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Nina Groven

A Thesis on Immune Differences in Chronic Fatigue Syndrome, Fibromyalgia and Healthy Controls

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
Faculty of Medicine and Health Sciences
Department of Mental Health

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Trondheim, January 2023

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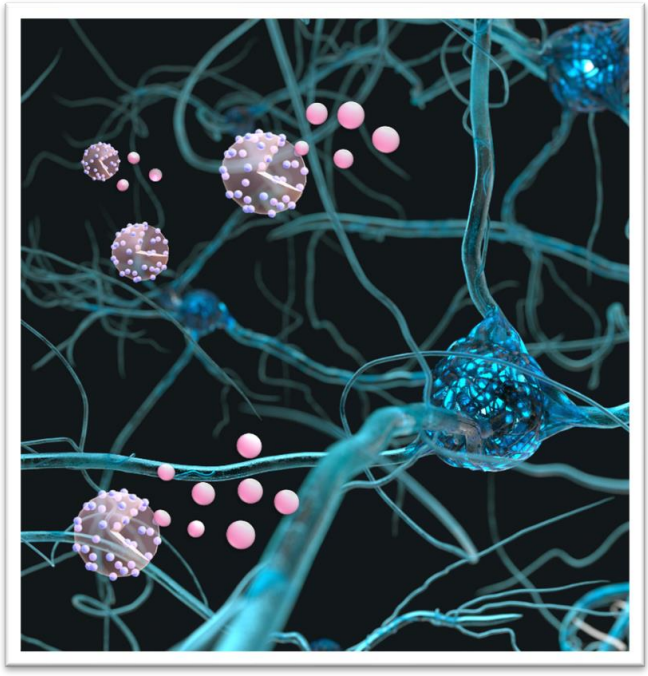

Norsk sammendrag (Summary in Norwegian)

Tittel: **En avhandling om immunforskjeller i kronisk utmattelsessyndrom, fibromyalgi og friske kontroller**

Kronisk utmattelsessyndrom (CFS) og fibromyalgi (FM) kjennetegnes ved ekstrem utmattelse og kroniske smerter. Selv om begge diagnosene er omdiskutert, er det ingen tvil om at disse pasientene lider og at dette utgjør en byrde for samfunnet i form av tapt arbeidsevne og liknende. CFS og FM har mange likhetstrekk, og begge har symptomer som minner om sykdommer knyttet til immunsystemet. Vi vet fremdeles lite om *hvilken* rolle immunsystemet kan ha i sykdomsbildet hos disse pasientene. Disse pasientene har ofte tilleggssymptomer på depresjon, noe som kan bety at sentralnervesystemet er involvert. En noe nyere tilnærming på sykdommer i sentralnervesystemet er knyttet mot kynureniner, men disse har i liten grad blitt studert i CFS- og FM-pasienter. Denne avhandlingen tar for seg en mer omfattende undersøkelse av immunologiske faktorer for å kunne kaste lys over eventuelle immunologiske avvik og/eller likheter mellom CFS- og FM-diagnosene.

Vi undersøkte blodet av 49 CFS-pasienter, 58 FM-pasienter og sammenlignet disse med de samme markørene hos 54 friske kontroller. Alle deltakerne var kvinner mellom 18 og 60 år. Funnene våre viste at begge pasientgruppene (CFS og FM) hadde høyere verdier av markører for betennelse: høysensitiv (eller mikro-) CRP (hsCRP) og monocyte chemoattractant protein (MCP)-1 i blodet sammenlignet med friske kontroller. Kroppsmasseindeks (BMI) hadde også sammenheng med nivåene av hsCRP. Begge pasientgruppene hadde ellers lavere verdier av flere cytokiner: Interleukin (IL)-1β, IL-4, IL-6, IL-10, IL17, transforming growth factor (TGF)-β1, TGF-β2, TGF-β3 og tumour necrosis factor (TNF)-α. For disse immunomarkørene kunne vi ikke skille CFS-gruppa fra FM-gruppa. FM hadde lavere verdier av Interferon (IFN)-γ sammenlignet med CFS og de friske kontrollene. Vi fant ingen forskjeller for IL-1ra, IL-8 og interferon gamma-induced protein (IP)-10. Det relative forholdet mellom kynureninene quinolininsyre (QA) og kynureninsyre (KA) [QA/KA] var lavere for CFS sammenlignet med friske kontroller; og det relative forholdet mellom KA og 3-hydroxykykynurenin (HK) [KA/HK] og xanturensyre (XA) og HK [XA/HK] var lavere for FM pasienter sammenlignet med friske kontroller. Sistnevnte ratio var også assosiert med BMI og smerte.

Konklusjon: I denne studien fant vi at begge pasientgruppene hadde høyere verdier av hsCRP og MCP-1 sammenlignet med friske kontroller. Lavere QA/KA kan være indikasjon på høyere neurotoksisitet i CFS-gruppa, og XA/HK kan være indikasjon på lavere aktivitet av enzymet som konverterer HK til XA (kynurenin-aminotransferase II). Vi fant assosiasjoner mellom fatigue og smerte for kynurenin og XA/HK, og dette belyser vanskeligheten med å skille symptomer fra diagnosene CFS og FM. Framtidig forskning bør implementere basalstudier og kliniske studier for å se nærmere på biologiske mekanismer knyttet mot kliniske symptomer på CFS og FM.

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Dear Cuneyt, Already before I started working with you, I knew you were all brains and that I would benefit immensely from your talent as a researcher and writer. I was nothing more than star-struck when you agreed to participate with my third article. It is an honour to have you as my official supervisor (long overdue). Your sense of humour is price-less, and having philosophical discussions with you, enlighten me and everyone around you. Your heart and professionalism are gold. I cannot give you enough credit – you deserve much more than just an acknowledgement in a humble thesis.

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Summary

Background: Chronic Fatigue Syndrome (CFS) and Fibromyalgia (FM) are debilitating disorders that significantly affect the daily lives of those suffering from them, as well as their loved ones. Both conditions have overlapping clinical features that resemble inflammatory disorders, and overlapping symptoms, such as depression, suggest central nervous system (CNS) involvement. The role of the immune system's soluble messengers in the pathogenesis of CFS and FM has been under investigation, but so far the results are inconclusive. In addition, there is growing evidence that the kynurenine pathway is involved in the pathology of diseases related to the CNS, yet the role of each metabolite is not clear. The relationship between kynurenine metabolism and CFS and FM has not been extensively explored. Few studies have simultaneously examined the immunological status in both CFS and FM, making this thesis the first to comprehensively evaluate the potential distinct immunological differences between the two disorders.

Objective: The objective of this study was to compare the CFS and FM with healthy controls, regarding the levels of several soluble blood markers related to the immune system. The markers chosen were:

- The inflammatory marker high-sensitive CRP (hsCRP)
- The following cytokines and chemokines: Interferon (IFN)-γ, Interleukin (IL)-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17, Interferon gamma-induced protein (IP)-10, Monocyte Chemoattractant Protein (MCP)-1, Transforming Growth Factor (TGF)-β1, TGF-β2, TGF-β3 and Tumour Necrosis Factor (TNF)-α
- The metabolites and their ratios of the kynurenine pathway: Tryptophan (Try), kynurenine (Kyn), kynurenic acid (KA), 3-hydroxykykynurenine (HK), anthranilic acid (AA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (HAA), quinolinic acid (QA) and picolinic acid (Pic).

Method: The population consisted of three groups: CFS patients (n = 49), FM patients (n = 58), and healthy controls (n = 54). All participants were females aged 18–60. Patients were recruited from a specialised university hospital clinic and controls were recruited by advertisement among the staff and students at the hospital and university. Plasma levels of hsCRP were analysed at the hospital. The cytokines and chemokines IFN-γ, IL-1β, IL-1ra,

IL-4, IL-6, IL-8, IL-10, IL-17, IP-10, MCP-1, TGF-β1, TGF-β2, TGF-β3, and TNF-α were analysed by multiplex. Kynurenine metabolites were analysed by LC-MS/MS. Linear regression models of log-transformed data for hsCRP and the kynurenine metabolites were conducted for comparison of the three groups CFS, FM and controls. The Kruskal-Wallis test was used to analyse differences of cytokines between the three groups. Main findings were controlled for age, body mass index (BMI), and symptoms of anxiety and depression.

Results: hsCRP levels were significantly higher for both the CFS and FM groups compared to healthy controls when adjusting for age and BMI ($p = .006$). There was no difference between the two patient groups. Level of hsCRP was affected by BMI ($p < .001$) but not age.

MCP-1 was significantly increased in both patient groups compared to healthy controls ($p < .001$). IL-1β, Il-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3 (all $p < .001$), IL-10 ($p = .003$) and IL17 ($p = .002$) all were significantly lower in the patient groups compared to healthy controls. IFN-γ was significantly lower in the FM group ($p < .001$). For IL-8, IP-10 and IL-1ra there were no significant difference.

QA differed between CFS and FM patients ($p = .036$) and was related to higher levels of BMI ($p = .002$). The KA/QA ratio was lower for CFS patients compared to healthy controls ($p = .016$). The KA/HK ratio was lower for FM patients compared to healthy controls, and this lower ratio was associated with increased symptoms of pain ($p = .002$). The kynurenine aminotransferase II (KAT II) enzymatic activity given by XA/HK was lower for FM patients compared to healthy controls ($p = .013$). In addition, BMI was negatively associated with enhanced KAT II enzymatic activity ($p = .039$).

Symptoms of anxiety and depression were not associated with any of the immune markers studied.

Conclusion: In our material hsCRP and MCP-1 are increased in patients both with CFS and with FM, while several other cytokines are either similar or significantly lower in patients than controls. Our study also indicates associations between kynurenine metabolism and CFS and FM. Kynurenine also is associated with single symptoms such as fatigue and pain. Forthcoming studies indicating interactions and causative effects, or restoration of the inflammatory status, may place cytokines and kynurenine metabolites as a target for treatment as well as prevention of these conditions in the future.

List of Articles

The present thesis is based on the following articles:

Article 1

Groven N, Fors EA, Reitan SK. Patients with Fibromyalgia and Chronic Fatigue Syndrome show increased hsCRP compared to healthy controls. *Brain, Behavior, and Immunity*. 2019;81:172-177. doi:10.1016/j.bbi.2019.06.010

Article 2

Groven N, Fors EA, Stunes AK, Reitan SK. MCP-1 is increased in patients with CFS and FM, whilst several other immune markers are significantly lower than healthy controls. *Brain, behavior, & immunity - health*. 2020;4:100067. doi:10.1016/j.bbih.2020.100067

Article 3

Groven N, Reitan SK, Fors EA, Guzey IC. Kynurenine metabolites and ratios differ between Chronic Fatigue Syndrome, Fibromyalgia, and healthy controls. *Psychoneuroendocrinology*. 2021;131:105287-105287. doi:10.1016/j.psyneuen.2021.105287

Abbreviations

5-HT	Serotonin
ACR	American College of Rheumatology
APA	American Psychiatric Association
BD	Bipolar disorder
BMI	Body mass index
BPI	Brief pain inventory
Cachexia	A wasting syndrome with metabolic derangements (i.e. resulting in weight loss and muscle wasting) due to chronic inflammation
CFS	Chronic fatigue syndrome
CFQ	Chalder fatigue questionnaire
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
FM	Fibromyalgia
FS score	Fibromyalgia severity score
FSDC	Fibromyalgia survey diagnostic criteria
GAT	Gradual exercise therapy
GP	General practitioner
HPA	Hypothalamo-pituitary axis
hsCRP	High sensitivity C-reactive protein
IASP	International Association for the Study of Pain
ICD-10	International Classification of Diseases, 10th revision.
ICD-11	International Classification of Diseases, 11th revision.
ICP-2	International Classification of Primary Care, version 2
ICP-3	International Classification of Primary Care, version 3
IBS	Irritable bowel syndrome
IQR	Interquartile range
MeSH	Medical subject headings

MDD	Major depressive disorder
NAD+	Nicotinamide adenine dinucleotide
NMDA	*N*-methyl-D-aspartate
NK cells	Natural killer cells
NLM	National Library of Medicine
N. Vagus	Nervus Vagus; the Vagus nerve
N. Sympathicus	*Nervus Sympathicus; the sympathetic nerve*
NRS	Numeric rating scale
OCD	Obsessive compulsive disorder
PEM	Pose exertion malaise
PTSD	Posttraumatic stress disorder.
REK	Regional Committee for Medical and Health Research Ethics
SNRI	Selective serotonin and noradrenaline inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TDO	Tryptophan 2,3-dioxygenase
Th1 response	T helper cell type 1 response; Cell-mediated immunity (inflammatory response, cytotoxicity etc.)
Th2 response	T helper cell type 2 response; Humoral immunity (e.g. IgE production and allergies)
TMJD	Temporomandibular joint disorders
T_{reg} cells	T regulatory cells
WHO	World Health Organization

Cytokines and chemokines

Abbreviation	Name	Chemokines		
		Motif family	Ligand (L)	Symbol
IFN-γ	Interferon γ			
IL-1β	Interleukin 1β			
IL-1ra	Interleukin 1 receptor antagonist			
IL-4	Interleukin 4			
IL-6	Interleukin 6			
IL-8	Interleukin 8	CXC	L8	CXCL8
IL-10	Interleukin 10			
IL-17A	Interleukin 17			
IP-10	Interferon γ-induced protein 10	CXC	L10	CXCL10
MCP-1	Monocyte chemoattractant protein 1	CC	L2	CCL2
TGF-β1	Transforming growth factor β1			
TGF-β2	Transforming growth factor β2			
TGF-β3	Transforming growth factor β3			
TNF-α	Tumour necrosis factor α			

Kynurenines

Abbreviation	Name
AA	Anthranilic acid
HAA	3-hydroxyanthranilic acid
HK	3-hydroxykynurenine
KA	Kynurenic acid
Kyn	Kynurenine
Pic	Picolinic acid
QA	Quinolinic acid
Try	Tryptophan
XA	Xanthurenic acid

Kynurenine ratios

Abbreviation	Name / Enzyme[i]	Ratio	Concentration ratio
IDO	Indoleamine 2,3-dioxygenase	$\frac{\mathrm{Kyn}}{\mathrm{Trp}}$	$\frac{[\mathrm{lnKyn}]}{[\mathrm{lnTrp}]}$
KAT	Kynurenine aminotransferase	$\frac{\mathrm{KA}}{\mathrm{Kyn}}$	$\frac{[\mathrm{lnKA}]}{[\mathrm{lnKyn}]}$
KAT II	Kynurenine aminotransferase II	$\frac{\mathrm{XA}}{\mathrm{HK}}$	$\frac{[\mathrm{lnXA}]}{[\mathrm{lnHK}]}$
KMO	Kynurenine 3-monooxygenase	$\frac{\mathrm{HK}}{\mathrm{Kyn}}$	$\frac{[\mathrm{lnHK}]}{[\mathrm{lnKyn}]}$
-	Kynurenase	$\frac{\mathrm{AA}}{\mathrm{Kyn}}$	$\frac{[\mathrm{lnAA}]}{[\mathrm{lnKyn}]}$
-	Kynurenase	$\frac{\mathrm{HAA}}{\mathrm{HK}}$	$\frac{[\mathrm{lnHAA}]}{[\mathrm{lnHK}]}$
NPR-1	Neuroprotective ratio 1	$\frac{\mathrm{KA}}{\mathrm{QA}}$	$\frac{[\mathrm{lnKA}]}{[\mathrm{lnQA}]}$
NPR-2	Neuroprotective ratio 2	$\frac{\mathrm{KA}}{\mathrm{HK}}$	$\frac{[\mathrm{lnKA}]}{[\mathrm{lnHK}]}$
KAT has several isoforms, and KAT I and KAT II are used here to differentiate between the KA/Kyn and XA/HK ratios. The metabolic turnover (given as ratio) is used as an indicator of these enzymes.			

[i] Our measurements do not include the enzymes. The metabolic turnover (given as ratio) is used in this study as an indicator of these enzymes.

1 Introduction

> *If, indeed we should ever be so happy, as to arrive at that degree of perfection, in the practice of physic, as to be able, at once, to discover a single mark in all diseases, that should certainly determine the disorder to be this or that particular disease; it would very much lessen the labour and difficulty of our profession.*[1(p79)]

These words from Sir Richard Manningham's *Febricula* express the same wish as many of the researchers today, myself included: Finding biological markers that can determine any disorder. For many diseases this is already the case, and clear diagnostic specificities exist. For others, finding a clear cause of the disease, is less obvious. The medical field has advanced dramatically since Manningham's days, but still there are some not so clear-cut disorders. Diagnoses are made based on symptoms or symptom clusters. Two of these disorders are chronic fatigue syndrome (CFS) and fibromyalgia (FM). These disorders are yet not possible to distinguish based on biological markers, which leads to a vast discussion and often disagreement amongst clinical professionals as to what is the "real cause" of these disorders. In line with lack of "biological evidence" it is even doubted whether they be regarded as "disorders". Similarly to Manningham, we still don't know much about the aetiology of these disorders.

