p**	**η^2**
IL-1α	2.0	82.0	25.60	**< 0.001**	0.38
IL-1β	2.0	78.0	15.29	**< 0.001**	0.28
IL-2	1.8	63.4	3.95	**0.024**	0.10
IL-4	1.6	64.3	15.45	**< 0.001**	0.27
IL-6	2.0	84.0	0.87	0.422	0.02
IL-8	1.8	73.9	0.57	0.545	0.01
IL-10	2.0	86.0	0.07	0.930	0.00
IFN-γ	2.0	78.0	17.55	**< 0.001**	0.31
TNF-α	2.0	86.0	10.24	**< 0.001**	0.19

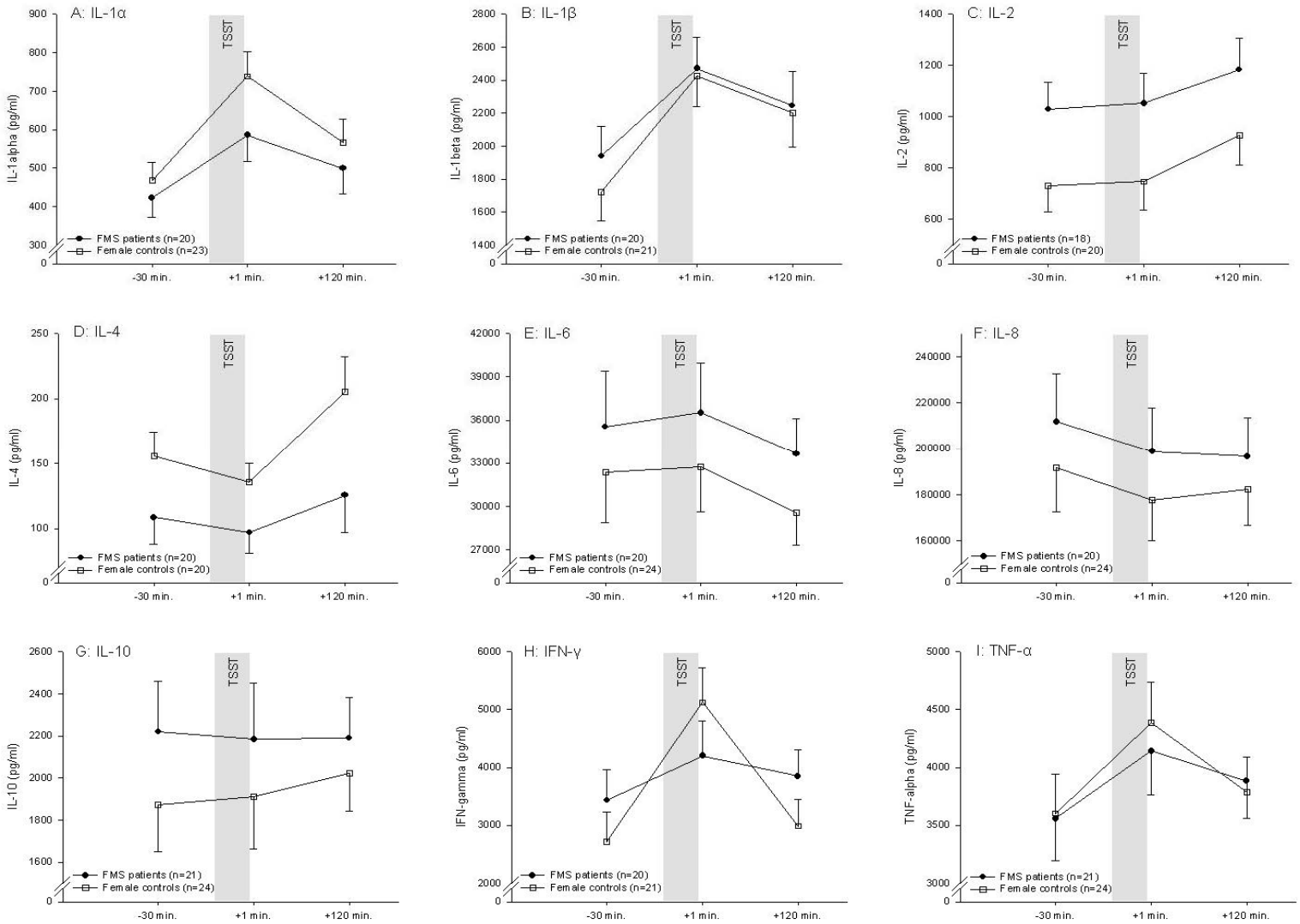

Figure 5.1 A-I: PHA-stimulated cytokine concentrations before and after the TSST in female FMS patients and healthy women. Bars indicate standard errors.

5.4.3 Comparison of cytokine levels between the FMS patients and healthy volunteers: IL-2, IL-4, and IL-2/IL-4 ratio

Beside the significant group*time interaction found for IFN-γ, further group comparisons revealed a significant group effect for IL-2 (*group*: $F_{1,36}$=4.30; p=0.045; η²=0.11) indicating elevated IL-2 levels in the FMS group. At all three time points, patients had higher IL-2 levels than healthy volunteers as demonstrated by t-tests (-*30 min*: t_{38} = 2.61, p=0.013; +*1 min*: $t_{25.2}$ = 2.13, p=0.043; +*120 min*: $t_{32.9}$ = 2.12, p=0.042; see Figure 5.2A). In addition, a marginally significant group effect and a trend towards a significant group*time interaction was found for IL-4 (*group*: $F_{1,41}$=3.74; p=0.060; η²=0.08; *group*time*: $F_{1.6,64.3}$=2.93; p=0.073; η²=0.07). FMS patients tended to have lower IL-4 levels than their healthy counterparts, which was most pronounced 120 min after the stress test (-*30 min*: $t_{34.8}$ = -1.67, p=0.11; +*1 min*: t_{41} = -1.79, p=0.08; +*120 min*: $t_{31.7}$ = -2.03, p=0.05; see Figure 5.2B). No further group differences or interaction effects were detected for the remaining cytokines over the three time points.

We next used the ratio of the stimulated IL-2 to IL-4 levels as an index of Th1/Th2 immune balance and found a significantly higher Th1/Th2 ratio in the FMS group (*group*: $F_{1,36}$=4.81; p=0.035; η²=0.12). T-tests revealed that the ratio was elevated at all time points (-*30 min*: t_{38} = 2.54, p=0.015; +*1 min*: t_{37} = 2.39, p=0.022; +*120 min*: t_{40} = 2.41, p=0.021; see Figure 5.2C).

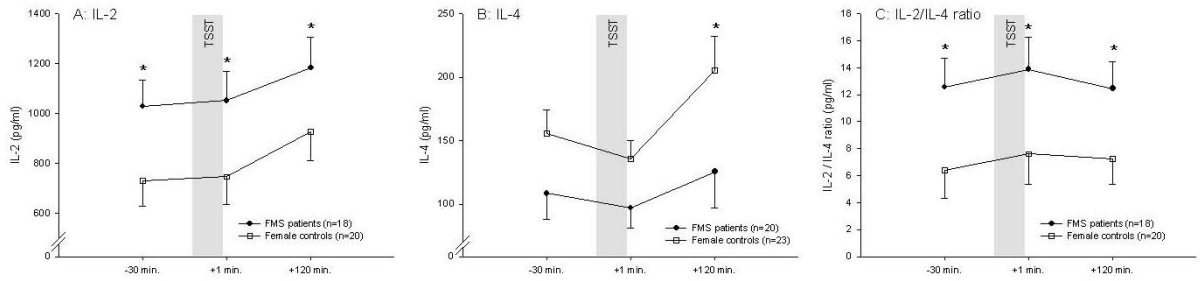

Figure 5.2: PHA-stimulated IL-2 (A) and IL-4 (B) concentrations before and after the TSST in women with FMS and female controls. FMS patients had significantly higher IL-2 levels than healthy controls (p<0.05) and a trend towards lower IL-4 levels (p=0.06), which was also reflected by an elevated IL-2/IL-4 ratio in the patient group (p<0.05) (C). Bars indicate standard errors. Asterisk: p<0.05.