This thesis is dedicated to the search of biological markers underpinning our understanding of disorders like CFS and FM. I use the word disorder about CFS and FM, but one could discuss whether diagnosis, illness, suffering or condition would be better words.

In the following section, I will first clarify the diagnostic and research criteria of CFS and FM used in this study (Section 1.1). Then I will give a brief history of CFS and FM that led to the diagnostic criteria we are working from today (Section 1.2). Important terms related to the core symptoms of CFS and FM are introduced followed by related symptoms of CFS and FM and the possible mechanisms involved (Section 1.3). Next, parallels are drawn to symptomatic interactions with activity in the immune system and the scientific field of neuro-immunopsychiatry (Section 1.4). Finally, I will make a short outline of the immune system and rationale for investigating immune markers as well as the present state of this research field (Section 1.5).

1.1 Symptoms and Characteristics of Chronic Fatigue Syndrome and Fibromyalgia

1.1.1 Chronic fatigue syndrome – CFS

The reported prevalence of CFS is ranging from 0.5–2.5 %.[4] CFS is characterised by severe fatigue with distinct onset, lasting more than 6 months, not necessarily connected to ongoing exertion, not relieved by rest, and causing reduced function. In addition, the occurrence of at least four of the following eight symptoms is observed: impairment in short-term memory or concentration, sore throat, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, headaches of a new type, pattern, or severity, unrefreshing sleep, and post-exertional malaise lasting more than 24 hours.[5]

Patients with CFS often report a sudden onset of the disease. The symptoms of CFS typically occur during stressful life-events, after infection or prolonged infection, or a combination. This isn't necessarily the case, and so far, there is no evidence of neither specific infections nor autoimmune conditions causing CFS. However, alterations in the immune system after infections have been postulated.[6]

The aetiology of CFS is not known, and additional symptoms and postulated features of CFS include: Infections at onset of disease, immunological disturbances, neuroendocrinological alterations, and neuropsychiatric symptoms.[2,7,8] Many of these symptoms are also common in FM.

1.1.2 Fibromyalgia – FM

The reported prevalence of FM is up to 5 % of the general population.[9] Symptoms of FM often develop over time[10]. According to the American College of Rheumatology (ACR) 1990 diagnostic criteria of Wolfe et al.,[11] FM is characterised by widespread pain lasting more than three months, in combination with tenderness at 11 or more tender points and affection of at least three out of four quadrants of the body (Figure 1.1).

Widespread pain is defined as[11]:

- Pain in the left side of the body
- Pain in the right side of the body
- Pain above the waist
- Pain below the waist

Tender points:

- 18 specific tender point sites (Figure 1.1)
- Digital palpation with an approximate force of 4 kg
- Positive tender point = subject states that palpation was painful
- In addition: axial skeletal pain must be present

The practical value of tenderpoints has been debated by researchers in this field. The challenge of measuring tenderpoints is applying the correct pressure, which shows great variation between physicians. Thus, new diagnostic criteria, listed in the form of a survey questionnaire, have been developed in three steps.[12,13] This newer diagnostic evaluation of FM is described in the method Section 2.3.2 The fibromyalgia survey diagnostic criteria and discussed in Section 4.3.14 Fibromyalgia severity score, but has not been commonly used FM diagnostic criteria until recently.

The aetiology of FM is not known, but it is probably multifactorial, and involvement of several mechanisms has been postulated, such as central sensitisation and inflammation. Additional symptoms similar to CFS are often reported, including fatigue, sleep disturbance, cognitive difficulties, headache, anxiety, and depression.[11,14]

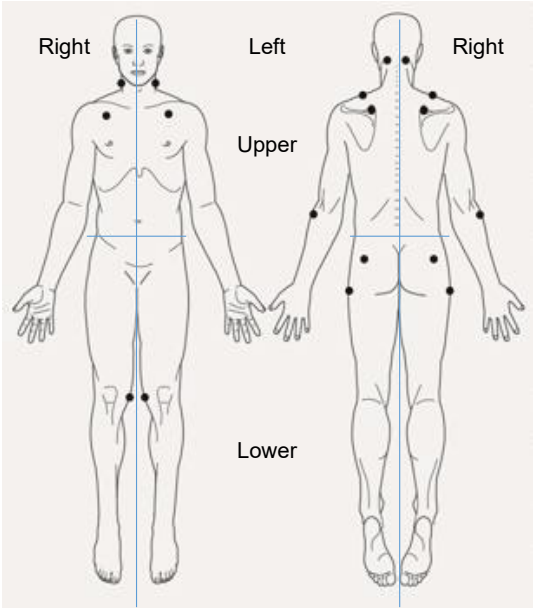


Figure 1.1.
Figure shows the axillary and mid lines (blue) separating the four quadrants of the body (upper right, upper left, lower right and lower left), and the locations of tender points (black dots). With the locations of 18 tenderpoints (black circles). Adapted from the translation of: Wigers SH, Finset A. Rehabilitation of chronic myofascial pain disorders. Tidsskr Nor Laegeforen. 2007;127(5):604-608. Figure 1, The American College of Rheumatology 1990 diagnostic criteria; p. 3.

1.2 A Historical Perspective of CFS and FM

Today, commonly used names for the disorders are "chronic fatigue syndrome" and "fibromyalgia".[15,16] There has been – and still is – considerable debate about the name(s) throughout history. This variety in nomenclature is based on what is considered the *cause, core symptom(s)* or *consensus* in research environments (or lack thereof).

In the following section, I will give a brief overview of some of the historically described disorders that resemble CFS or FM – or sometimes both – and some of the theories in the medical understanding of these disorders. The focus will be on western medicine, since this is what dominates the diagnostic classification system used in the medical professions today. Other paradigms might shed more light upon these disorders, but this is out of the scope of this thesis.

Although some of the following descriptions are seemingly more related to either CFS (*febricula, epidemic neuromyasthenia* or *benign myalgic encephalomyelitis*) or FM (*rheuma theory* or *muscular rheumatism*), *neurasthenia* was often used for a clinical picture covering both conditions.

1.2.1 Early historical explanations

Ancient Greece

Already in ancient Greek literature (approximately 500-300 BC), the terms *rheuma theory* and *febricula* are described by Hippocrates. Several observations and descriptions have been made up until the present diagnostic systems were developed in the previous century.

The Rheuma Theory

The *rheuma theory* was explained to be the influence of the brain on the body: "brain sends liquid [rheuma] to the limb, and where there is more liquid there is more pain".[17]

Febricula / The Little Fever

There are descriptions of an illness similar to *febricula* in Ancient Greek medical work associated by the father of medicine himself – Hippocrates – which dates back to 500–400 BC (Corpus Hippocraticum).[18] This disease was described as *"the little, low continued fever"*[1] and a section from this is presented in Box 1*Box 1*.

The Early Modern Period

Rheumatism

In 1592, Guillaime de Baillou was the first to describe a class of muscular pains that are not necessarily associated with arthritis.[19]

Febricula

In 1750, Manningham described a disease that was also known as *nervous* or *hysteric fever* (Box 2). He also used the terminology the "little, low or continued fever" (referring to Hippocrates' work) to describe an illness resembling CFS as we know it today.

Febricula[1]

The little, obscured fever carry one main symptom: distorted pulse. Accidental symptoms are often seen, such as: chilliness; doziness; anxiety; "a lifelessness, with great lassitude and weariness all over the body, frequent yawnings with little flying pains;" and many others.

The cause of the disease

(*Lentor*) *Fault* in the blood. Febricula can be caused by grief, severe solicitude, taking cold and the like.

The cure of the little fever

"The proper use of diaphoretical remedies, promoting only insensible perspiration, with a small portion of subastringents mixed with those remedies, is the method always found most effectual" Another remedy recommended that salvei absinthe be given regularly.

It was strongly advised against *bleeding* (commonly used to treat diseases at the time) for treating the *febricula* as it often lead to sudden death.

by Sir Richard Manningham, 1760

Box 1

The Modern Era

Muscular rheumatism

In 1815, William Balfour used the term *muscular rheumatism*, which he described as "a pain driven by inflammatory action," referring to an inflammation resulting from a deficiency in the lubrication of the surface of the muscles.[20] According to Perrot,[17] Balfour also labelled the resulting widespread pain *fibrositis*.

Neurasthenia

In 1869, George Beard used the term *neurasthenia* to describe an already recognised condition of "nervous exhaustion". Beard admits that the pathology of this disorder is not known. Beard believed neurasthenia to be disturbances of the nervous system and slight

morbid changes of the brain, spinal cord or the peripheral nerves, leading to a loss of nervous force.

Fibrositis

In 1904, William Gowers described muscular rheumatism is a form of inflammation of the fibrous tissue of the muscles – *fibrositis*. He also admits: "There is no indication of the formation of 'inflammatory products,' as we call them, but this is certainly not enough to justify a denial of its inflammatory nature."[21]

Epidemic Neuromyasthenia

Following epidemics, seemingly clusters of outbreaks of chronic illness – *epidemic neuromyasthenia*[22] – have occurred: either named after symptoms, e.g. *benign myalgic encepahomyeitis*[22]; or after the epidemic itself, such as *Icelandic disease*[23] or *Crimean fever*.[24]

Development of diagnostic classification systems

Up until the mid-1950s, diagnoses were mainly based on theoretical and published case reports. For better understanding and treatment of diseases, a more systematic approach for identifying disease was needed. Thus, diagnostic classification systems, where defining disease and health conditions was based on symptomatic, empirical data, were developed.

Diagnostic and Statistical Manual of Mental Disorders (DSM)

In 1952 the first disease classification system for mental disorders occurred by the development of the Diagnostic and Statistical Manual of Mental Disorders[ii] (DSM-1) by the American Psychiatric Association (APA).

International Classification of Primary Care (ICPC)

The diagnostic system used in primary care, i.e. commonly used by general practitioners (GPs) is the *International Classification of Primary Care, version 2* (*ICPC-2*).[25] This classification system was developed by the World Organization of National Colleges, Academies and Academic Associations of General Practitioners/Family Physicians (short name: World Organization of Family Doctors; WONCA) in 1972.[26] *Tiredness/weakness general* (A04) is mainly used for coding CFS; and *muscle pain* (L18) is used to code for FM.

[ii] DSM-5 is the latest version of this classification system. It is mainly used in North America, Australia and in research, and not Europe, and will not be discussed any further.

The term *neurasthenia*[iii] (P78) is still used in ICPC-2 and is occasionally used for coding of CFS.

International Classification of Diseases (ICD)

An international classification system of mortality was developing in the late 1800th and several revisions and editions were made regularly.[27] Following the creation of the World Health Organization (WHO) in 1948, WHO took over responsibility of this classification system. The *sixth edition* of the *ICD* (*ICD-6*) included a classification system for defining diseases and health conditions. The *ICD* is still in use both for clinical and research purposes.

The currently used edition of *ICD* (*ICD-10*) was implemented in 1994. In *ICD-10* the term *benign myalgic encephalomyelitis* (introduced by the Lancet in 1956)[22] is included in *postviral fatigue syndrome* (G93.3). The term *chronic fatigue syndrome* (CFS) was introduced in 1988[15] and it became a commonly recognised name for this disorder[28] although it does only appear under the clinical description of *malaise and fatigu*e (code R53) in *ICD-10*. Similar to *IPCP-2*, *neurasthenia* still remained a diagnostic term for CFS in ICD-10.[iv]

[iii] The term *neurasthenia* is still used in ICPC-2, and defined as:

> *[a.] Increased fatiguability with unpleasant associations, difficulties in concentration and a persistent decrease in performance and coping efficiency. [b.] The feeling of physical weakness and exhaustion after mental effort or after a minimal physical effort is often accompanied by muscular pain and an inability to relax.*

From this, it is apparent that neurasthenia (P78) in ICPC-2

a. resembles CFS, yet CFS is commonly coded "weakness/tiredness general" (A04); and
b. FM, yet FM is covered under the diagnosis "muscle pain" (L18)

and is possibly one reason for abandoning the term neurasthenia in ICPC-3.

[iv] The term *neurasthenia* is still used in ICD-10, and defined as:

> *Considerable cultural variations occur in the presentation of this disorder, and two main types occur, with substantial overlap. [a.] In one type, the main feature is a complaint of increased fatigue after mental effort, often associated with some decrease in occupational performance or coping efficiency in daily tasks. The mental fatiguability is typically described as an unpleasant intrusion of distracting associations or recollections, difficulty in concentrating, and generally inefficient thinking. [b.] In the other type, the emphasis is on feelings of bodily or physical weakness and exhaustion after only minimal effort, accompanied by a feeling of muscular aches and pains and inability to relax. In both types a variety of other unpleasant physical feelings is common, such as dizziness, tension headaches, and feelings of general instability. Worry about decreasing mental and bodily well-being, irritability, anhedonia, and varying minor degrees of both depression and anxiety are all common. Sleep is often disturbed in its initial and middle phases but hypersomnia may also be prominent.*

Neurasthenia was classified in Category V "mental and behavioural disorders" F48 "Other neurotic disorders" in ICD-10 but resembles

a. CFS, yet the equivalent – *myalgic encephalomyelitis* – was classified in Category VI "Diseases of the nervous system" G93 "Other disorders of brain"; and
b. FM, yet fibromyalgia was classified as a disease of the musculoskeletal system and connective tissue.

Currently (in 2022), the 11th Revision of ICD (ICD-11)[29] is to be implemented in all the (more than 100) countries[v] using the ICD system around the world. CFS is finally included as the main diagnostic term for this disorder in ICD-11[29] (Table 1.1).

As no evidence of inflammation had been shown in *fibrositis*, Philip Kahler Hench replaced this term with *fibromyalgia* in 1976.[16] Fibromyalgia still remains the most widely recognised name for this disorder, and this term also still remains in ICD-11 and is included in the diagnosis of *chronic widespread pain* (MG30.01).

Table 1.1 shows the different diagnostic codes that have developed for CFS and FM and illustrates the historical lack of agreement for these diagnoses and the future criteria. This summary also highlights the lack of a clear organic base. Because CFS and FM are diagnosed as two separate entities, and this is a research project, the definitions described in Sections 1.1.1 and 1.1.2 are used in this study:

- CFS: The Fukuda criteria[5]
- FM: The ACR 1990 criteria[11]

As mentioned, CFS and FM share many overlapping features. These are introduced next, followed by possible alternative biological explanations of the symptoms of CFS and FM.

[v] ICD-11 has yet to be translated into Norwegian and is not implemented for clinical use in Norway.

Table 1.1. *Codes from ICPC-2, ICPC-3, ICD-10 and ICD-11 for the Diagnosis of CFS and FM with Definitions Used in Primary and Secondary Care*

	ICPC-2		ICPC-3[a]		ICD-10		ICD-11	
Diagnosis	Code	Diagnosis	Code	Diagnosis	Code	Diagnosis	Code	Diagnosis
CFS	A04	Weakness/tiredness general (Includes: Chronic fatigue)	AS04 AS05	General weakness or tiredness Postviral fatigue (Includes: Chronic fatigue syndrome)	G93.3	Postviral fatigue syndrome (Benign myalgic encephalomyelitis)	8E49	Postviral fatigue syndrome (Includes: Chronic fatigue syndrome and Benign myalgic encephalomyelitis)
					R53 R53.52	Malaise and fatigue Chronic fatigue, unspecified		
	P78[a]	Neurasthenia[iii]			F48.0 Z73.0	Neurasthenia[iv] Burn-out		
FM	L18	Muscle pain (Includes: Fibromyalgia)	LS18	Chronic widespread pain (Includes: Fibromyalgia)	M79.7	Fibromyalgia Fibromyositis Fibrositis Fibrositis	MG30.01	Chronic widespread pain

ICPC-2 = International Classification of Primary Care, version 2.[25] ICPC-3 = International Classification of Primary Care, version 3.[30] ICD = International classification system. ICD-10 = ICD, version 10.[31] ICD-11 = ICD, version 11.[29]

[a]ICPC-3 is now under development and the code P78 is abandoned from the previous version.

1.3 The Core Symptoms

There are many symptoms and subjective complaints in patients suffering from CFS and FM (Table 1.2). Symptoms are, by definition, subjective, and here lies the challenge in conducting objective tests of disorders like CFS and FM. Still, some symptoms share resemblance to other disorders of known pathological origin, and before explaining the possible links between the biological underpinnings and these symptoms, a clarification of some important terms related to the core symptoms of CFS and FM is warranted: Pain and fatigue. The characteristics of pain and fatigue are very distinct, still these symptoms often co-occur. Post exertion malaise (PEM) is also a commonly reported symptom in CFS, as well as in FM. Measuring PEM is objectively challenging and beyond the scope of our research methods. Hence pain and fatigue are the two symptoms of focus in this thesis.

1.3.1 Pain

Pain is by definition unpleasant and is essential for the survival of any species. The main purpose of pain is to signal the presence of damaging stimuli and to guide the organism to escape or avoid the threat, i.e. guide us towards safety.

The International Association for the Study of Pain[32] defines pain as: "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."

This implies that pain sensation is more than just sensation caused by physical damage; and that the perception of pain is important to pain sensation. Physical stimuli such as physical damage, the potential to result in physical damage (e.g. too hot or too much pressure), inflammation, toxic compounds etc., are recognised by nociceptors (nerve cells that sensor pain). These signals are transduced to the central nervous system (CNS), where they are modulated, registered, and lastly interpreted into the conscious mind. The perception of pain is influenced by the physiological state of the individual. For example, during acute stress, an individual may escape a traumatic event with major injuries and still not feel the pain until he or she is in safety. Likewise, chronic stress can alter the pain processing mechanisms. Pain also relies on subjective experience because of the plasticity of the neurons and neural networks involved in the interpretation of pain. When the pain goes from being acute to becoming chronic, the mechanisms become even more complex. In the absence of any physical damage, the pain signal loses its main purpose. Chronic hyperalgesia (enhanced pain sensation) or allodynia (painful sensation upon normal, non-damaging stimuli such as touch) are examples of distortions that might arise. One potential mechanism for this is through sensitisation mediated by inflammation. After injury or trauma, inflammation often occurs locally (at the site of injury). Additionally, systematic inflammation may arise. Inflammatory mediators (such as certain cytokines) increase nociception in afferent neurons, thus becoming more responsive to pain stimuli. In addition, the pain-modulatory/inhibitory mechanisms in the CNS may be reduced by cytokines acting centrally. The end result being enhanced pain sensation.[33] Acute pain can become chronic, such as may be the case of FM. The mechanisms

for chronification of pain are not fully known, but may follow the same mechanisms of sensitisation. One aspect of this process may be mediated by chronic inflammation.

It is possible that experiencing chronic pain may also lead to development of chronic fatigue.

1.3.2 Fatigue

Fatigue can be defined as: “The state of weariness following a period of exertion, mental of physical, characterised by a decreased capacity for work and reduced efficiency to respond to stimuli.”[34]

Fatigue is evolutionary a signal to any organism that restoration is necessary. Following exertion, the organism needs to refill depleted energy reservoirs and rebuild any damage that may have been caused by this. By definition fatigue can be both physical and mental. Physical fatigue often follows strenuous exercise or trauma, and mental fatigue can for instance follow focus on a cognitive task and/or trauma. This is not to say that there are two distinct compounds of fatigue. Mental fatigue can follow physical exertion, and physical fatigue can follow intense cognitive tasks. Lack of nutrition will lead to lack of both physical and mental energy. Depletion of metabolites needed for cell energy production, of muscle cells will lead to diminished mobile activation and fatigue. Likewise, in neurons, this will lead to reduced focus and mental fatigue.

Chronic mental and physical stress can also lead to both physical and mental fatigue. The mechanisms behind this type of fatigue are not well understood, and there may be different as well as similar mechanisms involved. A disturbance in the cell energy production has been postulated in chronic forms of fatigue.[35-37] Other mechanisms directly or indirectly influencing energy production, are mediated by inflammatory compounds.[35]

Table 1.2. *Short Summary of Clinical and Physiological Findings in CFS and FM*

OVERLAPPING FEATURES

CFS	FM
Clinical	

• Fatigue
  ▪ Fatigue reported in ~50% of FM[11]
• Pain
  ▪ Muscle aches and pains: occurring in > 90% of CFS[38]
  ▪ Joint pain: occurring in > 80% of CFS[38]
• Cognitive and neuropsychological disturbances[39,40]	

Physiological	
• Peripheral inflammation[7,41] • Central inflammation[42-44] • HPA-axis disturbances[45,46] • Autonomic dysfunction[2,7] • Neurotransmitter alterations[46] • Anxiety and depression comorbidities[8,47] • Irritable bowel syndrome (IBS)	

POSSIBLE DIFFERENCES

CFS	FM
Clinical	**Clinical**
• More fatigue-specifically related • Post-external malaise	• More pain-specifically related • Chronic widespread pain
Physiological	**Physiological**
• Immunological disturbances[48] • Metabolic disturbances[35,36]	• Substance P in cerebrospinal fluid (CSF)[49] • Central sensitisation[33]

(Groven 2022)

1.4 Neuroendocrinology – Psychiatry – Immuno-psychiatry

Disturbances in the autonomic nervous system, neuroendocrine system (e.g. the HPA-axis), and reduced cognitive function are commonly found in CFS and FM (Table 1.2).

Childhood traumas or severe stressors in early life are reported by both CFS[50] and FM[51] patients. This is in line with the renowned ACE studies by Felitti et al.[52] Such events may lead to permanent changes in HPA function.[50,51,53] Endocrinological disturbances, such as high cortisol levels in chronic stress, or a hypofunction of the HPA-axis – and reduced cortisol production – has a direct influence on neurons and the production of neurotransmitters (see Section 1.5.3 Brain, behaviour and the immune system are closely interlinked). Chronic inflammation also follows chronic stress.[54]

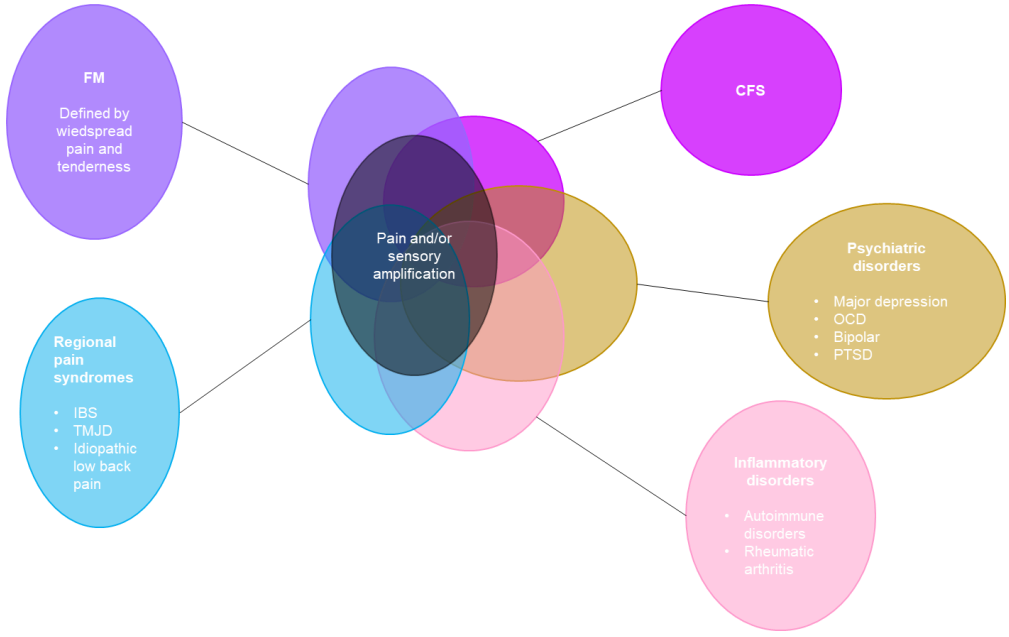


Figure 1.2.
Overlap between systemic syndromes. IBS = irritable bowel syndrome. TMJD = temporomandibular disorders. OCD = obsessive compulsive disorder. PTSD = posttraumatic stress disorder. Modified and adapted (by Groven) from Clauw, D. Perspectives on fatigue from the study of chronic fatigue syndrome and related conditions. PM R. 2010 May;2(5);414-30; Figure 1, Overlap between systemic syndromes; p. 417.[2]

It has also been speculated whether CFS and FM syndromes could be explained by personality, coping/adaptation strategies or depression.[55] Depression and anxiety are commonly co-occurring in both CFS[8,56] and FM patients.[47] Depression has many similarities with the most common symptoms of CFS and FM such as fatigue, disturbed sleep, occurrence of aches and pains.

None of these systems are mutually exclusive, and the neuro-endocrine system is closely linked to the immune system.

1.5 The Immune System

1.5.1 The innate and adaptive immune system

The immune system is not made up of two very distinct parts, yet a separation of the innate and adaptive immune system is made. This is merely based upon how the defence mechanisms work, and how invasive threats are encountered.

The cells of the innate immune system are activated by various general stimuli, e.g. infection by a pathogen, physical damage, malign tumour etc., and as a natural waste clear out of debris and dying cells.

For example, when the host gets infected by a pathogen, the cells of the innate immune system are the first to recognise the pathogen, and an immediate response to fight the invasion is induced. Immune cells, such as macrophages (the equivalent to microglia in the CNS) and dendritic cells survey the tissues of the body, and they will recognise common molecular structures shared by many pathogens, engulf the pathogen and call for help. The S.O.S. messages are cytokines and chemokines produced by this cell, and preferably these messengers are aimed at other cells of the immune system, such as recruiting and strengthening other macrophages and natural killer (NK) cells, thus enhancing the initial defence mechanisms. Macrophages and NK cells are able to kill pathogens, but they do so by unspecifically shooting towards the invader, and innocent bystanders, such as the nearby cells and structures, also are damaged. The cytokines released by this initial encounter, circulate the body, and stimulate cells of the liver to produce C-reactive protein (CRP; Box 2). In addition, their role is to prepare and strengthen the adaptive immune system to combat the pathogen.

CRP

C-reactive Protein (CRP) is produced by hepatocytes in response to certain cytokines such as IL-1β, TNF-α and IL-6. CRP is a potent biomarker of inflammation, and it is commonly measured to determine the inflammatory status in the population.

A more accurate measurement of CRP is done by high sensitivity CRP (hsCRP), and is commonly used for reference values < 5 mg/L.

CRP/hsCRP > 10 mg/L is indicative of inflammation or infection of known cause.

The American Heart Association recommends the following levels of CRP for risk assessment (for cardio-vascular disease).[42]

Low	< 1.0 mg/L
Average	1.0–3.0 mg/L
High	> 3.0 mg/L

Box 2

The adaptive immune system creates a more specific attack on the invader. Specific T-cells and B-cells are activated based on receptor specificity for the pathogen. The activated cells are then stimulated by cytokines and other surface receptors and develop into cell lines with specific antigen receptors (T-cell receptors and B-cell antibodies) aimed to recognise molecular structures specific for the microbe or substance that entered the host. This response "marks" the invader, aiding the precise elimination/destruction of the pathogen by the immune cells adapted for this task. Interestingly, T- and B-cells develop a "memory" towards these antigens. The memory is based on a multiplication of the number of cells specific for a particular pathogen so that when the host gets attacked again by the same antigen (same virus, microbe etc.), it will be able to respond quickly and with great accuracy and force.

There are several types of immune cells and compounds of the immune system, but for the scope of this thesis, only a brief overview on how lymphocytes operate is introduced. The following section describes the cytokines, also produced by lymphocytes, which, together with CRP and kynurenines, are used to examine the inflammatory status in our study population.

1.5.2 Cytokines

The word *cytokine* derives from Greek and refers to a protein made by a cell (*cyto* = cell) that acts on target cells (*kinein* = to move/act on).

There are over 200 known cytokines. They can be divided into groups based on molecular structure, and often are grouped as pro-inflammatory, anti-inflammatory or regulatory based on known function. Cytokines are produced by a variety of cells both peripherally (e.g. by cells of the immune system) and centrally (e.g. by glial cells of the CNS).

Cytokines are secreted in response to a wide range of stimuli, such as infection (bacterial, viral etc.) and physical trauma. Production of cytokines is also influenced by the stress responses, i.e. the HPA-axis (see Section 1.5.3 Brain, behaviour and the immune system are closely interlinked) which is commonly disturbed in both CFS and FM (Table 1.2).

The cytokines bind to receptors that are either soluble or cell bound. Receptors for cytokines are found on many cells throughout the body. They are also found in various brain regions,[57] and nerve cells, astrocytes and leukocytes express cytokine receptors.

Cytokines influence cells and tissues both locally and at longer distances, activating intracellular transduction pathways of the target cell, and thus potentially change the properties of the cell. Each cytokine usually binds to more than one receptor, and this is why one cytokine can have opposing effects on different target cells. Thus, cytokines may act solitary, synergistic or antagonistic – modulating the effect of other cytokines.

The main known physiological responses to cytokines are: Induce the production and secretion of inflammatory proteins, including other cytokines and CRP; induce, control and regulate the intensity and duration of the immune response; control and regulate cellular proliferation and differentiation.

The influence of cytokines is not limited to regulation of the immune response. Cytokine also influence behaviour upon cytokine-brain interactions as discussed in the next section.

1.5.3 Brain, behaviour and the immune system are closely interlinked

Cytokines stimulate physiological responses throughout the CNS. Since the brain is the administrative centre for behaviour, emotions and cognition, cytokines are closely linked to behaviour. These mechanisms linking cytokines to behaviour, are either mediated directly or indirectly, and could involve:

- HPA-axis modulation (through the effect of cortisol on immune cells and cytokine production)
- Neurotransmission
  - Cytokine binding to neurons
    - Peripherally or centrally
- Neurotransmitter availability by:
  - Cytokine binding to glial cells
  - Cytokine activation of enzymes in the CNS

Administration and induction of pro-inflammatory cytokines in human study participants have led to profound behavioural changes, such as fatigue, depressed mood, and decrease in psychomotor speed.[58-60]

In all species throughout evolution, an adequate, adaptive response to infections and acute injuries are mostly beneficial for survival. When combating pathogens and injuries, a quick recovery relies upon energy conservation and withdrawing so that the body can use the resources to fight the invasion. Thus, the host's behavioural response includes fatigue, hyperalgesia and reduced mobility of the injured site. This behavioural change is termed "sickness behaviour", and is mediated by cytokines,[61] either directly by binding to neurons in the CNS or peripherally (through *N. Vagus* and *N. Sympathicus*; the autonomic nervous system); indirectly enhancing production of cytokines mediated by microglia; or potentially indirectly by influencing the availability of neurotransmitters, such as serotonin. The latter mechanism is explained next.

1.5.4 Tryptophan and the kynurenine pathway

Tryptophan (Try) is an essential amino acid, i.e. it cannot be produced in humans, and has to be consumed. Main sources are protein rich foods, such as meat, milk and nuts. Try has several metabolic pathways. In addition to building proteins there are two pathways that may be extra important for brain functioning (Figure 1.3):

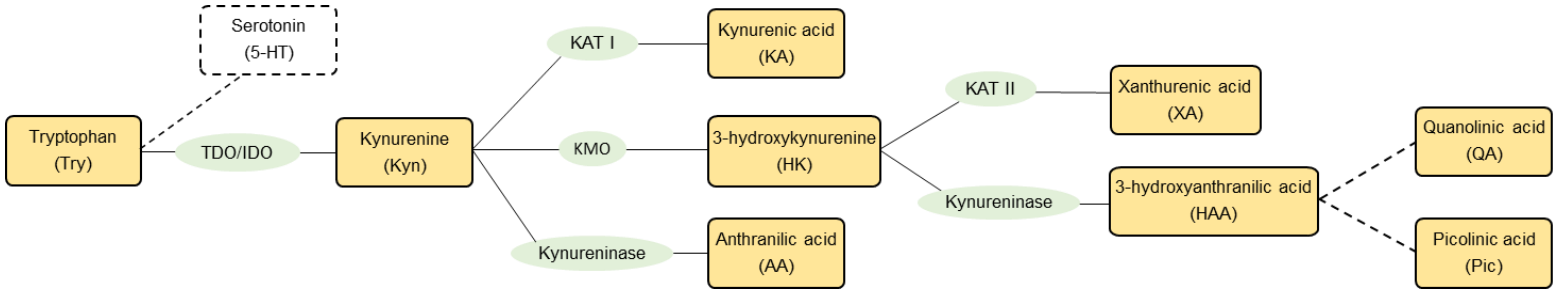


Figure 1.3. *The Kynurenine Pathway*

Tryptophan (Try) is either transferred into serotonin (5-HT), or kynurenine (Kyn) and its downstream metabolites. Enzymes involved are illustrated as green ellipses. Activity of enzymes involved could be described as the ratio of the converted metabolite over the previous metabolite.
Enzymes: TDO = Tryptophan 2,3-dioxygenase. IDO = Indoleamine 2,3-dioxygenase. KAT = Kynurenine aminotransferase. KMO = Kynurenine 3-monooxygenase. (Groven 2022.)