5.4.4 The role of T cell concentrations on IL-2, IL-4, and the IL-2/IL-4 ratio

In addition to cytokine levels, changes in CD3 cells in response to the stress test were investigated and compared between the patient and the healthy control groups. As demonstrated in Figure 5.3, there was a significant increase in T cells in response to the stress test (*time*: $F_{2,94}$=9.01; p<0.001; η^2=0.16), while no significant group differences occurred (*group*: $F_{1,47}$=0.06; p=0.805; *group*time*: $F_{2,94}$=0.31; p=0.736).

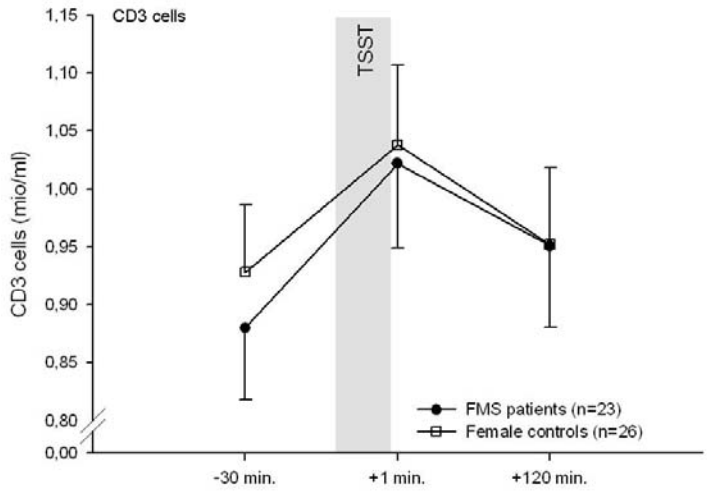

Figure 5.3: T cell levels before and after the TSST in female FMS patients and healthy women. While T cells increased significantly after the stress test (p<0.001), no group differences could be detected. Bars indicate standard errors.

Given the role of PHA as a potent T cell stimulator, we next investigated the impact of CD3 cell concentrations at each time point on the respective IL-2 and IL-4 levels and on the IL-2/IL-4 ratio in order to find out if the reported group differences between FMS patients and healthy controls were still evident when T cell levels were taken into consideration. ANCOVAs were conducted with group affiliation (FMS, healthy controls) as independent variable, IL-2 levels, IL-4 levels, and the IL-2/IL-4 ratio at the three time points, respectively, as dependent variables, and the respective CD3 cell levels 30 min before, as well as 1 min and 120 min after the TSST as covariates. Results are shown in Table 5.4.

The ANCOVAS revealed that T cell concentrations had no significant impact on IL-2 levels, whereas IL-4 levels at baseline were marginally ($F_{1,41}$=3.24, p=0.079), and 1 min and 120 min post-TSST significantly effected by T cell concentrations ($F_{1,40}$=5.35, p=0.026 and $F_{1,41}$=4.73, p=0.035, respectively). In terms of the IL-2/IL-4 ratio, a significant effect only occurred at baseline ($F_{1,37}$=5.97, p=0.019).

Table 5.4: Effects of group and T cell concentrations on PHA-stimulated IL-2 and IL-4 concentrations and on the IL-2/IL-4 ratio 30 min before and 1min and 120 min after the TSST

	df effect	df error	F	p
IL-2 30 min pre-TSST				
Group	1	37	7.84	**0.008**
CD3 pre-TSST	1	37	1.69	0.202
IL-2 1 min post-TSST				
Group	1	36	4.36	**0.044**
CD3 1 min post-TSST	1	36	0.35	0.557
IL-2 120 min post-TSST				
Group	1	39	4.56	**0.039**
CD3 120 min post-TSST	1	39	0.66	0.420
IL-4 30 min pre-TSST				
Group	1	41	4.00	*0.052*
CD3 pre-TSST	1	41	3.24	*0.079*
IL-4 1 min post-TSST				
Group	1	40	4.26	**0.045**
CD3 1 min post-TSST	1	40	5.35	**0.026**
IL-4 120 min post-TSST				
Group	1	41	5.17	**0.028**
CD3 120 min post-TSST	1	41	4.73	**0.035**
IL-2/IL-4 30 min pre-TSST				
Group	1	37	8.64	**0.006**
CD3 pre-TSST	1	37	5.97	**0.019**
IL-2/IL-4 1 min post-TSST				
Group	1	36	4.67	**0.037**
CD3 1 min post-TSST	1	36	0.01	0.946
IL-2/IL-4 120 min post-TSST				
Group	1	39	6.73	**0.013**
CD3 120 min post-TSST	1	39	2.79	0.103

As also illustrated in Table 5.4, the group differences for IL-2 and for the IL-2/IL-4 ratio as observed earlier in the GLMs and in the t-tests (Section 5.4.3) were still significant at all three time points despite some influence of T cell levels on PHA-stimulated cytokine production. After Bonferroni adjustment of p levels to p=0.017, baseline IL-2 levels and the IL-2/IL-4 ratio at baseline and 120 min post-TSST remained significantly lower in the FMS group. In addition, the reported trend towards lower IL-4 levels in the patients was still detectable.

5.4.5 The role of psychosocial variables on IL-2 and IL-4 levels, and on the Th1/Th2 ratio in FMS patients

Within the patient group, total FIQ-G and PSQI scores ranged between 23 and 66 and between 4 and 20, respectively, thus showing a broad range of symptom burden and sleep disturbances. Tertiles were built for both scores, and the subgroup of patients in the highest tertile was compared to a subgroup of those in the lowest and the middle tertiles. We suggested that highest subjective symptom burden and lowest sleep quality would be related to cytokine levels in terms of elevated pro-inflammatory activity and decreased anti-inflammatory activity.

As depicted in Figures 5.4 A and B, FMS patients with a higher symptom burden tended to have elevated IL-2 levels and decreased IL-4 levels. In both cases, however, statistical analyses did not reach significance (IL-2: *group*: $F_{1,16}$=1.49; p=0.240; η^2=0.09; IL-4: *group*: $F_{1,18}$=1.44; p=0.245; η^2=0.07). On the other hand, the IL-2/IL-4 ratio was significantly higher in FMS patients in the upper tertile of FIQ-G scores (IL-2/IL-4 ratio: *group*: $F_{1,16}$=4.35; p=0.05; η^2=0.21; Figure 5.4C).