1. Conversion into serotonin (5-HT)
    a. 5-HT is a neurotransmitter involved in several brain and body functions. Mood, sleep and pain regulation are important behaviours mediated by 5-HT. 5-HT is also abundantly found in the gut.
    b. 5-HT seems to be relevant in CFS and FM pathology.[62,63]
2. Conversion into kynurenine (Kyn); the kynurenine pathway

Indoleamine-2-3-dioxygenase (IDO) is an enzyme converting tryptophan into kynurenine.

1. IDO is converting tryptophan into kynurenine at the cost of serotonin transformation.
2. IDO activation is enhanced during inflammation
3. The breakdown products of Kyn can be both neuroprotective and neurotoxic.
4. The kynurenine pathway been postulated as a contributing factor in both CFS and FM,[64] only a few studies exist,[65-67] and studies *comparing both* CFS and FM are lacking.

The enzyme IDO is expressed in astrocytes and activated by proinflammatory cytokines such as IFN-γ, IL-1β and TNF-α. Cytokine activation of IDO thus shunts the transformation of tryptophan away from serotonin and towards the kynurenine pathway. The equivalent enzyme tryptophan-2-3-dioxygenase (TDO), mainly expressed in hepatocytes, is activated by cortisol (another compound involved in inflammation). Since Kyn and its downstream metabolites are produced both in the brain and in the periphery, and under certain circumstances can cross the blood brain barrier (BBB)[68] all these analytes can be measured in blood.

The metabolites of the kynurenine pathway (and the enzymes involved) are illustrated in Figure 1.3, and the breakdown products of Kyn can be both neuroprotective and neurotoxic.

Two important metabolites of kynurenine are the kynurenic acid (KA) and the quinolinic acid (QA). QA is a neurotoxic agent, serving as an agonist to NMDA receptors, and thus simulating the toxicity of excessive production and release of glutamate. KA is considered a neuroprotective compound by inhibiting N-methyl-D-aspartate (NMDA) receptors, preventing the neurotoxic effects of QA. KA also serves as an antioxidant agent and free radical scavenger, another neuroprotective property of KA.[69] An imbalance between the neurotoxic and neuroprotective metabolites is described in several neurodegenerative disorders and depression.[70,71]

Kynurenine and its metabolites seem to be related to the two core symptoms relevant for the patient groups in this study: fatigue[72]; and pain perception.[73] Disturbances in the tryptophan-serotonin-pathway are also found in major depression.[74] There is growing evidence of the involvement of the immune system in aetiology of depression,[75] and a pro-inflammatory state is found in depression and other psychiatric disorders.[76]

Bearing in mind that depressive symptoms are often present in both disorders, there might be important similarities with depression, the kynurenine pathway and inflammation in the pathology of CFS and FM.

1.6 Aims and Objectives of the Study

The rationale for studying inflammation in CFS and FM is not necessarily obvious neither from the historical nor modern diagnostic criteria (see Section 1.2.1 Early historical explanations and the Sub-Section Development of diagnostic classification systems). By introducing the complexity of the immune system, it becomes more obvious that the symptoms of CFS and FM mimic those seen in different inflammatory disorders.[77] Indeed, inflammatory mechanisms have been postulated in the pathology of both CFS and FM patients.[7,41]

Altered cytokine levels have been reported in both CFS[78] and FM[79] but the results are inconclusive. And although the role of Try and its metabolites have long been postulated as contributing factors in both disorders,[64] the kynurenine pathway has been poorly studied in CFS and FM.

Only a few studies have compared immunological markers between CFS and FM,[80-83] but most studies focus on the immune system for each disorder separately, and do not consider the heterogenicity in, and overlap between, the two conditions.

Given the behavioural characteristics influenced by mediators of the immune system, CRP (a general inflammatory marker), cytokines and chemokines (indicative of immune activity), and metabolites of the kynurenine pathway (as a possible response to inflammation), are all relevant candidates to investigate the pathophysiology of CFS and FM.

As described above, there are certain subjective, diagnostic and clinical overlaps between CFS and FM. Their characteristics include symptoms also seen in depression. For depression there are several studies reporting altered immune activity[75] – but this will not be elaborated further in this thesis. Hence, the primary aim was to compare the two patient groups CFS and FM versus controls on biological markers of the immune system that may share overlapping features.

The objectives were:

1. Compare immunological biomarkers in CFS and FM outpatients, in relation to healthy control subjects.
2. Examine the potential influence of the psychiatric symptoms anxiety and depression in the outcome of the a sample of CFS patients, FM patients and healthy controls.

The objectives for each individual article were as follows:

Article 1

Primary objective: To study the potential differences of the inflammatory marker hsCRP in CFS and FM patients compared to controls. Secondary objective: To examine the association between inflammation, anxiety and depression.

Article 2

To study the potential differences of a set of cytokines and chemokines in CFS and FM patients compared to healthy controls

Article 3

To study the potential differences of Try and its downstream metabolites of the kynurenine pathway in CFS and FM patients compared to healthy controls, controlling for confounding factors (age, Body Mass Index, anxiety and depression).

2 Method

2.1 Sampling and Procedure

2.1.1 Sample population

The patients in this study were recruited from the Department for Pain and Complex Disorders at St. Olav's Hospital.

The Department for Pain and Complex Disorders consists of two sub-clinics:

1. CFS outpatient clinic
2. Pain outpatient clinic

2.1.2 Patient flow

All physicians / medical doctors in Mid-Norway (a population of 750.000), Norway, may refer patients with pain or fatigue of unknown aetiology aged 18 to 60 years to the Multidisciplinary Pain Center at St. Olav's Hospital, Norway for evaluation and treatment according to ordinary hospital routine.

CFS outpatient clinic

a. Patients suffering from fatigue of unknown aetiology were referred to the CFS outpatient clinic of the Multidisciplinary Pain Center. Before admission, patients filled out an extensive questionnaire provided by the clinic.
b. An expert team of medical doctors, physiotherapists and clinical psychologists evaluated the patients separately. Each consultation was scheduled for 1–1 ½ hours. After the three examinations the team of consultants met and evaluated each case for diagnostic and treatment purposes.
c. When symptoms according to the Fukuda criteria were met and other medical or mental explanation for their condition was ruled out (Fukuda, Straus et al. 1994), patients would fall into the category of CFS for the purpose of this study.
d. Where appropriate, the patients would get referred on to necessary further evaluation and/or treatment for their condition by the clinicians.
e. All patients were informed about the evaluation including diagnosis and further treatment options by consultation or letter. In addition a letter with this information was sent to their general practitioner (GP) and (if another) referring physician.

Pain outpatient clinic

a. Patients suffering from various complex symptom disorders often accompanied by a chronic pain state were referred to the pain outpatient clinic of the Multidisciplinary Pain Center. Before admission, patients filled out an extensive questionnaire provided by the clinic.
b. All patients were examined by a medical doctor as part of the clinical procedure, and this consultation was scheduled for 1–1 ½ hours. Further evaluation by a physiotherapist and/or psychologist were individually scheduled according to the patients' needs. After the examination, the team of consultants meet and evaluate each case for diagnostic and treatment purposes.
c. The medical doctor evaluated each patient according to the 1990 ACR criteria. Where in doubt, they consulted a physiotherapist who would evaluate the patient for a second opinion. If the patient fulfilled the 1990 ACR criteria[84] they would fall into the category fibromyalgia for the purpose of this study.[vi]
d. The expert team met with the patient for discussion and suggested possible further follow-up. The follow-up would either be done by the specialized clinicians at the Multidisciplinary Pain Center, or the patients would be referred on to necessary further evaluation and/or treatment for their condition.
e. All patients were informed about the evaluation including diagnosis and further treatment options. In addition a letter with this information was sent to their general practitioner (GP) and referring physician.

In this study all CFS patients were recruited by the CFS clinic, and the FM patients were recruited from both the CFS clinic and pain clinic (Figure 2.1).

2.1.3 Recruitment procedure and inclusion

Chronic fatigue syndrome patients

The CFS patients were recruited from the CFS outpatient clinic where they had been evaluated by the expert team and diagnosed and fulfilling the Fukuda criteria for CFS.[5]

The first patients were then contacted, usually by phone by one of the staff members, informed about the study and asked for participation. In the beginning of the recruitment period, this was done after receiving a CFS diagnosis. Those who agreed would be scheduled for a date and time for the data collection.[vii]

This proved to be a slow way of recruiting the number of participants needed for the study. Therefore, half-way into the collection of data (CFS $n = 22$), the inclusion procedure was changed (and the changes approved by Regional Committee for Medical and Health Research Ethics [REK]).

[vi] Multicenter Criteria Committee in 1990[8] and have been the widest used evaluation tool for assessment of fibromyalgia in research.

[vii] The actual number of eligible patients is not known as we only had permissions from the Regional Committee for Medical and Health Research Ethics (REK) to register those accepting.

The patients scheduled for evaluation at the CFS clinic were informed about the study in a letter sent to the patients in advance. They were then contacted by a member-of-staff and asked to participate in the study. Those who agreed, would be scheduled for a date and time for the data collection. This meant that the patients still had not been fully evaluated by the team of experts at the time of data collection. The patients were later categorized into groups according to diagnostic criteria as either "idiopathic fatigue" or CFS cases (Figure 2.1. Flowchart). Only CFS cases were included in the analyses of this study. Participating in this study did not lead to any benefits nor disadvantages, i.e. it did not affect the timing or procedure of clinical evaluation of the patients.

Fibromyalgia Patients

The fibromyalgia patients were recruited from both the pain outpatient clinic and the CFS outpatient clinic (Figure 2.1).

If the patient fulfilled the 1990 ACR criteria, they were given information about the study by the clinician and asked if one of the staff members could get in touch with them for inclusion and further information.[viii] Those who agreed, would then be contacted by phone a few days later by a member-of-staff and asked to participate in the study. Upon agreement, they were scheduled for a date and time for the data collection.

Healthy controls

Information about the study seeking healthy volunteers was posted on the NTNU and St. Olav's Hospital intranet. The posts were open to everyone and could also be shared across social platforms. The information post stated that the volunteers be female 18–60 years of age, not suffering from any known medical disorder interfering with the results, and not pregnant. Information about the contact details (e-mail and phone number) of the PhD candidate was given in the announcement. Women interested in participating in the study would contact the candidate, and time and date was arranged for the data collection.

2.1.4 Exclusion criteria

The exclusion criteria were: Inflammatory disease (including auto-immune diseases and immune-suppressive medication used as treatment for such), on-going infection, and pregnancy (based on participant report). Any psychological, psychiatric or somatic disorders that could explain the symptoms also lead to exclusion from this study. These are already classified as exclusion criteria of the Fukuda 1994 criteria.[5]

Also, participants were screened for deviating white blood cells and CRP (Table 2.2). Participants with serology indicating active infection of mycoplasma pneumonia, cytomegalovirus, Epstein-Barr virus, hepatitis B virus (HBsAg and anti-HBcore), hepatitis C virus and borrelia burgdorferi would be excluded.

[viii] The actual number of eligible patients is not known as we only had permissions from the Regional Committee for Medical and Health Research Ethics (REK) to register those accepting.

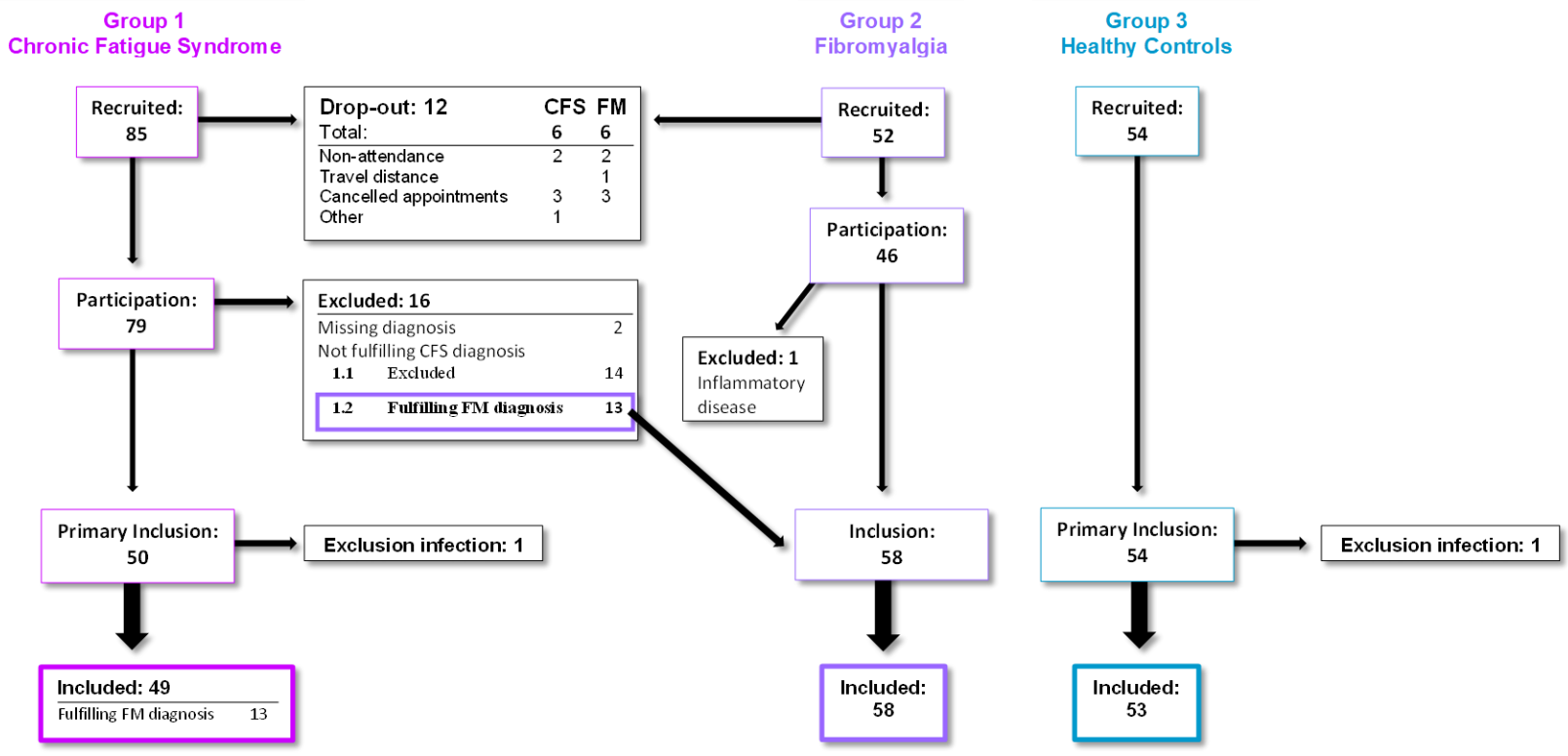


Figure 2.1. *Flowchart*

2.2 Ethics

2.2.1 Consent

All participants had to sign an informed written consent before participating in the study. The study and inclusion procedures were approved by the local Regional Committee for Medical and Health Research Ethics (REK 2014/711).

2.2.2 Follow-up

The results of all the tests were examined and evaluated by a trained psychiatrist (SKR). If the findings showed any abnormalities or other cause of concern, the subjects/GPs would be informed.

This study did not qualify as a clinical trial and hence was not registered in the Clinical Trials Register.

2.3 Data Collection

A one-day in-hospital assessment of approximately 45 minutes included an interview, questionnaires and blood sampling. Data were collected in the period March 2015 until December 2016. Sampling was done consecutively, and the order of assessment was random.

2.3.1 Interview

In addition to recording the time and date of the sampling, a structured clinical interview about present health state was conducted for each participant. All interviews were performed by the same person (NG). Basic population descriptives and medication were part of the interview questionnaire items:

1. Age
2. Height (in cm)
3. Weight (in kg)
4. Subjective symptoms of infection
5. “Feverishness”
6. Comorbid disorders/diagnoses
7. Allergies
8. Medication
9. Menstrual cycle
10. Use of contraceptives
11. Menopause
12. Level of physical activity
13. Nicotine use
14. Duration of illness

Items and question asked are found in (Supplementary Table 1).

2.3.2 Questionnaires

For data management and collection of psychometric and symptom evaluation, an online questionnaire was constructed in *SelectSurvey.* SelectSurvey is a system developed by the Faculty of Research and Educational Sciences, Research Section (previously Faculty of Social Sciences and Technology, IT Section, NTNU) for online questionnaires. Questions were added manually into SelectSurvey to create the desired set of questionnaires appropriate for the research question.

A specific link was provided for accessing the SelectSurvey questionnaire designed for this study (https://survey.svt.ntnu.no/Login.aspx).

Participants were given access to a laptop for filling out the SelectSurvey questionnaire online. Responses were punched manually into SelectSurvey by the participant, and the data was collected at an internal server of the faculty. The responses would later be extracted directly into spreadsheets such as Excel and SPSS.

All data plotted into SelectSurvey were anonymous, since any tracking of the IP-address would be this laptop, owned by NTNU. Only the study identity number was added for linking data.[ix] The participant was given the option of asking questions for clarification but was otherwise left alone when filling out the questionnaire.

For four participants (FM patients only) technical failure resulted in no internet access, and these participants were given a printed version of the questionnaire which was filled out by hand. These data were plotted into SelectSurvey by the candidate at a later date.

Four questionnaires were provided:

1. The hospital anxiety and depression scale (HADS) for psychometric evaluation (anxiety and depression)
2. A numeric rating scale (NRS) for pain intensity evaluation
3. The Chalder fatigue questionnaire (CFQ)
4. The fibromyalgia survey diagnostic criteria (FSDC) for fibromyalgia diagnosis according to ACF2016,[13] and symptomatic evaluation (fatigue, fibromyalgia, and pain, respectively)

1. The hospital anxiety and depression scale – Anxiety and depression score

HADS is a validated, self-complete scale[85,86] for monitoring depressive and anxiety symptoms. The total potential HADS score ranges from 0 to 42. The cut-off scores for non-cases, doubtful cases and definite cases are found in Table 2.1, respectively. It is possible to evaluate the total score of both depression and anxiety.[85,87] HADS can be subdivided into depression scores and anxiety scores. The scores in both subdivisions range from 0 to 21; with high scores being suggestive of more symptoms (Table 2.1).[85]

In this study we used HADS as a continuous variable if not otherwise specified.

[ix] The link between personal data and the study identity number was stored in a fire safe locked at St. Olav's hospital where only the PhD candidate had access.

Table 2.1. *HADS cut-off Scores*

	non-case	doubtful case	definite case
HADS total	<15	15–18	>18
HADS anxiety	<8	8–10	>10
HADS depression	<8	8–10	>10

The rationale for choosing HADS for assessing anxiety and depression in this study was that HADS does not contain any somatic symptom items. Based on the assumption that the patient groups in this study, and in particular the CFS patients, report many somatic symptoms, this bias (overlapping with depressive and anxiety symptoms) was reduced. The HADS is a reliable instrument for assessing anxiety and depression, but should not be used for making a specific diagnosis of major depression.[87]

2. Numeric rating scale – Pain

A numeric rating scale (NRS) was used to evaluate pain. Three of these items were taken from the brief pain inventory (BPI),[88,89] and describes the subjective sensation of pain experienced over the last week (BPI 1–BPI 3). NRS is a continuous variable measured on a Likert scale ranging from 0 ("no pain") to 10 ("maximal possible pain"). Three additional items were added (NRS 1–NRS 3) where the participant was asked to evaluate the pain, fatigue and level of stress experienced at the time of answering the questionnaire on a scale from 0 to 10. The pain score was used as a continuous variable in this study, and no cut-off scores is given.

- BPI 1: Highest experienced pain during the last week
- BPI 2: Lowest experienced pain during the last week
- BPI 3: Average pain during the last week
- NRS 1: Pain experienced during the time of assessment
- NRS 2: Fatigue experienced during the time of assessment
- NRS 3: Perceived stress experienced during the time of assessment

3. The Chalder fatigue questionnaire – Fatigue score

The Chalder fatigue questionnaire (CFQ)[90,91] consists of 11 items measuring fatigue. The items are divided into physical and mental fatigue. Each question on fatigue is answered with four options: "better than usual", "no more than usual", "worse than usual" and "much worse than usual," and is scored 0–3 on a Likert scale. The total sum for all 11 items ranges from 0 to 33 with higher scores imply more severe fatigue. This continuous scale was used for most analyses in this study.

Cases of fatigue are rated from a bimodal version (item scores 0–1 = 0; and item scores 2–3 =1) of the CFQ. If the total score is 6 or more, this is defined as a positive "fatigue case".[92] We included both of these parameters in our study, although it is recommended that fatigue is viewed as a dimension rather than category.[90]

4. The fibromyalgia survey diagnostic criteria – Fibromyalgia severity score

To assess FM, the 1990 ACR criteria[11] have been the gold standard used in research and clinical evaluation/diagnosis of FM. The fibromyalgia survey diagnostic criteria (FSDC) were developed in 2010[93,x] (with small corrections made in 2011)[94] and later revised in 2016[13,xi] with the aim of constructing criteria for fibromyalgia status without the use of tenderpoints.

From this questionnaire (FSDC)[13] comes the fibromyalgia symptom scale which consists of two sub-scales (bullet points 1 and 2 below), and are summarised into a third, symptom severity score (bullet point 3):

1. Widespread pain index (WPI)
   - Scores 0–9
2. Symptom severity scale (SSS)
   - Scores 0–12
3. Fibromyalgia severity (FS) score[xii]
   - This is the level of *fibromyalgianess*
   - Range: 0 *(no symptoms)* to 31 (*most severe symptoms*).
     - If needed, it was suggested a cut-off score for FM cases ≥ 12 vs. non-cases < 12.

The latter (FS score) is the score we used in this study controlling for the lack of FM assessment in the CFS patients (see Sections 2.1.2 and 2.1.3). The FS scores is a quantitative tool for assessing the severity of fibromyalgia, with the presumption that FM is more of a continuous disorder with varying degrees of symptoms and severity, *i.e.* that FM is not a clear cut-off categorical disorder. This was termed *fibromyalgianess* scores by the authors[95] (and is equivalent to the FS score).

Equipment for Blood Collection

Blood collection

- Alcohol swabs (Alkotip®)
- Butterfly needle (BD Vacutainer® 0.8 x 19 mm x 178 mm)

Blood collection tubes

- 5 Serum Gel (Vacuette® 5 ml Z Serum Sep Clot Activator)
- 1 Lithium Heparin Gel (Vacuette® 3 ml LH Lithium Heparin Sep)
- 2 EDTA Plasma (Vacuette® 3 ml K2E K2EDTA)
- 2 EDTA Plasma (Vacuette® 6 ml K2E K2EDTA)
- Storage tubes for freezer:
  - 4 Corning cryogenic vials 2.0 ml (no. 430488)

Box 3

2.3.3 Blood sampling – General

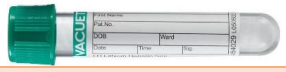
Blood samples were taken the same day shortly before or after the interview and questionnaires. In addition to blood for immune markers (CRP, cytokines/chemokines, and kynurenines), general blood samples (clinical, chemical and serology) were collected for health assessment. The general blood samples were collected and sent to the St. Olav's hospital clinical laboratories for further analysis. A detailed list of analytes is found in Table 2.2.

[x] Referred to as the FM 2011 criteria throughout this thesis.
[xi] Referred to as the FM 2016 criteria throughout this thesis.
[xii] Also called the polysymptomatic distress (PSD) scale.

Table 2.2. *Procedure and Analytes of the General Blood Samples Collected*

5 tubes		Serum Gel (Vacuette® 5 ml Z Serum Sep Clot Activator)
Blood component	**Procedure**	**Analytes**

Serum	Sent via internal transport systems to hospital lab: Laboratioremedisinsk klinikk, Avdeling for Medisinsk Biokjemi, St. Olav's Hospital. Analysis conducted according to procedures in the lab.	• hsCRP
		• Total IgE
		• ANA screening (antinuclear antibody test)
		• RF IgM (rheumatic factor)
		• Tissue anti-transglutaminase IgA
		• Anti-gliadin IgG
		• Folate
		• Chromatogranin A
		• Mycoplasma pneumonia
		• Cytomegalovirus
		• Epstein-Barr virus
		• Hepatitis B virus:
		  ○ HBsAg (current hepatitis B infection)
		  ○ anti-HBcore
		• Hepatitis C virus
		• Borrelia burgdorferi

1 tube		Lithium Heparin Gel (Vacuette® 3 ml LH Lithium Heparin Sep)
Blood component	**Procedure**	**Analytes**
Plasma	Sent via internal transport systems to hospital lab: Laboratioremedisinsk klinikk, Avdeling for Medisinsk Biokjemi, St. Olav's Hospital. Analysis conducted according to procedures in the lab.	• IgG • IgM • IgA • hsCRP • TSH (thyroid-stimulating hormone) • fT_4 (unbound thyroxine) • FSH (follicle-stimulating hormone) • LH (luteinizing hormone) • Prolactin • Cortisol • Glucose • Ferritin • Fe (iron) • TIBC (transferrin iron-binding capacity) • Cobalamin (vitamin B_{12}) • Na (sodium) • K (potassium) • Mg (magnesium) • Ca (calcium) • Albumin • ALAT (alanine transaminase) • GT (gamma-glutamyltransferase) • ALP (alkaline phosphatase) • CK (creatine kinase) • Creatinine

2 tubes		EDTA plasma (Vacuette® 3 ml K2E K2EDTA)
Blood component	**Procedure**	**Analytes**

Plasma	Sent via internal transport systems to hospital lab: Laboratioremedisinsk klinikk, Avdeling for Medisinsk Biokjemi, St. Olav's Hospital. Analysis conducted according to procedures in the lab.	• Differential count of leukocytes:
		  ○ Neutrophils
		  ○ Eosinophils
		  ○ Basophils
		  ○ Lymphocytes
		  ○ Monocytes
		• Haemoglobin
		• MCH (mean corpuscular haemoglobin)
		• Thrombocytes
		• HbA1c (glycated haemoglobin)

2 tubes		EDTA plasma (Vacuette® 6 ml K2E K2EDTA)
Blood component	**Procedure**	**Analytes**
Plasma	EDTA plasma tubes immediately put on ice. Centrifuged (1500g, 15 min, 4 °C) within maximum 30 minutes. Aliquoted plasma by 1 ml into cryogenic vials and frozen at -80 °C until assayed.	• IFN-γ • IL-1β • IL-1ra • IL-4 • IL-6 • IL-8 • IL-10 • IL-17 • IP-10 • MCP-1 • TGF-β1 • TGF-β2 • TGF-β3 • TNF-α
4 tubes		Corning cryogenic vials 2.0 ml (430488)
Blood component	**Procedure**	**Storage**
Plasma	Pipetting of 1 ml EDTA plasma into vials and immediately frozen.	-80°C Stored in biobank (REK: 2014/711; and REK: 18127)

2.3.4 Blood samples – Immune markers explored in this study

The main focus of this study was to explore the immune markers CRP and cytokines/chemokines in blood in two patient groups (CFS and FM) and one control group. Other markers of interest were the kynurenines.

hsCRP

Serum samples were sent to the St. Olav's hospital clinical laboratory for hsCRP analysis. The method described for analysing can be found in the following link: http://data.stolav.no/labhandboker/Medisinsk_biokjemi/a.html (accessed 17.03.2017).

The method of analysis in this laboratory was changed during our data collection. Splitting and comparing the samples collected before and after this date did not change any of the results, and further considerations were not taken.

Cytokines and chemokines

The blood samples for the 14 cytokines and chemokines interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), interleukin 1 receptor antagonist (IL-1ra), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 17 (IL-17A), interferon γ-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1; also known as chemoattractant protein-2 [CCL2]), transforming growth factor β1 (TGF-β1), transforming growth factor β2 (TGF-β2), transforming growth factor β3 (TGF-β3) and tumour necrosis factor α (TNF-α) were collected in two 6 ml EDTA tubes. The samples were immediately put on ice and centrifuged, (1500g, 15 min, 4 °C). Plasma was aliquoted by 1 ml into cryogenic vials and frozen at -80 °C until assayed.

Multianalyte profiling / Multiplex was used to analyse the collected plasma for:

- IFN-γ, IL-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17A, IP-10, MCP-1, TNF-α (Box 4, a.)
- TGF-β1, TGF-β2, and TGF-β3 (Box 4, b.)

All analyses were performed according to the manufacturer's protocol.

Cytokine and chemokine profiling were analysed with the BioPlex 2000 multiplex testing platform and the BioPlex manager software (Biorad, Hercules, CA 94547, USA). This is an immunoassay with dual-laser, multiplex flow detection method. Magnetic 8 µm beads, infused with varying ratios of fluorescent dyes are used to create bead sets. Beads within each set are coated with a ligand (i.e. antigen, antibody, analyte, etc.) specific to the particular assay. Bead sets are then mixed in a single reagent pack, allowing for simultaneous detection of multiple analytes from a single sample.

Multiplex Plasma Cytokine Analysis

Cytokines were analysed with the following equipment:

a) MILLIPLEX® MAP immunoassay from EDM Millipore (Merck KGaA, Darmstadt, Germany; EMD Millipore Corporation, Billerica, MA 01821, USA) with the Human Cytokine / Chemokine Magnetic Bead Panel, 96 Well Plate Assay, Catalog # HCYTOMAG-60K, HCYTMAG-60K-PX29, HCYTMAG60PMX29BK, HCYTMAG-60K-PX30, HCYTMAG60PMX30BK, HCYTMAG-60K-PX38, HCYTMAG60PMX38BK, HCYTMAG-60K-PX41, HCYTMAG60PMX41BK

b) Bio-Plex Pro™ TGF-β Assays from Bio-Rad Laboratories, Inc. was used to analyse the collected plasma for: TGF-β1–TGF-β3.

Box 4

All samples analysed for IP-10, MCP-1, TNF-α, TGF-β1 and TGF-β2 had levels above the manufacturer's detection limits (8.6 pg/mL, 1.9 pg/mL, 0.7 pg/mL, 3.9 pg/mL and 1.9 pg/mL, respectively). Detection limits for IFN-γ, IL-1β, IL-1ra, IL-4, IL-6, IL-8, TGF-β3, IL-10 and IL-17A were based on the lowest detected concentrations in our samples: 0.43 pg/mL, 0.05 pg/mL, 1.6 pg/mL, 0.31 pg/mL, 0.40 pg/mL, 0.05 pg/mL, 0.91 pg/mL, 1.15 pg/mL, 6.36 pg/mL, respectively. Samples below the detection limits for these cytokines were set to half of the detection limits (0.215 pg/mL, 0.025 pg/mL, 0.8 pg/mL, 0.155 pg/mL, 0.20 pg/mL, 0.025 pg/mL, 0.46 pg/mL, 0.58 pg/mL and 3.18 pg/mL, respectively).

Kynurenines

Blood samples for tryptophan (Try) and its metabolites kynurenine (Kyn), kynurenic acid (KA), 3-hydroxykynurenine (HK), anthranilic acid (AA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (HAA), quinolinic acid (QA) and picolinic acid (Pic) were collected in EDTA plasma tubes, immediately put on ice, centrifuged (1500g, 15 min, 4 °C) and aliquoted into cryovials and frozen at -80°C until further analyses. The frozen samples were shipped to Bevital AS, Bergen, Norway, and analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) according to the company's protocols (bevital.no).

2.4 Statistics

Number of patients was calculated based on a significance level of 5% and a power of 80%. The pilot study[96] showed a SD of 1 for the most important considered cytokine in the pilot study: TNF-α. The smallest difference between the smallest and largest mean between the 3 groups worth detecting was 0.6 pg/mL of TNF-α. The numbers needed in each group became 55, and the aim was to include a total of 165 participants in the current study.

This is an observational study including variance analysis and linear regression. For linear regression, the total number of participants would be 130 for three groups. Since variance analysis estimates are 165 participants, the higher number is chosen for this study.

The statistical analyses were performed using the Statistical SoftWare Package (SPSS) Statistics for Windows, version 22. All variables were tested for normality and homogeneity by using the Shapiro-Wilk tests and visual inspection of histograms and Q-Q-plots.

2.4.1 Statistical analysis Article 1

For the variable hsCRP, natural log transformed data (ln-CRP) fulfilled the criteria for parametric statistical methods, and linear regression was applied for analysis when comparing the three groups CFS, FM and controls. Post-hoc pair-wise comparison between each group was conducted by means of *Student's t-test*. For comparison of age and BMI between groups, the Kruskall-Wallis test was applied. Mann-Whitney *U* was used for post-hoc analysis of pair-wise comparison.

2.4.2 Statistical analysis Article 2

The data consisted of a considerable number of samples below the detection limit. Transformation of the data did not improve this bias, and the Kruskal-Wallis ranks test was applied for comparison between groups. Dunn's test was used for post-hoc analysis of pair-wise group comparisons. A conservative approach was taken to account for multiple comparisons between groups, and these results were considered significant at $p < .01$. Associations between variables were analysed by Spearman's rho (ρ). Confounding factors were defined as variables with significant associations of $p < .05$.

2.4.3 Statistical analysis Article 3

The criteria for using parametric statistics were met when all variables were transformed into natural log (ln), i.e. lnTry, lnKyn, lnKA, lnHK, lnAA, lnXA, lnHAA, lnQA, and lnPic, and these ln-values were used throughout this study. The metabolite ratios were the ratios between one log transformed metabolite over another log transformed metabolite: [lnKyn]/[lnTry], [lnKA]/[lnKyn], [lnXA]/[lnHK], [lnHK]/[lnKyn], [lnAA]/[lnKyn], [lnHAA]/[lnHK], [lnKA]/[lnQA], and [lnKA]/[lnHK]. Less is known on effects of the kynurenines compared to the other markers studied (hsCRP and cytokines/chemokines), and thus comparing ratios were considered useful. The chosen ratios were based on the assumption that they indirectly represent enzyme activity of the kynurenine pathway or are indicative of a neuroprotective or neurotoxic state (Table 2.3).