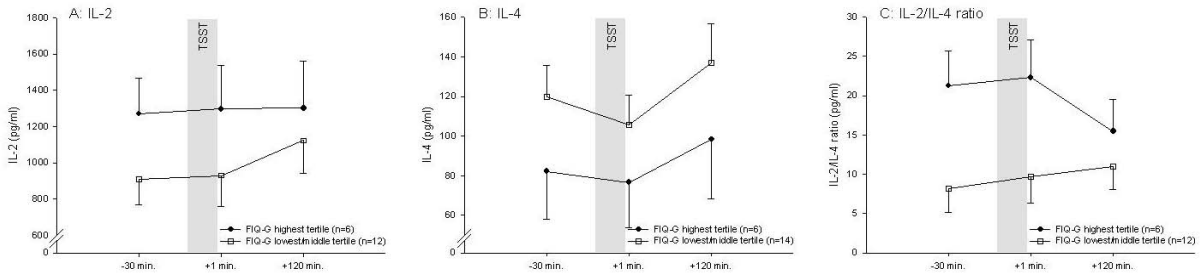

Figure 5.4: PHA-stimulated IL-2 (A) and IL-4 (B) levels and the IL-2/IL-4 ratio (C) before and after the TSST in FMS patients with highest symptom burden (highest FIQ-G tertile) compared to patients with less symptom burden (lowest/middle FIQ-G tertile). Patients with the highest FMS impact on functioning had a significantly elevated IL-2/IL-4 ratio (p=0.05).

Considering the effect of sleep disturbances on PHA-stimulated cytokine levels, patients reporting worst sleep quality had significantly higher IL-2 concentrations than patients in the lowest and middle tertile (IL-2: *group*: $F_{1,15}$=4.40; p=0.05; η^2=0.23). While no significant differences were found in IL-4 levels between the two FMS subgroups (IL-4: *group*: $F_{1,17}$=0.10; p=0.760), a trend was observed towards a higher IL-2/IL-4 ratio in the patient group with most severe sleep difficulties (IL-2/IL-4 ratio: *group*: $F_{1,15}$=3.91; p=0.067; η^2=0.21, Figure 5.5A-C).

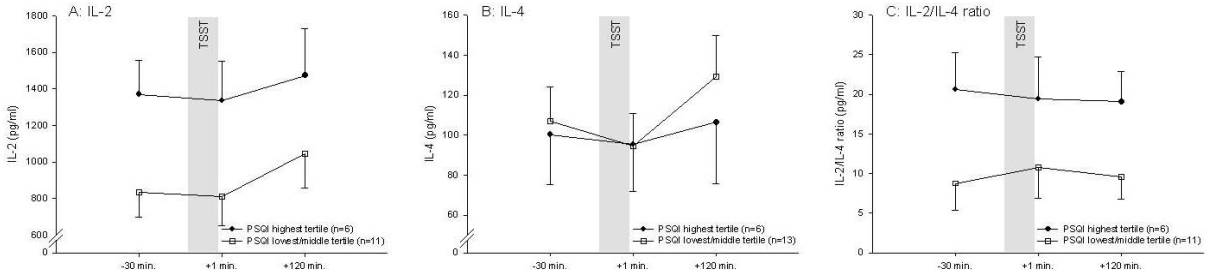

Figure 5.5: PHA-stimulated IL-2 (A) and IL-4 (B) levels and the IL-2/IL-4 ratio (C) before and after the TSST in FMS patients with severe sleep disturbances (highest PSQI tertile) compared to patients with less severe sleep disturbances (lowest/middle PSQI tertile). In patients with worst sleep difficulties, significantly increased IL-2 levels (p=0.05) and a trend towards a significantly elevated IL-2/IL-4 ratio (p=0.067) was observed.

5.4.6 The impact of salivary free cortisol responses to the TSST on IL-2, IL-4, and the IL-2/IL-4 ratio: cortisol responders and non-responders

As illustrated in Chapter 3 (Sections 3.3.5 and 3.4.2.1), the FMS and the healthy control groups were subdivided into TSST responders and non-responders based on positive/negative AUCi values. It was shown that FMS responders had significantly lower salivary cortisol levels in the TSST than their healthy counterparts supporting the assumption of decreased adrenal activity in FMS patients. Based on these findings, we hypothesized in the present chapter that hypocortisolemic features as observed in the FMS responder group were accompanied by a shift towards an increased post-stress Th1 activity. In order to investigate this hypothesis, we focused on IL-2 and IL-4 levels and on the IL-2/IL-4 ratio as an index of Th1/Th2 immune balance because we could show differences in these parameters between the FMS and the healthy control groups (see Section 5.4.3). MANOVAs were conducted with the post-TSST (+1 min, +120 min) cytokine levels and ratios, respectively, as dependent variables and the factor group (FMS non-/responders, controls non-/responders) as independent variable. As shown in Table 5.5, a significant group difference was seen for the IL-2/IL-4 ratio 120 min after the stress test, while no such group differences were found for IL-2 and IL-4.

Table 5.5: Effects of group affiliation (FMS non-/responders, controls non-/responders) on PHA-stimulated IL-2 and IL-4 concentrations and on the IL-2/IL-4 ratio 1min and 120 min after the TSST

	df effect	df error	F	p
IL-2				
1 min post-TSST	3	35	1.52	0.227
120 min post-TSST	3	35	1.22	0.316
IL-4				
1 min post-TSST	3	39	2.01	0.128
120 min post-TSST	3	39	1.69	0.186
IL-2/IL-4 ratio				
1 min post-TSST	3	35	2.13	0.115
120 min post-TSST	3	35	3.02	**0.043**

Interestingly, pair-wise comparisons revealed significantly lower IL-4 levels 1 min (p=0.021) and 120 min (p=0.035) after the stress test in the FMS responder group than in the healthy responder group; however, these differences were no longer significant in Bonferroni post-hoc tests after adjusting the alpha level to the multiple comparisons. A similar observation was made for the IL-2/IL-4 ratio immediately after the TSST that was significantly higher in the FMS responder group compared to the healthy responder group in pair-wise comparisons (p=0.025). But again, after Bonferroni adjustment, the difference was no longer significant. In contrast to this, the IL-2/IL-4 ratio 120 min post-TSST remained significantly different in the FMS responder group than in the healthy responder group in the Bonferroni post-hoc test (p=0.05) indicating a higher ratio in the patient subgroup. None of the other subgroups differed significantly in any parameter from each other. IL-2 and IL-4 levels and the IL-2/IL-4 ratio 1 min and 120 min post-stress in the four subgroups are presented in Figure 5.6.

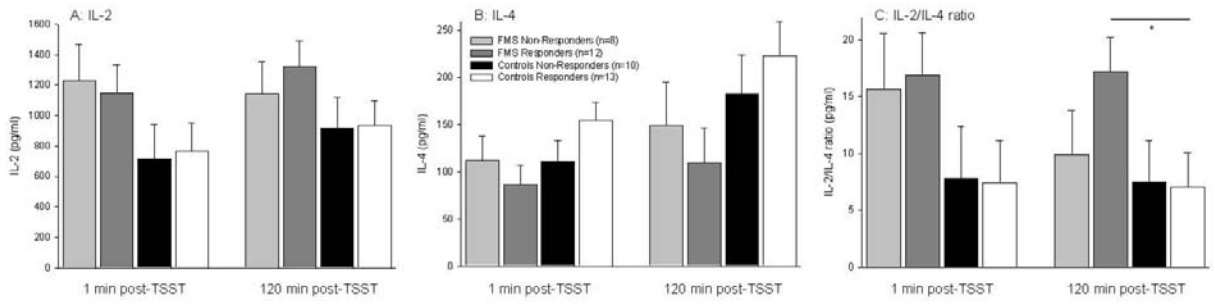

Figure 5.6: PHA-stimulated IL-2 (A) and IL-4 (B) levels and the IL-2/IL-4 ratio (C) 1 min and 120 min after the TSST in the four subgroups FMS non-responders, FMS responders, controls non-responders, controls responders, which were based on negative/positive AUCi values for salivary cortisol up to 60 min after the TSST. The IL-2/IL-4 ratio was significantly increased in the FMS responder group compared to the healthy responder group 120 min after the stress test (p<0.05). Bars indicate standard errors. Asterisk: p<0.05.