Table 2.3. *The Enzymes of the Kynurenine Pathway and the Kynurenine Ratios Representing Those Enzymes*

Name / Enzyme	Abbreviation	Ratio	Concentration ratio
Indoleamine 2,3-dioxygenase	IDO	$\frac{\text{Kyn}}{\text{Try}}$	$\frac{[\ln\text{Kyn}]}{[\ln\text{Try}]}$
Kynurenine aminotransferase	KAT I	$\frac{\text{KA}}{\text{Kyn}}$	$\frac{[\ln\text{KA}]}{[\ln\text{Kyn}]}$
Kynurenine aminotransferase II	KAT II	$\frac{\text{XA}}{\text{HK}}$	$\frac{[\ln\text{XA}]}{[\ln\text{HK}]}$
Kynurenine 3-monooxygenase	KMO	$\frac{\text{HK}}{\text{Kyn}}$	$\frac{[\ln\text{HK}]}{[\ln\text{Kyn}]}$
Kynureninase	-	$\frac{\text{AA}}{\text{Kyn}}$	$\frac{[\ln\text{AA}]}{[\ln\text{Kyn}]}$
Kynureninase	-	$\frac{\text{HAA}}{\text{HK}}$	$\frac{[\ln\text{HAA}]}{[\ln\text{HK}]}$
Neuroprotective ratio 1	NPR-1	$\frac{\text{KA}}{\text{QA}}$	$\frac{[\ln\text{KA}]}{[\ln\text{QA}]}$
Neuroprotective ratio 2	NPR-2	$\frac{\text{KA}}{\text{HK}}$	$\frac{[\ln\text{KA}]}{[\ln\text{HK}]}$
The enzymes are not actually included in this study. KAT has several isoforms, and KAT I and KAT II are used here to differentiate between the KA/Kyn and XA/HK ratios. The metabolic turnover (given as ratio) is used as an indicator of these enzymes.			

The group overall effect shows to what extent the group variable contributed to the model. The overall group variable would be the whole study population. Each diagnostic groups CFS and FM were compared against the control group. *Student's t-test* was used for post-hoc pair-wise comparison between the CFS, FM and control groups, which was the built-in comparison feature of the statistical program with significance levels set to $p < .05$.

In addition, the intercept was included based on the primary setting of the statistical program, which automatically includes this in the model (but can be opted out). We decided that it would not be of any disadvantage to include the intercept. Interpreting the intercept, however, makes no sense, since all the covariates added would take on the value "zero". E.g. all participants were already born (and no infants were included), and the body mass was intact throughout the sampling period.

2.4.4 Explorative approach to Article 3

In this thesis an additional, different, and more explorable approach to that presented in the article (Article 3: Kynurenine metabolites and ratios differ between Chronic Fatigue Syndrome, Fibromyalgia, and healthy controls)[3] is made: The three groups (CFS, FM and controls) were put into three regression models, which then were compared to find the best explanatory factor(s) of any differences in kynurenines or the ratios that may not be explained by the diagnostic group. The three regression models 0, 1 and 2 are presented in Box 5.

Regression models for the tryptophan-kynurenine pathway

- Model 0 (Baseline):
  - Fixed factors: Group (CFS, FM, controls)
- Model 1 (Hypothesis):
  - Fixed factors: Group (CFS, FM, controls)
  - Co-factors: age, BMI, HADS anxiety, HADS depression
- Model 2 (Explorative):
  - Fixed factors: Group (CFS, FM, controls)
  - Co-factors: age, BMI, HADS anxiety, HADS depression, fatigue, pain, FS scores, nicotine, IgE

Box 5

3 Results

3.1 Summary of the articles

3.1.1 Summary of Article 1

Patients with Fibromyalgia and Chronic Fatigue Syndrome show increased hsCRP compared to healthy controls

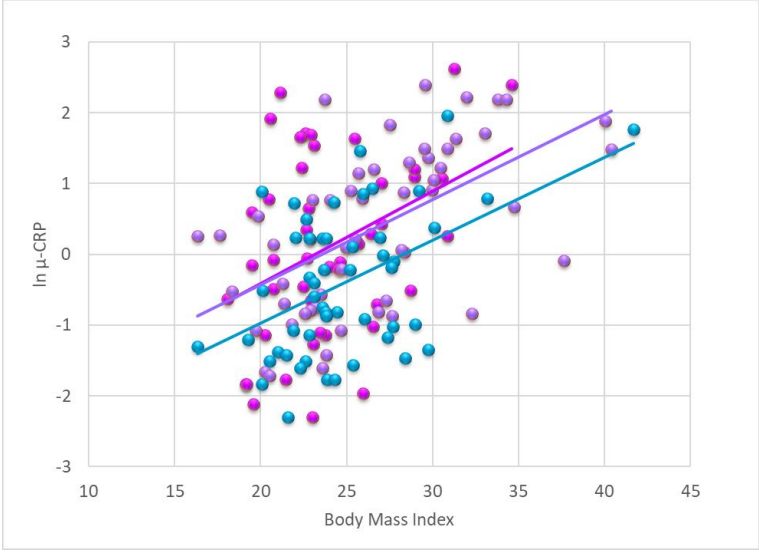
Groven N, Fors EA, Reitan SK

Brain, Behavior, and Immunity. 2019;81:172-177.

The purpose of this study was to compare levels of the inflammatory marker high sensitivity CRP (hsCRP) between CFS and FM compared to healthy controls. Blood samples of 49 CFS patients, 57 FM patients and 54 healthy controls were analysed.

Main findings:

- hsCRP levels were significantly higher for both the CFS and FM groups compared to healthy controls when adjusting for age, smoking and BMI ($p = .006$).
- There was no difference between the two patient groups.
- Level of hsCRP was affected by BMI ($p < .001$) but not age and smoking.



3.1.2 Summary of Article 2

MCP-1 is Increased in Patients with CFS and FM, whilst several other immune markers are significantly lower than healthy controls

Groven N, Fors EA, Stunes AK, Reitan SK.

Brain, behavior, & immunity - health. 2020;4:100067.

This article explored the levels of immunomarkers in 49 CFS patients, 57 FM patients and 54 healthy controls. Plasma levels of IFN-γ, IL-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17, IP-10, MCP-1, TGF-β1, TGF-β2, TGF-β3 and TNF-α were analysed by multiplex.

Main findings:

- MCP-1 was significantly increased in both patient groups compared to healthy controls ($p < .001$).
- IL-1β, Il-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL17 all were significantly lower ($p < .003$) in the patient groups than healthy controls.
- IFN-γ was significantly lower in the FM group ($p < .001$).
- For IL-8, IP-10 and IL-1ra there were no significant difference between the groups.

3.1.3 Summary of Article 3

Kynurenine Metabolites and Ratios Differ Between Chronic Fatigue Syndrome, Fibromyalgia, and Healthy Controls

Groven N, Reitan SK, Fors EA, Guzey IC.

Psychoneuroendocrinology. 2021;131:105287-105287.

This study explored the plasma kynurenine metabolite status in the 49 CFS and 57 FM patients as well as 54 healthy controls eligibly enrolled in this study, and how the overlapping symptoms such as anxiety and depression may be correlated to these metabolites and their ratios in these patient groups.

Main findings:

- Quinolinic acid (QA) differed between CFS and FM patients ($\beta = .144$, $p = .036$) and was related to higher levels of BMI ($p = .002$).
- The neuroprotective ratio given by kynurenic acid (KA) and QA: KA/QA was lower for CFS patients compared to healthy controls ($p = .016$).
- The neuroprotective ratio given by KA and 3-hydroxykykynurenine (HK): KA/HK was lower for FM patients compared to healthy controls ($p = .048$).
- Lower neuroprotective ratio KA/HK was also associated with increased symptoms of pain ($p = .002$).
- The kynurenine aminotransferase II (KAT II) enzymatic activity given by the ratio of xanthurenic acid (XA) and HK: XA/HK was lower for FM patients compared to healthy controls ($p = .013$).
- KAT II (XA/HK) was negatively associated with BMI ($p = .039$).
- Symptoms of anxiety and depression were not associated with the metabolites or ratios studied.

Because of the complexity of the data, this following section is an extensive representation of the results, upon which the discussion is also based.

3.2 Sampling

As seen in the flowchart (Figure 2.1) a total of 160 participants were enrolled in this study. There were 49 patients that fulfilled the CFS diagnosis according to the Fukuda 1994 criteria.[5] All but three CFS patients were additionally evaluated for FM diagnosis,[11] and 13 CFS patients fulfilled the ACR 1990 criteria in the clinic. These 13 CFS patients were still included in the CFS group. There were 58 patients diagnosed with FM according to the ACR 1990 criteria,[11] of which none were evaluated according to the Fukuda 1994 criteria for possible CFS diagnosis. The 53 healthy controls did not have any history of CFS nor FM diagnoses.

3.3 Interview

3.3.1 Time and date

Data were collected between March 2015 and December 2016. Patients were recruited consecutively, and the data collection was distributed throughout the year, with most patients being tested in the spring season (both years; Figure 3.1). Participants in the control group were recruited mostly during the spring of 2015. Time of year did not seem to influence any of the (analyses run, data not shown).

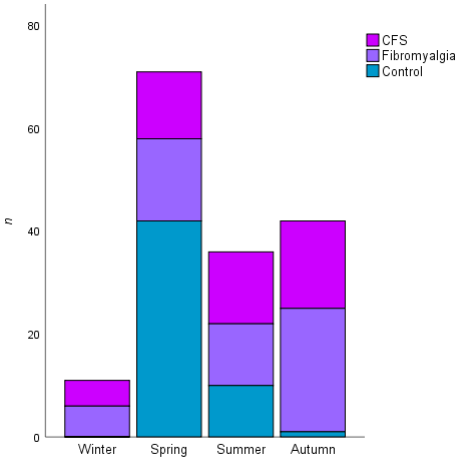


Figure 3.1.
The seasons of which the participants in this study (CFS, FM and controls) were tested (data collected).

3.3.2 Age and sex

The age for all participants ranged from 18 to 60 years ($M = 39$ years, $N = 160$). The groups differed in age (Table 3.1) with the CFS group being significantly younger than both the FM group ($U = 786.5$, $z = 3.97$, $p < .001$, $r = .38$) and the control group ($U = 916.0$, $z = 2.56$, $p = .010$, $r = .25$). The FM group and control group were within the same age ($U = 1314.5$, $z = 1.32$, $p = .189$, $r = .13$). All participants in this study were female.

3.3.3 BMI

BMI was calculated based on height and weight:

$$BMI = \frac{weight\ (kg)}{[height\ (m)]^2}$$

BMI was not equally distributed between the groups, with the FM group being significantly higher than CFS patients ($U = 928.0$, $z = 2.69$, $p = .007$, $r = .26$), and the control group ($U = 1182.0$, $z = 1.97$, $p = .049$, $r = .19$). The CFS group was not different from the control group ($U = 1095.0$, $z = 1.04$, $p = .299$, $r = .10$).

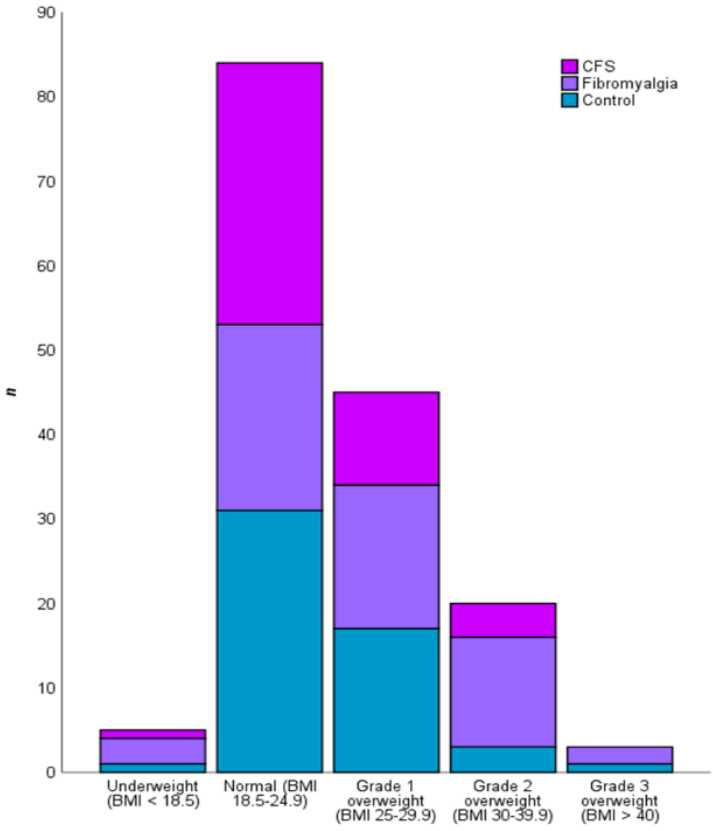


Figure 3.2.
Cases from CFS, FM and controls within the 5 BMI categories: underweight (BMI < 18.5); normal (BMI 18.5-24.9); overweight (BMI 25-29.9); obese (BMI 30-39.9); and severely obese (BMI > 40).

BMI categories[97] and frequency for the total study population ($N = 160$):

- Underweight (BMI < 18.5) 3%
- Normal weight (BMI 18.5-24.9) 53%
- Grade 1 overweight (BMI 25-29.9) 28%
- Grade 2 overweight / Obese (BMI 30-39.9) 11%
- Grade 3 overweight / Severely obese (BMI > 40) 3%

In the total study population, 15 % were considered obese or severely obese (BMI > 30, of which 3% were CFS patients, 9% were FM patients, and 3% were controls.

3.3.4 Subjective symptoms of infection and feverishness

Some patients reported always feeling "ill" due to their symptoms of either CFS ($n = 4$) or FM ($n = 11$), and one control person reported having symptoms of an oncoming cold or flu. A current feeling of "feverishness" was reported in 11 CFS patients, 13 FM patients and one control. The subjective symptoms of infection were compared to the leukocyte count and hsCRP. Slight abnormal findings in the leukocyte count were only found in four of the FM patients (and none of the other subjects), and only one of them had hsCRP > 5 (6.20). No participants were excluded based on the subjective symptoms of infection and feverishness due to the discrepancy between subjective symptoms and objective findings of infection.

Table 3.1. *Descriptives of Age, Body Mass Index (BMI), and Psychometrics: Depression, Anxiety, Fatigue, Fibromyalgia Severity (FS), and Pain*

	CFS (n = 49)				FM (n = 58)				Control (n = 53)					
Parameter	Missing (n)	*M* (*SD*)	*Mdn*	*Range* (*min–max*)	Missing (n)	*M* (*SD*)	*Mdn*	*Range* (*min–max*)	Missing (n)	*M* (*SD*)	*Mdn*	*Range* (*min–max*)	*H*	*p*[a]
Age	0	33.8 (11.3)	35.0	18–60	0	42.0 (9.1)	42.5	22–60	0	39.4 (10.4)	39.0	23–59	15.974	< .001
BMI	2	24.0 (3.6)	23.1	18.1–34.6	1	26.7 (5.6)	25.7	16.3–40.4	0	24.7 (4.0)	23.8	16.3–41.7	8.188	.017
HADS total	0	11.9 (7.8)	11.0	0–29	1	14.8 (7.3)	14.0	1–31	1	4.5 (3.7)	4.0	0–14	53.197	< .001
HADS depression	0	6.0 (4.2)	5.0	0–17	1	6.4 (3.9)	6.0	0–16	1	1.3 (1.8)	1.0	0–8	60.963	< .001
HADS anxiety	0	5.9 (4.6)	5.0	0–19	1	8.4 (4.1)	8.0	0–17	1	3.2 (2.6)	3.0	0–10	39.979	< .001
NRS pain	0	2.9 (2.1)	3.0	0–8	0	4.9 (2.2)	5.0	0–8	1	0.5 (1.2)	0.0	0–6	83.958	< .001
Fatigue score	0	25.5 (5.3)	26.0	12–33	1	22.5 (5.5)	23.0	7–33	1	10.3 (3.2)	10.0	3–22	97.897	< .001
FS score	0	15.2 (5.5)	13.0	7–29	1	20.1 (5.2)	20.0	3–30	1	3.1 (2.5)	3.0	0–11	110.455	< .001

BMI = Body mass index. HADS = Hospital anxiety and depression scale. NRS pain = Numeric rating scale (0–10) of pain severity experienced at present. Fatigue scores are the total scores of the Chalder fatigue questionnaire (CFQ). FS = Fibromyalgia severity; based on the fibromyalgia survey diagnostic criteria (FSDC).
[a]Statistical difference (Kruskall-Wallis test: *H*).

3.3.5 Comorbid disorders/diagnoses

Comorbid disorders and diagnoses were seen in all groups of participants. In the CFS group, 49% reported having one or more comorbid disease/disorders in addition to their CFS diagnosis. Of these, the most common comorbidities were hypothyroidism (in which cases this was stabilised by medication; *n* = 7), migraine (*n* = 4) and asthma (*n* = 3). In the FM group, 80% reported having comorbid disease/disorders, with the most common being asthma (*n* = 8), migraine (*n* = 7) and IBS (*n* = 3). For the control group, 19% (*n* = 10) reported having a disease or medical diagnosis of which only migraine was reported by more than one participant (*n* = 2). Individual diagnoses for the others (*n* = 8) cannot be publicised due to ethical considerations.

3.3.6 Allergies

Allergies were commonly reported (not published) and varied from confirmation from medical doctors to conclusions from alternative consultations or personal perspective. To avoid any bias, active allergies were partly controlled for by serum concentrations of IgE (see Section 3.5 Blood samples – General; IgE)

3.3.7 Medication

Patients using immunosuppressive medication for treatment of autoimmune disease were excluded from the study (see Section 2.1.4 Exclusion criteria). The total number of any medication used in the CFS group, FM group and controls was 36, 126, and 16, respectively. (Notice that some patients were using several medications simultaneously, which explains the higher number compared to participants). Approximately half of the CFS patients (47%), the majority of FM patients (86%), and one quarter of the controls (26%) were on some sort of medication. The highest frequency of medication used by all groups (*N* = 46 using 70 medications) were medication targeting the CNS (ATC code N06A; FM: 30 medications registered; CFS: 5 medications registered; and controls: 3 registered medications; Table 3.2). (The second most used group of medication were antihistamines (FM: 18 medications registered; CFS: 11 medications registered; and controls: 6 medications registered). Thirty-six percent of the study population had been taking medication on the day of sampling (CFS = 20%, FM = 69%, and controls = 15%). No participants were taking mood stabilisers or antipsychotic medication, as these would have been excluded prior to enrolment in this study.

The medications potentially influencing the results (Table 3.2) were the regular use of antidepressants (ACT-code N06A) and immunomodulatory medication (ACT-code H02A, M01A and N02B E01) taken on the day of sampling.

Table 3.2. *Frequencies of Immunomodulatory and Anti-depressive Medication*

	Regular use (*n*)			Day of sampling (*n*)		
Medication (ACT-code)	CFS	FM	Controls	CFS	FM	Controls
Immunomodulatory medication:	**4**	**17**	**1**	**4**	**9[a]**	**1**
Corticosteroids (H02A)	0	1	0	0	1	0
Anti-inflammatory medication (M01A)	1	8	1	1	6	1
Paracetamol (N02B E01)	3	8	0	3	3	0
Anti-depressive medication (N06A):	**3**	**14**	**1**	**3**	**14**	**1**
NSRI	0	8	0	0	8	0
SSRI	2	3	1	2	3	1
Other	1	3	0	1	3	0

NSRI = Non-selective monoamine reuptake inhibitors. SSRI = Selective serotonin reuptake inhibitors.
[a]Participants could be registered taking more than one drug.

The cytokines, chemokines, hsCRP and IgE were tested for the influence of medication by the following measures:

1. The original analyses of the cytokines were re-run by excluding cases that were using immunomodulatory medication (ACT-code H02A, M01A and N02B E01) and/or antidepressants (ACT-code N06A).
2. Conducting a Mann-Whitney *U* test and comparing each cytokine in each group (CFS, FM and control) and the total study population, comparing:
    a. The participants who were on either immunomodulatory and/or anti-depressive medication
    b. The participants who were not on any of these medications

Comparing the participants on anti-depressants (N06A; $n = 14$) with all participants not on anti-depressants ($n = 44$) in the FM group, showed higher IL-4 levels in the group on anti-depressive medication (M (SD) = 22.58 (39.21), 95% CI = −1.12–46.28, $Mdn = 6.42$) compared to the group not on medication (M (SD) = 24.64 (111.37), 95% CI = −10.06–59.35, $Mdn = 0.84$), ($U = 189.5$, $z = -2.22$, $p = .027$).

None of the other tests had any effect on the results (data not shown).

3.3.8 Hormones

All participants were asked about the status of their menstrual cycle, menopause and any type of contraceptives. 10% of the study population reported having reached menopause, 10% were in the transition of reaching menopause, and 80% were still fertile. Approximately 39% of the females were using hormonal contraceptives. The Kruskall-Wallis test showed there was no difference in distribution of cases with menopause between the CFS, FM and control group ($H(2) = 3.15$, $p = .207$). Similarly, there were no differences between the groups in the use of hormonal contraceptives and menstrual cycle ($H(2) = 0.261$, $p = .878$).

3.3.9 Physical activity

One patient in the CFS group reported being bedridden due to the disorder. The number of participants conducting regular exercise were six in both the CFS group and FM group (12 total), and 38 in the control group. A binominal scale of activity showed that both patient groups had lower activity levels (43% of CFS and 30% of FM), whilst all control subjects reported high activity levels. This means there was a significantly higher activity level in the control group compared to both CFS and FM patients ($\chi^2(2) = 27.38$, $p < .001$), whereas the CFS and FM patients were equally less active ($\chi^2(1) = 2.13$, $p = .145$).

3.3.10 Nicotine

From the total study population 29% reported a regular use of nicotine substances (Table 3.3). Compared to controls, more of the CFS and FM patients were using nicotine regularly ($\chi^2(1) = 4.36$, $p = .037$; and $\chi^2(1) = 7.65$, $p = .006$, respectively). There were no differences in the number of nicotine users in the two patient groups CFS and FM ($\chi^2(1) = 0.41$, $p = .525$).

Table 3.3. *Frequencies of Smoking or Nicotine Use*

	CFS		FM		Control		Total	
	n	%	*n*	%	*n*	%	*n*	%
No	33	67.3	35	60.3	45	84.9	113	70.6
Yes	16	32.7	22	37.9	8	15.1	46	28.8
Missing	0	0	1	1.7	0	0	1	1.7

3.3.11 Duration of illness

The requirement for FM diagnosis is having the symptoms lasting for three months or longer.[11] One patient in the FM group reported duration of less than one year since the onset of symptoms. It was still over 6 months, which is the requirement for CFS diagnosis.[5] The frequency distribution of the patient groups according to the duration is found in Table 3.4. In the CFS group, 27% reported that the disorder started less than three years ago. In the FM group, this was 18% of the patients. The majority of CFS and FM patients reported to have had the disorder longer five years (53% and 72% respectively). There were no differences in the number of patients in the CFS and FM group with shorter illness (< 3 years) and longer lasting illness (> 3 years) ($\chi^2(1) = 2.13$, $p = .145$).

Table 3.4. *Number of Years Since Debut of the Disease for Chronic Fatigue Syndrome (CFS) and Fibromyalgia (FM) Patients*

	CFS		FM	
Onset	*n*	%	*n*	%
< 1 year	0	0	1	2
< 3 years	13	27	9	16
3-5 years	8	16	6	10
> 5 years	26	53	42	72
Missing	2	4	0	0
Total	49	100	58	100

3.4 Questionnaires

The data for the questionnaires are found in Table 3.1.

3.4.1 The hospital anxiety and depression scale

HADS total scores

The HADS total scores were significantly lower in the control group ($M = 4.5$, $SD = 3.7$) compared to CFS and FM patients ($M = 11.9$, $SD = 7.8$, $U = 558.5$, $z = -4.87$, $p < .001$, $r = .48$; and $M = 14.8$, $SD = 7.3$, $U = 302.0$, $z = -7.17$, $p < .001$, $r = .69$, respectively). There was a difference, although not significant, with the FM group having the highest HADS total scores compared to CFS ($U = 1094.0$, $z = 1.92$, $p = .055$, $r = .19$). The same pattern was found for the sub-scales of HADS.

HADS anxiety scores

The lowest HADS anxiety scores were reported in the control group ($M = 3.2$, $SD = 2.6$), and this was significantly lower than the CFS and the FM group ($M = 5.9$, $SD = 4.6$, $U = 850.0$, $z = -2.90$, $p = .004$, $r = .29$; and $M = 8.4$, $SD = 4.1$, $U = 435.5$, $z = -6.37$, $p < .001$, $r = .61$, respectively). The highest anxiety scores were reported in the FM group were also significantly higher compared to the CFS patients ($U = 904.0$, $z = -3.13$, $p = .002$, $r = .30$).

HADS depression scores

The lowest depression scores were reported by the controls ($M = 1.3$, $SD = 1.8$), and this was significantly lower than the CFS and FM group ($M = 6.0$, $SD = 4.2$, $U = 369.5$, $z = -6.23$, $p < .001$, $r = .62$; and $M = 6.4$, $SD = 3.9$, $U = 297.0$, $z = -7.26$, $p < .001$, $r = .69$, respectively). The two patient groups CFS and FM did not differ from each other for the HADS depression scores ($U = 1302.5$, $z = -0.60$, $p = .550$, $r = .06$).

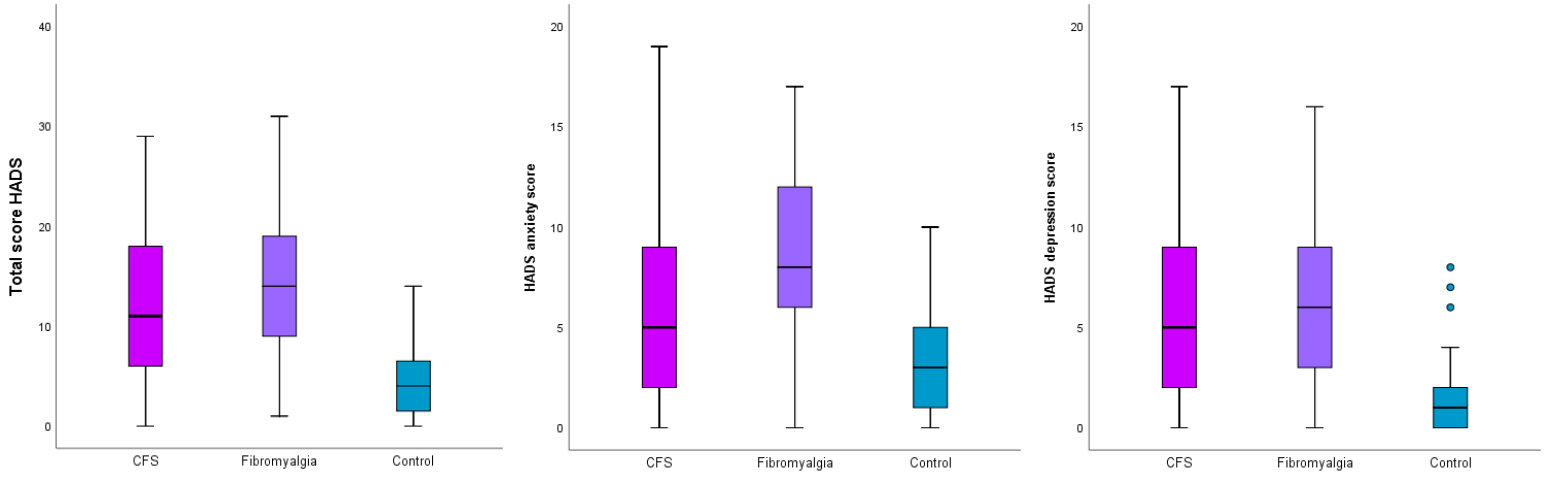


Figure 3.3. *Boxplots of HADS Scores for CFS, FM and Controls*

3.4.2 Numeric rating scale – Pain

Pain was measured on an NRS scale, and the results are shown in Table 3.1 and Table 3.5. Three questions were taken from the brief pain inventory (BPI)[98] and three self-constructed questions were added (NRS 1–3) of which NRS 1 was used continuously throughout the analyses:

- BPI 1: For all four pain items listed above, the FM group was significantly higher than both the CFS group ($U = 733.5$, $z = 4.36$, $p < .001$; $U = 730.5$, $z = 4.39$, $p < .001$; $U = 628.0$, $z = 5.01$, $p < .001$; and $U = 737.0$, $z = 4.31$, $p < .001$, $r = .42$,) and the control group ($U = 85.5$, $z = 8.52$, $p < .001$; $U = 244.0$, $z = 7.76$, $p < .001$; $U = 69.5$, $z = 8.66$, $p < .001$; and $U = 146.5$, $z = 8.36$, $p < .001$, $r = .80$).

The CFS group scored significantly higher on the same pain items compared to controls ($U = 190.0$, $z = 7.36$, $p < .001$; $U = 559.0$, $z = 5.13$, $p < .001$; $U = 243.5$, $z = 7.05$, $p < .001$; and $U = 363.5$, $z = 6.49$, $p < .001$, $r = .65$).

The two additional NRS items measured fatigue and stress:

For both items (NRS 2 and NRS 3) the control group scored lower than both the CFS group ($U = 78.5$, $z = -8.21$, $p < .001$; and $U = 713.5$, $z = -3.97$, $p < .001$) and FM patients ($U = 101.5$, $z = -8.50$, $p < .001$; and $U = 802.5$, $z = -4.40$, $p < .001$). There were no differences between the CFS and FM patients for the same variables ($U = 1235.5$, $z = 1.13$, $p = .241$; and $U = 1261.5$, $z = 1.01$, $p = .312$).

Table 3.5. *Descriptives of the Numeric Rating Scale (NRS) items*

	CFS (*n* = 48)			FM (*n* = 58)			Control (*n* = 52)				
Parameter	*M (SD)*	*Mdn*	*Range (min–max)*	*M (SD)*	*Mdn*	*Range (min–max)*	*M (SD)*	*Mdn*	*Range (min–max)*	*H*	*p*[a]
BPI 1	5.8 (2.0)	6	1–10	7.4 (1.8)	8	0–10	1.7 (1.7)	1	0–7	94.542	< .001
BPI 2	1.7 (1.7)	1	0–7	3.2 (2.2)	3	0–9	0.4 (0.7)	0	0–3	69.104	< .001
BPI 3	3.9 (2.0)	4	0–9	6.0 (1.8)	6	0–10	0.9 (1.2)	1	0–5	96.546	< .001
NRS 1	2.9 (2.1)	3	0–8	4.9 (2.2)	5	0–8	0.5 (1.2)	0	0–6	83.958	< .001
NRS 2	5.8 (2.0)	6	1–10	6.3 (2.2)	6	0–10	1.1 (1.2)	1	0–5	93.041	< .001
NRS 3	2.3 (2.2)	2	0–8	3.0 (2.7)	2	0–9	0.8 (1.3)	0	0–5	23.448	< .001

BPI = Brief Pain Inventory. BPI 1 = Highest pain experienced during the last week. BPI 2 = Lowest pain experienced during the last week. BPI 3 = Average pain experienced during the last week. NRS 1 = Current experienced pain. NRS 2 = Current experienced fatigue. NRS 3 = Current experienced level of stress. All scale ranges: 0–10.

[a]Statistical difference (Kruskall-Wallis test: *H*).

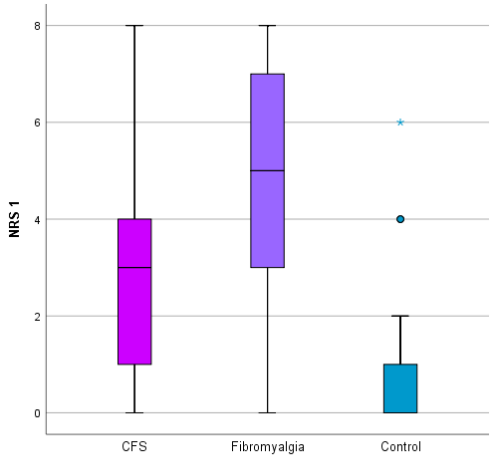


Figure 3.4.
Boxplots of Experienced Pain During Sampling Assessment (NRS 1) for CFS, FM and Controls

3.4.3 Chalder fatigue questionnaire – Fatigue score

Fatigue scores were highest for the CFS group (*Mdn* = 25.5, *SD* = 5.3), followed by the FM group (*Mdn* = 22.5, *SD* = 5.5) and lowest for the control group (*Mdn* =10.3, *SD* = 3.2). There were highly significant differences between CFS patients and controls ($U = 30.0$, $z = -8.47$, $p < .001$, $r = .82$) and FM patients and controls ($U = 112.0$, $z = -8.33$, $p < .001$, $r = .78$). The differences in the fatigue scores just about reached significance levels between CFS patients and FM patients ($U = 957.0$, $z = -2.79$, $p = .005$, $r = .28$).