5.5 Discussion

In the present study, the production of cytokines by PHA-stimulated lymphocytes was investigated before and after a psychosocial stress test in female FMS patients and healthy, age-matched women. A biochip protein array was used for the simultaneous quantification of different pro- and anti-inflammatory cytokines. Significant increases of cytokine production in response to the stress test were found for IL-1α, IL-1β, IL-2, IL-4, IFN-γ, and TNF-α. In terms of IFN-γ production, changes after the stress test were only significant in healthy subjects.

Comparisons between the FMS and healthy control groups revealed a significantly higher IL-2 production and a trend towards a lower IL-4 production in the patients. The shift to elevated Th1 activity was also reflected by a significantly increased IL-2/IL-4 ratio which was used as an index of Th1/Th2 immune balance. T cells as one of the main targets of PHA stimulation significantly increased in response to the TSST. However, no group differences in the levels and stress reactivity of T cells were detected between the patient and the control groups. This indicates that the observed Th1/Th2 alterations were most likely not attributable to changes in T cell levels but rather to a modified rate of IL-2 and IL-4 synthesis and secretion by the T cells of FMS patients. These findings were supported by results from ANOVAS revealing in part significant group effects on the individual IL-2 levels and the IL-2/IL-4 ratios, respectively, that were controlled for T cell levels.

It has been reported that patients with cancer who underwent an IL-2 therapy experienced myalgias, fatigue, malaise, concentration difficulties, sleep disturbances, and depression (Cleeland, Bennett, Dantzer, Dougherty, Dunn, Meyers, Miller et al., 2003; Lee et al., 2004; Wichers & Maes, 2002; Yirmiya et al., 2000), symptoms that are similar to those experienced in FMS patients. Since the symptoms in cancer patients usually disappear shortly after termination of cytokine treatment, studies on cytokine therapies and their effects in humans most evidently reveal a causative role for cytokines in the initiation of sickness symptoms. In the present study, we were able to show an elevated IL-2 production in FMS patients whose symptoms resemble much those of the sickness response. In addition, when the patient group was subdivided in tertiles according to scores on the FIQ-G and the PSQI, we found that a greater impact of FMS symptoms in the patients was associated with slightly elevated IL-2 levels. Statistical analyses did not reach significance when differences in IL-2 levels were considered, which might be explained by very small group sizes: only six

patients comprised the group with the highest symptom burden. Despite this limitation, a significantly higher IL-2/IL-4 ratio was found in the patients with highest FIQ-G scores indicating an increased pro-inflammatory activity in this patient subgroup. In terms of the impact of sleep disturbances as one prominent symptom in FMS, we observed an elevated IL-2 production in patients in the highest tertile of sleep disturbance scores. The shift towards augmented Th1 immunity was also indicated by a trend towards a higher IL-2/IL-4 ratio. Therefore, in spite of the small sample size presenting a clear limitation to our analyses, we were able to demonstrate an increased pro-inflammatory activity in a subgroup of patients reporting worst health status and most pronounced sleep difficulties when compared to patients with less severe symptoms. Even though a causative link cannot be drawn from our findings, we were thus able to show a relationship between a disorder whose symptoms resemble much those of the sickness response and an elevated pro-inflammatory activity.

As reported in Chapter 3 (Section 3.4.2), we found lower cortisol levels in the FMS patients than in the healthy subjects. These differences were even more pronounced when subgroups consisting of FMS responders and non-responders and of controls responders and non-responders were built based on the AUCi for salivary cortisol in the TSST. Healthy responders had significantly higher cortisol levels than patient responders, while no significant differences were found between the two non-responder groups. In the present chapter, we investigated if a blunted adrenal activity as observed in the FMS responder group was reflected by changes in cytokine profiles in terms of an elevated IL-2 and a decreased IL-4 production in response to the stress test. After Bonferroni adjustment for multiple comparisons, the IL-2/IL-4 ratio 120 min after the TSST was still significantly increased in the FMS responder group compared to the healthy responder group. The results support the recommendation of other scientists that post-stress cytokine assessments should be extended to at least two hours in order to increase the chance of revealing stress-induced cytokine changes as well as variations in the rate of changes between groups (Brydon, Edwards, Mohamed-Ali & Steptoe, 2004; Steptoe et al., 2001). For example, Brydon and colleagues (2004) compared the immunological and cardiovascular reactivity to a mental stress test between men with high socioeconomic status (SES) and men with low SES assuming that the latter were more prone to coronary artery disease. A prolonged stress-induced increase of IL-6

was found that was still present 120 min post-stress in the low SES group, whereas IL-6 levels stabilized in the high SES group after 75 min. The authors concluded that men in the low SES group showing an impaired post-stress recovery were less able to adapt to stress than men in the high SES group. In the present study, we made a similar observation of impaired post-stress recovery when we looked at stress-induced cytokine changes in conjunction with free cortisol reactions in the psychosocial stress test. Hypocortisolemic FMS patients, who showed a cortisol response in the TSST that was significantly lower than that of healthy responders, displayed a significantly higher IL-2/IL-4 ratio 120 min after cessation of the stress test. Consequently, a sustained disturbance of the Th1/Th2 immune balance might be present in FMS patients who showed inappropriately low adrenal activity in response to an acute stressor.

A line of evidence suggests that FMS might be related to chronic stress, such as stress at work (Kivimaki et al., 2004), and also to traumatic stress and early life stress. For example, higher rates of sexual and/or physical abuse as well as emotional neglect on the part of the parents have been reported in FMS patients (Goldberg et al., 1999; Imbierowicz & Egle, 2003; Van Houdenhove et al., 2001). Hellhammer and Wade (1993) postulated that hypocortisolism might develop after prolonged periods of stress that are at first accompanied by a hyperactivity of the HPA axis and excessive glucocorticoid release, followed by a change in HPA axis activity from hyper- to hypocortisolism. The mechanisms underlying this process are discussed elsewhere (Fries et al., 2005; Heim et al., 2000). Consequently, FMS might constitute a stress-related disorder with past and/or current chronic stress causing a permanent downregulation of HPA activity at least in a subgroup of patients. The term “stress” might also include stress with regard to immunological offenses. According to Raison and Miller (2003), prolonged exposure to immune stimuli might predispose an individual to diminished glucocorticoid signalling thus freeing bodily defences from the inhibitory control of glucocorticoids in the face of ongoing infections. Similar to the sickness response that is considered an adaptive strategy of the body to fight infection and inflammation, a reduction of inhibitory cortisol activity on inflammatory processes might be adaptive under certain conditions in which recurrent infection is likely and immune readiness is an attendant requirement (Raison & Miller, 2003).

The assumption of elevated pro-inflammatory activity in hypocortisolemic patients is supported by one study comparing PHA-stimulated IL-2 secretion in patients with Cushing's syndrome with IL-2 secretion in healthy subjects and hypocortisolemic patients suffering from primary and secondary adrenal insufficiency including Addison's disease (Sauer et al., 1994). IL-2 secretion was inhibited in patients with Cushing's syndrome, whereas it was significantly elevated in the hypocortisolemic patients indicating an increased pro-inflammatory activity in association with a clinically relevant glucocorticoid deficit. We are aware that the type of hypocortisolism investigated in the present study was only a mild, subclinical form. Nevertheless, we were able to demonstrate a significantly elevated IL-2/IL-4 ratio in our hypocortisolemic FMS patients. However, further studies with larger sample sizes are needed that replicate our finding of increased pro-inflammatory activity in a subgroup of patients with reduced adrenal activity. In addition, it might be interesting to extend post-stress monitoring even further in order to learn more about the duration of the Th1/Th2 disequilibrium in hypocortisolemic FMS patients and to find out if this post-stress disequilibrium is related to worsening of FMS symptoms.

Taken together, our results indicate a higher IL-2 production and an elevated IL-2/IL-4 ratio in female FMS patients as compared to healthy, age-matched women. Fibromyalgia symptoms that resemble those of the sickness response such as pain, fatigue, and sleep disturbances, seem to play an important role in cytokine disturbances because the shift to Th1 immunity was especially pronounced in patients with highest symptom burden as assessed by two questionnaires. In support of the assumption that FMS constitutes a stress-related disorder that goes along with hypocortisolemic features associated with potential immune alterations, we found that a subgroup of patients who responded to the TSST with reduced cortisol levels (FMS responders) displayed an increased IL-2/IL-4 ratio two hours after the stress test in comparison to their healthy counterparts (controls responders). Since no differences in cytokine production were found between the FMS and healthy non-responder groups who failed to react to the TSST in terms of cortisol increases, this is the first study that shows a relationship between altered endocrine responses to a stressor and post-stress changes in cytokine production in FMS patients. Further studies are needed replicating our findings of alterations in endocrine-immune interactions in larger samples of FMS patients.

Chapter 6

6 General Discussion

Within the framework of this thesis, possible alterations in HPA, SNS, and immune system activity were investigated in female FMS patients compared to healthy, age-matched women. Data was obtained from saliva and blood samples that were assessed before and after a psychosocial stress test. Three hypotheses were tested that will be resumed individually in the following sections.

6.1 Summary of results

6.1.1 Hypothesis 1

This hypothesis was subdivided into two parts: the first part examined the incidence of hypocortisolemic features in FMS patients in combination with an exaggerated SNS activity. In the second part, the occurrence of low NK cell levels as potential consequence of chronically increased norepinephrine concentrations was investigated. For these purposes, cortisol, norepinephrine, and NK cell levels and their changes in response to a psychosocial stress test, the TSST, were analyzed. Additional analyses included ACTH and further lymphocyte subpopulations such as T cells, cytotoxic T cells, T helper cells, and B cells.

In support of the hypothesis, decreased salivary cortisol levels were found in the patient group that were especially pronounced when only cortisol responders with regard to a positive AUCi were compared between the patient and control groups (see Chapter 3, Section 3.3.5, for the definition of responder groups). Additionally, plasma cortisol levels were significantly lower in the patient group, a finding that was also applicable when only the two responder groups were compared. These findings confirm previous studies that have observed a mild hypocortisolism in FMS patients. In addition, no differences were found in ACTH levels between the patient and the control groups, thus further underscoring the assumption of a reduced HPA activity on adrenal level associated with FMS.

As hypothesized, norepinephrine levels were significantly elevated in the female FMS group before and after the stress test. Unfortunately, no conclusions can be drawn from the present results on the chronicity of the observed alterations in SNS activity because only one baseline sample was assessed 30 min before the onset of the TSST. The determination of 24-h urinary norepinephrine concentrations would have been an appropriate method to shed more light on the chronicity of SNS alterations. However, since norepinephrine levels were already elevated at the pre-stress time

point in the FMS group, one might carefully interpret this observation as an indication of a persistently increased SNS activity that does not only occur in response to an acute stressor. This interpretation would be in accordance with the hypothesis that FMS constitutes a “sympathetically maintained pain syndrome”, in which permanent sympathetic hyperactivity results in sensitization of primary nociceptors thus evoking typical FMS symptoms such as widespread pain and allodynia (Martinez-Lavin, 2001; Martinez-Lavin et al., 2002; Martinez-Lavin, 2004). One should be aware, however, that the observed basal norepinephrine elevations in the patient group might as well have been the result of an increased agitation against the forthcoming stress test being more pronounced in the FMS group than in the healthy control group.

A potential indirect support of the assumption of permanent alterations in SNS activity in FMS patients was provided by the observation that NK cell levels were significantly lower in the FMS group than in the healthy control group. As described in Chapter 2, Section 2.2.3, and in Chapter 3, Section 3.2, chronic SNS activity might lead to decreased NK cell numbers in the peripheral blood. Blunted NK cell concentrations in the FMS patients despite higher norepinephrine levels might thus be a possible consequence of prolonged SNS activity.

In summary, it was shown that FMS patients display a reduced cortisol activity before and after a psychosocial stress test that is accompanied by increased norepinephrine levels and blunted NK cell concentrations. Although causalities cannot be drawn from these findings, the study might provide a first indication of the consequences of disturbed HPA activity on the SNS and the immune system in FMS patients.

6.1.2 Hypothesis 2

The second objective of the present work was to examine the response of the pro-inflammatory cytokine MIF to the TSST and to compare these responses between the FMS and the control groups. Since MIF has been considered to play an important role in a number of disorders that share some symptoms with FMS, increased levels were expected in the FMS group. Accordingly, a decreased cortisol/MIF ratio in the patient group was hypothesized.

All in all, the TSST induced significant increases in MIF levels in the whole study group. Unlike hypothesized, no differences were detected in total serum MIF levels between FMS patients and healthy subjects. However, net MIF increases in response to the TSST as compared to baseline levels were significantly higher in the FMS

group than in the healthy control group indicating an increased MIF reactivity after the stress test in the patients. With regard to the cortisol/MIF ratio, the hypothesis could partially be confirmed in that the ratio was lower in the FMS group. This result, however, remained only marginally significant when BMI was entered as control variable in the statistical analyses. The latter observation is in accordance with previous studies reporting positive correlations between body mass and MIF levels (see Chapter 4, Section 4.2, for references).

In consideration of the role of MIF as potent counter-regulator of glucocorticoid activity and as important inducer of inflammatory mediators, the results might suggest that elevated MIF levels in response to stress and a trend towards a reduced cortisol/MIF ratio indicate an increased post-stress inflammatory activity in FMS patients.

6.1.3 Hypothesis 3

The production of different pro- and anti-inflammatory cytokines before and after the TSST was simultaneously measured with a biochip array in order to test the hypothesis of increased pro-inflammatory and decreased anti-inflammatory cytokine activity in FMS patients. On the part of pro-inflammatory cytokines, an increased IL-2 production was observed in the FMS group, while IL-1, IL-6, TNF-α, and IFN-γ levels did not differ between the two study groups. With regard to anti-inflammatory cytokine production, IL-4 levels were slightly lower in the patients, whereas no differences were observed in IL-10 levels. In addition, IL-8 concentrations were similar in the FMS and control groups. In an attempt to obtain an index of Th1/Th2 immune balance, the IL-2/IL-4 ratio was calculated revealing significantly higher ratios in the patient group. Hence, in further analyses, only IL-2 and IL-4 levels and the IL-2/IL-4 ratio were taken into consideration because of the missing group differences in the other cytokines studied.

As part of the hypothesis, associations between FMS symptoms assessed by the FIQ-G and sleep disturbances assessed by the PSQI with cytokine levels were analyzed in the FMS group. Patients in the highest tertile of symptom burden displayed an elevated IL-2/IL-4 ratio as compared to patients in the lowest and middle tertile. With regard to sleep disturbances, IL-2 levels were increased and the IL-2/IL-4 ratio was slightly elevated in the patients whose PSQI scores were in the highest tertile. These results suggest a partial role of altered pro- and anti-

inflammatory cytokine activity, as represented by IL-2 and IL-4 levels, in the extent of symptom severity in FMS patients.

Finally, associations between salivary cortisol responses to the TSST and cytokine concentrations were studied by comparing post-stress IL-2 and IL-4 levels and the IL-2/IL-4 ratio between the cortisol responder and non-responder groups that were defined in Chapter 3 (Section 3.3.5). Results revealed no group differences in IL-2 and IL-4 production; however, the FMS responder group had a significantly elevated IL-2/IL-4 ratio two hours after the TSST compared to the healthy responder group. Therefore, blunted cortisol concentrations in the context of psychosocial stress might be related to a Th1/Th2 disequilibrium and a worsening of Th1-induced sickness behavior occurring with some delay after stress cessation.

In summary, the third hypothesis was partly confirmed in that levels of the pro-inflammatory cytokine IL-2 were elevated in the FMS group, whereas IL-4 levels were slightly decreased, which was also reflected by an increased IL-2/IL-4 ratio in the patients. However, no other cytokine was affected by group affiliation. The extent of sickness-response-like symptoms in the patient group was related to IL-2 levels and the IL-2/IL-4 ratio, and an increased IL-2/IL-4 ratio 120 min after the TSST was associated with blunted cortisol responses in a subgroup of FMS patients.

6.2 Model of neuro-endocrine-immune interactions in FMS

Based on the present results, an integrative model is proposed combining the observed HPA, SNS and immune system alterations in FMS patients (Figure 6.1). Figure 6.1A exemplarily depicts neuro-endocrine-immune interactions as expected in healthy individuals, while Figure 6.1B describes the biological changes observed in the patient group, as well as additional potential changes concerning the HPA axis that might arise from the present study results. The central role of cortisol in the course of this thesis is reflected by its central position in the two graphics.

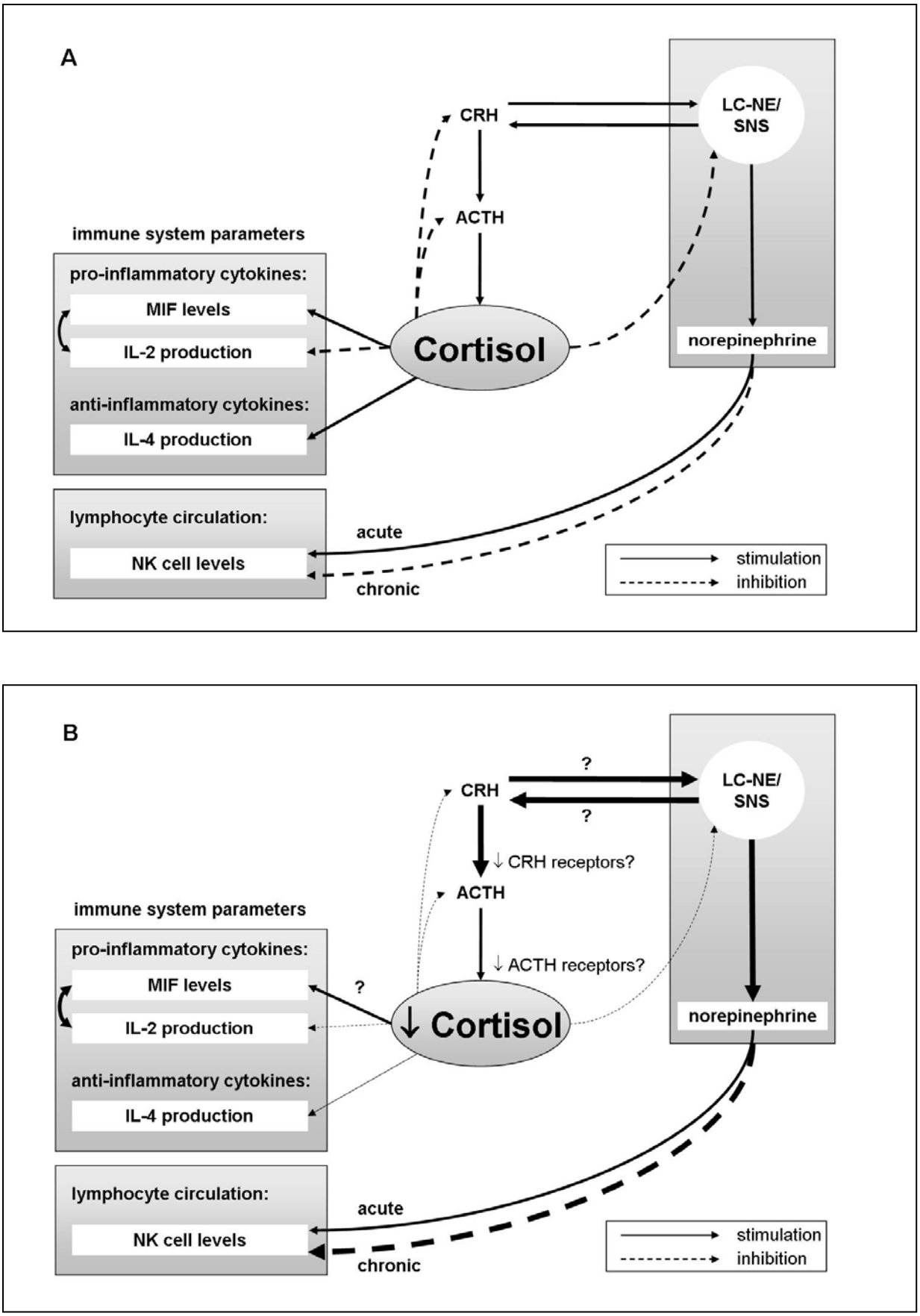

Figure 6.1: Selected neuro-endocrine-immune interactions as assumed in healthy individuals (A) and possible alterations in FMS patients derived from the present study results (B).

In the present study, as indicated by different arrow thicknesses in Figure 6.1B compared to Figure 6.1A, reduced cortisol levels in the FMS group were accompanied on the part of SNS activity by elevated norepinephrine concentrations potentially resulting in decreased NK cell numbers. On the part of cytokine activity, diminished cortisol levels went along with enhanced MIF increases in response to the TSST, a disinhibited IL-2 production, and a trend towards a blunted IL-4 production.

Focusing on the potential role of hypocortisolism in altered SNS activity, two explanations seem most feasible for the observed augmented norepinephrine release in the patient group: firstly, an exaggerated SNS activation might be the direct result of diminished negative cortisol feedback on the LC-NE/SNS system (Kvetnansky et al., 1993; Pacak et al., 1993). Alternatively, a reduced negative feedback of glucocorticoids at the hypothalamic level in hypocortisolemic FMS patients might cause CRH disinhibition and consequently an enhanced CRH release within the LC like observed in adrenalectomized rats (Pavcovich & Valentino, 1997). With regard to the consequences of altered CRH activity on the HPA axis, a disinhibition of CRH neurons might hypothetically have caused excessive ACTH release resulting in downregulation of ACTH receptors on the adrenal level. This stage might have been followed by a downregulation of CRH receptors in the pituitary, thus explaining normal ACTH and blunted cortisol responses despite potentially enhanced CRH activity.

Although we were unable to test the hypothesis of increased CRH activity within the scope of this thesis, CRH elevations have been repeatedly linked to FMS by another working group (Neeck & Riedel, 1999; Neeck & Crofford, 2000; Neeck, 2002; Riedel et al., 1998; Riedel et al., 2002). As pointed out by Neeck and Riedel, hyperactivity of CRH neurons in FMS seems to not only effect HPA activity but also causes changes in the set point of other hormonal axes such as the growth-hormone-insuline-like-growth-factor-1 (GH-IGF-1) axis. CRH stimulates somatostatin secretion at the hypothalamic level, which in turn causes inhibition of the GH-IGF-1 axis (Katakami, Arimura & Frohman, 1985). Consequently, in FMS patients with CRH hyperactivity, decreased GH and/or IGF-1 levels would be expected, which has indeed been observed in several studies (Bagge, Bengtsson, Carlsson & Carlsson, 1998; Bennett, Cook, Clark, Burckhardt & Campbell, 1997; Landis, Lentz, Rothermel, Riffle, Chapman, Buchwald & Shaver, 2001; Leal-Cerro, Povedano, Astorga, Gonzalez, Silva, Garcia-Pesquera, Casanueva et al., 1999). As reviewed by Bennett, GH

deficiency occurs in approximately 30% of FMS patients (Bennett, 1998; Bennett, 2002), an observation that might indirectly support the assumption of CRH hyperactivity in these patients.

In addition to the central effects of hypothalamic CRH, it has been shown that CRH can also be secreted peripherally at inflammatory sites thus directly influencing the immune system through local modulatory actions. Importantly, while the effects of hypothalamic CRH on the immune system are mainly anti-inflammatory and mediated by glucocorticoids, immune-derived CRH is considered a pro-inflammatory agent (Crofford, Sano, Karalis, Webster, Goldmuntz, Chrousos & Wilder, 1992; Karalis, Sano, Redwine, Listwak, Wilder & Chrousos, 1991; Webster, Barrientos, Contoreggi, Isaac, Ligier, Gabry, Chrousos et al., 2002). Data suggests that mast cells are a major target of immune CRH. For example, studies have shown that both acute stress resulting in local CRH increases and intradermal CRH injection induce a marked increase in vascular permeability and mast cell degranulation (Lytinas, Kempuraj, Huang, Boucher, Esposito & Theoharides, 2003; Theoharides, Singh, Boucher, Pang, Letourneau, Webster & Chrousos, 1998). Since mast cells are able to release inflammatory mediators including the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Kwon, Lee, Moon, Jung, Lin, Nam, Baek et al., 2002; Shin, Song, Kim & Shin, 2004), exaggerated immune-derived CRH activity might cause an increased release of mast cell derived cytokines resulting in FMS-related symptoms. In this context, Lucas and colleagues have recently hypothesized that FMS constitutes a neuro-immuno-endocrine disorder where increased release of CRH and substance P from neurons in specific muscle sites triggers local mast cells to release pro-inflammatory and neurosensitizing molecules that contribute to the symptoms of pain experienced by FMS patients (Lucas, Brauch, Settas & Theoharides, 2006). This idea needs further elaboration in future studies.

Unlike our expectations, pro-inflammatory cytokine production did not differ between FMS patients and healthy subjects with the exception of increased IL-2 levels in the patient group. The fact that IL-2 levels were already elevated before the stress test might indicate a chronic alteration of IL-2 activity in the FMS patients. Hypothetically, blunted cortisol levels as observed in the patient group might partly explain higher IL-2 levels occurring due to an attenuated glucocorticoid-mediated inhibition of pro-inflammatory activity. Our results do not allow for this conclusion because data on

cortisol and IL-2 levels over an extended period of time ideally starting before the onset of the disease would have been needed to test this hypothesis.

Importantly, although pro-inflammatory cytokines that are typically linked to the sickness response such as IL-1, TNF-α, and IL-6 did not differ in the present study between FMS patients and healthy subjects, the role of IL-2 in the induction of symptoms of the sickness response should not be underestimated. Data from studies with cancer patients undergoing IL-2 treatment underline the role of this cytokine in eliciting sickness symptoms such as myalgias, fatigue, malaise, concentration difficulties, sleep disturbances, and depression (Cleeland et al., 2003; Lee et al., 2004; Yirmiya et al., 2000). These symptoms usually disappear shortly after termination of IL-2 administration thus circumstantiating the causative role of this cytokine in the generation of the sickness response. Our observation that IL-2 levels were elevated in FMS patients with worst sleep disturbances and that the IL-2/IL-4 ratio was increased in FMS patients with highest symptom burden seems to support our suggestion of an involvement of IL-2 in FMS symptoms that resemble those of the sickness response.

Besides an elevated IL-2 production, IL-4 levels were slightly decreased in the FMS group, which was further reflected by a significantly elevated IL-2/IL-4 ratio. IL-4 constitutes a pleiotropic anti-inflammatory cytokine that is mainly produced by activated Th2 lymphocytes and mast cells (Janeway et al., 2001). In addition to its effects on several immune cells including the activation and support of B cell growth and the initiation of IgE production, IL-4 inhibits macrophage activation and directs T helper cell differentiation to the Th2 side (Okada, Banchereau & Lotze, 2003), which might in part be achieved by the suppression of pro-inflammatory cytokines (Hart, Vitti, Burgess, Whitty, Piccoli & Hamilton, 1989). Another important property of IL-4 is its performance of antihyperalgesic effects. As shown in a rat model of mechanical hyperalgesia, IL-4 is capable of inhibiting hyperalgesic responses to carrageenin, bradykinin, TNF-α, and IL-1β (Cunha, Poole, Lorenzetti, Veiga & Ferreira, 1999). In addition, pre-treatment of mice with IL-4 inhibits the writhing response induced by intraperitoneally application of acetic acid or zymosan (Vale, Marques, Moreira, Rocha, Ferreira, Poole, Cunha et al., 2003). In a recent study using a rat model of neuropathic pain, transgene-mediated expression of IL-4 reduced mechanical allodynia and thermal hyperalgesia in response to spinal nerve ligation and reversed up-regulation of IL-1 and PGE_2 in the spinal cord (Hao, Mata, Glorioso & Fink, 2006).

As addressed in Chapter 2, Section 2.2.5.2, pro-inflammatory cytokines released by glial cells in the spinal cord during sickness might be critically involved in the creation and maintenance of pathological pain (Wieseler-Frank et al., 2005). Consequently, slightly blunted IL-4 activity as observed in our patient group might result in a reduction of its antihyperalgesic effects and a disinhibition of spinal pro-inflammatory activity, thus potentially explaining some of the chronic pain symptoms experienced by our FMS patients.

In summary, our results suggest that FMS is the result of an interplay between different bodily systems, in which subtle alterations have been observed. Thus, pain and other symptoms experienced by FMS patients might be triggered by elevated sympathetic activity, weakened innate immune responses, and/or increased pro-inflammatory and decreased anti-inflammatory activity as indicated by a blunted cortisol/MIF ratio and a Th1/Th2 imbalance with regard to IL-2 and IL-4 production. The role of the observed hypocortisolism as potential origin of sympathetic and immune alterations resulting in sickness-response-like symptoms in FMS patients needs to be elucidated in future studies.

6.3 Limitations of the studies and resulting implications

The presented studies have several limitations of which some will be discussed in the present section.

Most importantly, the reported results do probably not apply to the whole population of FMS patients for a number of reasons: firstly, we included only patients without concomitant chronic viral and/or autoimmune disorders in order to make sure that potential immunological changes in the patient group were not due to co-diagnosed disorders with demonstrable involvement of the immune system. For example, with regard to the comorbidity between FMS and chronic HCV infection, a subgroup of approximately 15-16% of FMS patients (Buskila et al., 1997b; Buskila et al., 1998; Rivera et al., 1997) was not taken into consideration in our studies. In addition, rheumatoid arthritis patients also fulfilling the diagnosis of FMS, which is the case in about 17% of patients (Wolfe & Michaud, 2004), were excluded from the study as well as patients with other autoimmune disorders. Thus, the study results might not apply to FMS patients with additional diagnoses considering that neuro-endocrine-immune interactions might be different in these patients. In order to investigate if

potential biological alterations in this patient subgroup are attributable to FMS or to underlying autoimmune disorders and/or chronic viral infections, large sample sizes are needed allowing for the comparison of biological markers between patients who are only diagnosed with FMS and those who are co-diagnosed with additional disorders.

Other research groups have emphasized the importance of building FMS subgroups based on psychosocial and behavioral characteristics. In one study, FMS patients were sub-classified as dysfunctional, interpersonally distressed, or adaptive copers based on responses to the Multidimensional Pain Inventory (MPI). Depending on subgroup affiliation, the patients showed substantial differences in clinical symptom presentation (Turk, Okifuji, Sinclair & Starz, 1996). Similarly, Thieme and colleagues (2005) used the same subgroup distribution as Turk and colleagues. They reported highest pain behaviors such as groaning and pain-based refusal of activities in FMS patients classified as dysfunctional. Interestingly, pain behavior was significantly negatively associated with cortisol levels in this patient subgroup (Thieme, Spies, Sinha, Turk & Flor, 2005). In summary, these results underscore the importance of regarding FMS as a heterogeneous disorder. As pointed out by both Thieme and Turk, treating patients with FMS as a homogeneous group might compromise research results, impede understanding of the mechanisms underlying this condition, and result in inappropriate and inadequate treatment (Thieme et al., 2005; Turk et al., 1996).

A second reason for the limited generalizability of results is the fact that we included only data from female FMS patients in the analyses implying that the results are most likely not applicable to male patients. Unfortunately, we were unable to recruit more than 5 male FMS patients, which can be explained by a much lower prevalence of the disease among men comprising only 10% of the FMS population (Yunus, 2002). Since gender differences in HPA (re)activity were reported in some studies under basal conditions (reviewed by Kirschbaum & Hellhammer, 1999) and more pronounced in response to an applied stressor such as the TSST (Kajantie & Phillips, 2006; Kirschbaum, Kudielka, Gaab, Schommer & Hellhammer, 1999; Uhart, Chong, Oswald, Lin & Wand, 2006), we decided that data from male and female study participants should be examined differently. This led to the exclusion from the male data due to the limited sample size that was too small for statistical analyses.

Interestingly, the impact of gender as an important determinant of human health has lately received growing attention based on the observation that men are usually more susceptible to cardiovascular and infectious diseases, whereas women develop more often FMS, other chronic pain conditions, and autoimmune disorders. While cardiovascular diseases and susceptibility to infection have been associated with increased HPA activity (Elenkov, 2004; Reynolds, Walker, Syddall, Andrew, Wood, Whorwood & Phillips, 2001), blunted cortisol concentrations have been observed in association with FMS (see Chapter 3), other chronic pain conditions (Heim et al., 2000), and autoimmune disorders such as rheumatoid arthritis (Cutolo, Sulli, Pizzorni, Craviotto & Straub, 2003). According to these findings, one might suggest that men are more susceptible to disorders associated with increased cortisol secretion, whereas women might be more vulnerable for disorders characterized by a mild hypocortisolism.

In this context, the role of the fetal environment on health throughout the life course has been increasingly recognized (de Rooij, Painter, Phillips, Osmond, Michels, Godsland, Bossuyt et al., 2006; Kajantie, Osmond, Barker, Forsen, Phillips & Eriksson, 2005; Phillips, 2004; Ward, Moore, Steptoe, Cockington, Robinson & Phillips, 2004). While the understanding of possible differences in the consequences of maternal stress between human male and female fetuses and its impact on gender-dependent disease vulnerability in adult life is still in its infancy, first results indicate a discrepant cortisol responsivity in intrauterine growth restricted healthy young males and females (Buss, Wadiwalla, Hellhammer, Meaney, Lupien & Pruessner, in prep.). In Buss' study, male subjects born small for gestational age showed a significantly higher salivary cortisol response after a psychosocial stress test than female subjects in the same prenatal risk group. Compared to subjects born appropriate for gestational age, the cortisol stress response in the male risk group was slightly elevated, whereas cortisol levels in the female risk group were rather blunted. These results strengthen the hypothesis of the adrenocortical stress response being programmed in a gender-specific way in that men with impaired fetal growth are more prone to HPA hyperreactivity and women in the same risk group more vulnerable to HPA hyporeactivity. With regard to FMS, it would be interesting to follow-up those women in the prenatal risk group in order to investigate if the discrepancies in the stress response might in some cases result in the development of FMS – a disorder showing an increased prevalence in older-aged women (Wolfe et

al., 1995b). Interestingly, preliminary evidence suggests a higher incidence of prenatal stress in female FMS patients in terms of increased family conflicts and financial constraints during pregnancy as well as shorter gestational length and less maternal appreciation of the pregnancy (Klingmann, Kugler & Hellhammer, in prep.). Doubtlessly, this issue and its underlying biological mechanisms consequently leading to the development of FMS symptoms will be of growing interest in future studies.

Finally, another limitation of our studies is the rather small sample size that certainly has prevented the detection of small effects due to lack of statistical power. This became especially relevant in the context of the generation of patient subgroups according to the extent of symptom burden and sleep disturbances. When FMS patients in the lowest/middle tertiles of FIQ-G and PSQI scores were compared with patients in the highest tertile, the latter group consisted of only 6 patients. This might have contributed to the fact that descriptively apparent group differences, e.g. in terms of IL-2 and IL-4 levels with regard to FIQ-G scores (see Chapter 5, Figure 5.4), did not reach statistical significance. A significantly elevated IL-2/IL-4 ratio was found in patients with highest symptom burden and significantly increased IL-2 levels were detected in those with greatest sleep disturbances (Chapter 5, Section 5.4.5) despite limited group sizes indicating the relevance of the involvement of cytokine alterations in symptom severity of FMS patients.

In conclusion, despite several limitations, our results have shed light on biological alterations in three strongly interacting bodily systems in female FMS patients. In the future, longitudinal studies with greater patient cohorts are needed disentangling the complexity of changes in neuro-endocrine-immune interactions in FMS and related disorders. The investigation of causalities, specifically concerning the chronological order of biological alterations observed in FMS patients, as well as the contribution of prenatal risk factors in this context will be of special relevance.

7 References

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Hiermit versichere ich, dass ich die vorliegende Dissertationsschrift selbständig verfasst und keine anderen als die angegebenen Hilfsquellen verwendet habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Erkrath, 20. Oktober 2006

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Dipl.-Psych. Judith Heße-Husain