Table 3.6 shows the number of fatigued cases. The cut-off score for fatigue is the bimodal score of ≥ 6. The sensitivity of this score to correctly recognise CFS cases was .96, whilst the specificity of discriminating between not fatigued cases when there was a positive CFS diagnosis was .93

Table 3.6. *Fatigued Cases for CFS and FM According to Bimodal Fatigue Scores ≥ 6*

		n	%
CFS	Not fatigue	4	8.2
	Fatigue	45	91.8
	Missing	0	0.0
FM	Not fatigue	9	15.5
	Fatigue	48	82.8
	Missing	1	1.7
Control	Not fatigue	50	94.3
	Fatigue	2	3.8
	Missing	1	1.9

3.4.4 Fibromyalgia survey diagnostic criteria – Fibromyalgia severity score

The FS (fibromyalgia severity) score was highest for the FM group ($M = 20.1$, $SD = 5.2$) followed by CFS ($M = 15.2$, $SD = 5.5$), and lowest for the control group ($M = 3.1$, $SD = 2.5$). The FM group had significantly higher FS score than both CFS and controls ($U = 680.5$, $z = -4.64$, $p < .001$, $r = .45$; and $U = 29.5$, $z = -8.87$, $p < .001$, $r = .85$, respectively). The CFS group also had significantly higher FS scores compared to controls ($U = 21.0$, $z = -8.53$, $p < .001$, $r = .85$).

Table 3.7 shows the number of FM positive cases with the use of the FSDC (FS scores ≥ 12). The sensitivity of correctly recognise FM cases that also fulfilled the ACR 1990 criteria was discriminating true FM cases was .88, and the specificity of discriminating between non-cases was .87.

Table 3.7. *FM Criteria 2016*

		n	%
CFS	Fulfilling criteria	21	42.9
	Not fulfilling criteria	28	57.1
FM	Fulfilling criteria	49	84.5
	Not fulfilling criteria	9	15.5
Control	Fulfilling criteria	0	0.0
	Not fulfilling criteria	52	98.1
	Missing	1	1.9

3.5 Blood samples – General

3.5.1 Serology

No participants had any active infections for the analytes from serology: antinuclear antibody test (ANA) screening, rheumatic factor (RF) IgM, tissue anti-transglutaminase IgA, anti-gliadin IgG, Mycoplasma pneumonia, Cytomegalovirus, Epstein-Barr virus, Hepatitis B virus, Hepatitis C virus, or Borrelia burgdorferi.

3.5.2 Leukocytes

The analysis of leukocyte count and the differential count of neutrophils and lymphocytes can be indicative of infection or inflammation. High leukocyte count > 9,8 × 10^9/L was found in two participants (one CFS patient and one control; none was found in FM), and both were excluded from the study (see Figure 2.1. Flowchart). Normal leukocyte count with deviating neutrophil and lymphocyte count was found in six FM patients (none was found in CFS or controls). Any deviating leukocyte (including neutrophil and lymphocytes) counts were compared to levels of hsCRP (see Section 3.6 hsCRP). A combination of abnormal leukocyte count and hsCRP > 10 was considered a clear-cut for infection. The highest hsCRP concentration registered in cases with abnormal leukocyte count was 6.20 mg/L. Any slight deviations in leukocyte count were also compared with the interview (Section 2.3.1 Subjective symptoms of infection and "Feverishness"), in response to questions 4 and 5 (Supplementary Table 1). No such symptoms interfering with the results were found, and no participants were excluded from further analyses due to the results of leukocyte count.

3.5.3 IgE

As mentioned in Section 3.3.6 Allergies, it was decided to use serum concentrations of IgE when controlling for allergies. The descriptive data for IgE is found in Table 3.11. IgE values were skewed in a manner that fitted a logarithmic scale, therefore absolute serum concentrations of IgE were converted into the natural log (ln): ln-IgE. There were no differences in the ln-IgE between the three groups CFS, FM and controls ($F(2,154) = 0.111$, $p = .895$, $r = .001$).

3.6 Blood samples – Immune markers explored in this study

3.6.1 hsCRP

hsCRP was not normally distributed, and a natural log (ln) transformation was conducted to this variable (ln-CRP). After visual inspection of the log transformed data and the standardised residual plots for the groups in the regression model, we concluded that it was

good enough to conduct a regression analysis for ln-CRP (Table 3.8). In addition, both the Kolmogorov-Smirnov and Shapiro-Wilk tests of normality were not significant for ln-CRP, and the same conclusion could be made.

The original non-transformed data of hsCRP is shown in Table 3.11. The interquartile range (IQR) of hsCRP for each group were as follows:

- CFS: 0.49–2.98 mg/L
- FM: 0.50–3.91 mg/L
- Controls: 0.27–1.27 mg/L

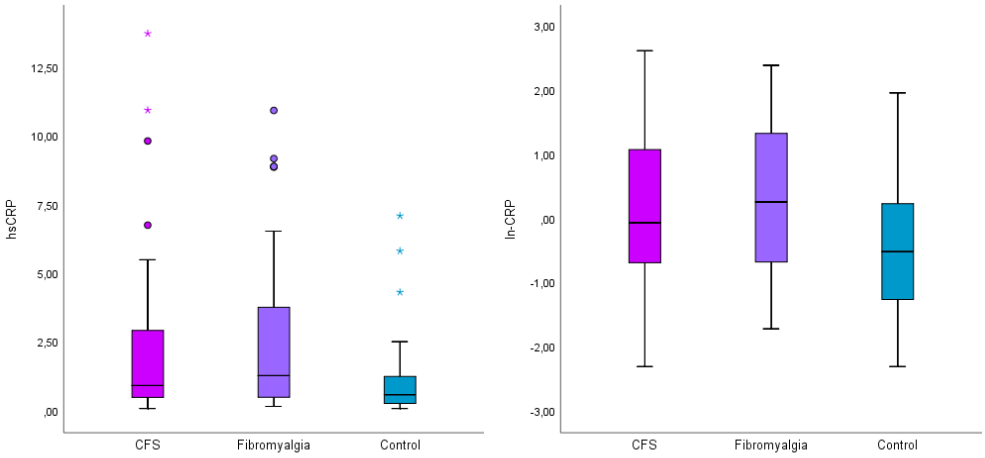


Figure 3.5.
Distribution of the non-transformed hsCRP (left), and the log transformed ln-CRP (right) for CFS, FM and controls.

Table 3.8. *Linear Regression Model for the Dependent Variable ln-CRP*

	β	95% CI		*df*	*F*	*t*	*p*	ΔR^2
Intercept	24.450	−4.168	−1.946	1	23.46		< .001	.139
Age	−0.011	−0.027	0.006	1	1.66		.200	.011
BMI	0.124	0.087	0.161	1	43.62		< .001	.231
Group (overall)				2	5.37		.006	.069
CFS	0.563	0.141	0.986			2.63	.009	
FM	0.591	0.188	0.993			2.90	.004	
Control[a]	0							

Univariate, linear regression model with forced entry.
[a]The control group was set as the reference group
R^2 = .292 (Adjusted R^2 = .273)

Diagnostic group

A pairwise t-test from the regression model for ln-CRP (Table 3.9), showed that ln-CRP was lower in the control group the CFS group and FM patients. There were no differences of ln-CRP between the CFS and FM patients.

Table 3.9. *Pairwise Comparisons of ln-CRP Between the Groups*

Group		Mean Difference	*p*	95% CI	
CFS	FM	-0.027	.902	−0.467	0.412
	Control	0.563	.009	0.141	0.986
FM	CFS	0.027	.902	−0.412	0.467
	Control	0.591	.004	0.188	0.188
Control	CFS	−0.563	.009	−0.986	−0.141
	FM	−0.591	.004	−0.993	−0.188

Pairwise t-tests from the linear regression model (**Table 3.8**).

The average age for this sample of the variable ln-CRP was 38.5 years, and the average BMI was 25.2. By transforming the ln-CRP values back according to the formula:

$$[hsCRP] = e^{[lnCRP]}$$

The mean ln-CRP for the average participant of 38.8 years and BMI of 25.2 is equivalent to the hsCRP concentration of 1.23 mg/L for the CFS group; 1.26 mg/L for the FM group; and 0.70 mg/L the control group, respectively. The mean difference of ln-CRP, using the same formula above, shows that the hsCRP for the CFS group is 76% higher than the hsCRP for the control group; and 81% higher in for the FM group than for the controls, given that the subject is 38.5 years old with a BMI of 25.2.

Overall, the group parameter could explain 6.9% of the variance of ln-CRP.

Confounding factors not included in model

The confounding factors associated with hsCRP in addition to BMI were:

- Nicotine
- Fatigue

The results for the confounding factors from the structured interview (except hormonal status and medication) and questionnaires that reached statistical significance levels of $p \leq .05$ with the correlational analyses of Spearman's ρ are found in Supplementary Table 2.

3.6.2 Cytokines and Chemokines

Visual inspection of the data of the cytokines and chemokines showed that normal distribution occurred in TGF-β2 for the CFS group; TNF-α, IL-10 and IL-17A for the FM group; and IL-10, TGF-β1, TGF-β2 and TGF-β3 for the control group. The same data also showed many outliers, and the variance of all these immune markers were not equally distributed. An attempt of log-transforming (into the natural log ln) the data, did show improvement for IP-10 (lnIP-10) and TGF-β1 (lnTGF-β1) in the CFS group; IP-10 (lnIP-10) in the FM group; and TNF-α (lnTNF-α) in the control group. The variance of the transformed data was also not equally distributed.

The limit of the analyse kit was its ability to measure low concentrations for the cytokines/chemokines. Each analyse kit came with specifications of the suggested detection limit (Table 3.10). The proportion of cases in our study population below the detection limit ranged from 0% to 29%. Some of the lowest detectable concentrations for the cytokines in our sample were lower than the detection limit suggested by the manufacturer of the analyse kit. For samples with non-detectable values an arbitrary value, that equalled half of the lowest detectable concentration, was chosen (Table 3.10).

Because none of the cytokines or chemokines (non-transformed as well as ln-transformed) were equally distributed between the groups, and also due to the many cases below the detection limit, the non-parametric Kruskall-Wallis H test was used for group comparison (Table 3.11).

Table 3.10. *Minimum Detectable Concentrations (in pg/mL) for Cytokines and Chemokines in the Sample Population (N = 159), and the Proportion of Cases Below the Detection Limit*

	IFN-γ	IL-1ra	IL-1β	IL-4	IL-6	IL-8	IP-10	MCP-1	TNF-α	TGF-β1	TGF-β2	TGF-β3	IL-10	IL-17A
dtl. analyse kit	0.8	8.3	0.8	4.5	1	0.4	8.6	1.9	0.7	3.9	1.9	0.5	1.1	0.7
Lowest concentration recorded	0.43	1.6	0.05	0.31	0	0.05	125	32.3	1.05	379.4	68.76	0.91	1.15	6.36
Value set for cases < *dtl.*[a]	0.22	0.8	0.03	0.16	0	0.03	4.3[b]	0.95[b]	0.35[b]	1.95[b]	0.95[b]	0.455	0.58	3.18
n < *dtl.*	5	42	16	46	37	36	0	0	0	0	0	10	1	5
% < dtl.	3.1	26.4	10.1	28.9	23.3	22.6	0.0	0.0	0.0	0.0	0.0	6.3	0.6	3.1

dtl. = Detection Limit.

[a]The detection limit was set to half of the lowest concentration in the sample (*dtl.*/2). [b] No samples were below the detection limit so this value is redundant. No and percentage < *dtl.* are given for the total study population.

Table 3.11. *Descriptives of the Immune Markers IgE in (kU/L), hsCRP (in mg/L), Cytokines and Chemokines (in pg/L)*

	CFS $n = 49^a$			FM $n = 58^b$			Control $n = 53^c$				
Parameter	*M* (*SD*)	*Mdn*	*Range* (*min*[d] – *max*)	*M* (*SD*)	*Mdn*	*Range* (*min*[d] – *max*)	*M* (*SD*)	*Mdn*	*Range* (*min*[d] – *max*)	*H*	*p*
IgE	105.35 (366.53)	25.00	1–2543	89.84 (249.59)	29.00	2–1626	82.38 (219.78)	19.00	2–1427	*0.404*	.817
hsCRP	2.34 (3.00)	0.94	0.10–13.74	2.62 (2.74)	1.30	0.18–10.94	1.13 (1.39)	0.60	0.10–7.11	12.179	.002
INF-γ	175.01 (879.68)	29.51	0.22–6123.03	35.33 (90.19)	17.59	0.22–640.03	92.00 (318.18)	30.31	0.22–239.33	13.964	< .001
IL-1ra	226.92 (1052.08)	20.40	0.80–7314.77	113.74 (255.37)	25.39	0.80–1428.93	135.73 (243.92)	61.10	0.80–1227.83	6.646	.031[e]
IL-1β	28.08 (143.74)	4.23	0.03–998.63	3.86 (3.68)	2.75	0.03–17.77	10.53 (16.26)	7.07	0.03–119.8	23.686	< .001
IL-4	43.07 (219.89)	5.31	0.16–1530.79	24.66 (96.43)	1.57	0.16–722.18	47.72 (48.56)	34.51	0.16–285.71	44.666	< .001
IL-6	13.69 (49.4)	2.28	0.20–336.07	5.22 (6.27)	3.45	0.20–28.36	13.35 (23.17)	8.04	0.20–169.80	25.592	< .001
IL-8	16.75 (61.55)	1.74	0.03–423.33	10.49 (23.27)	1.55	0.03–140.10	14.27 (28.33)	3.41	0.03–126.78	3.168	.205
IP-10	382.69 (204.40)	334.80	125.15–1521.68	381.84 (152.68)	326.63	174.72–785.81	374.76 (191.74)	310.43	178.96–1363.06	0.576	.750
MCP-1	221.13 (62.08)	210.78	137.88–500.72	209.97 (71.69)	202.62	32.27–561.03	190.17 (75.71)	183.25	97.09–680.53	15.865	< .001
TNF-α	37.28 (155.64)	14.81	5.60–1092.17	13.37 (5.23)	12.66	1.05–28.4	22.95 (26.03)	18.59	6.71–200.07	20.479	< .001

Descriptives of the Immune Markers IgE in (kU/L), hsCRP (in mg/L), Cytokines and Chemokines (in pg/L) (cont).

Parameter	CFS n = 49[a]			FM n = 58[b]			Control n = 53[c]			H	p
	M (*SD*)	*Mdn*	*Range* (*min*[d] – *max*)	*M* (*SD*)	*Mdn*	*Range* (*min*[d] – *max*)	*M* (*SD*)	*Mdn*	*Range* (*min*[d] – *max*)		
TGF-β1	3806.11 (3007.46)	2783.11	379.36–14394.67	3334.18 (2174.34)	2400.00	936.27–9432.22	5436.44 (1899.64)	5650.65	1366.48–8759.66	27.010	< .001
TGF-β2	390.72 (156.38)	414.70	68.76–709.93	404.48 (146.21)	341.07	204.62–693.68	559.94 (116.38)	545.91	252.38–853.75	34.466	< .001
TGF-β3	32.20 (32.68)	18.42	0.46–138.60	30.91 (27.85)	17.70	0.46–90.76	57.90 (30.46)	55.78	0.46–133.23	22.293	< .001
IL-10	19.50 (27.75)	15.32	0.58–196.94	14.50 (9.03)	13.56	1.15–53.68	19.20 (6.14)	18.56	5.51–33.89	11.798	.003
IL-17A	114.58 (92.66)	115.56	3.18–599.26	98.51 (43.06)	104.66	3.18–171.06	130.61 (40.12)	128.44	48.75–324.52	12.302	.002

[a]CFS: hsCRP n = 47 (missing 2); cytokines n = 48 (missing 1). [b]FM: IgE n = 56 (missing 2); hsCRP n = 55 (missing 3). [c]Control: IgE n =52 (missing 1); hsCRP n = 51 (missing 2). [d]Values below the detection limit were set to half detection limit. Statistical difference (Kruskall-Wallis test: H). [e]Not significant after correcting for multiple tests.

Table 3.12. *Pairwise Comparison of Cytokines and Chemokines Between Groups*

	CFS vs. FM (*N* = 106)				CFS vs. control (*N* = 101)				FM vs. controls (*N* = 111)			
	U	*z*	*p*	*r*	*U*	*z*	*p*	*r*	*U*	*z*	*p*	*r*
IFN-γ†	1014.0	-2.40	.016	.23	1154.5	-0.80	.424	.08	905.0	-3.73	< .001	.35
IL-1ra*	1371.0	-0.14	.892	.01	978.0	-2.01	.044	.20	1131.5	-2.41	.016	.23
IL-1β*	1264.0	-0.81	.416	.08	791.5	-3.27	.001	.33	715.0	-4.85	< .001	.46
IL-4*	1339.5	-0.34	.731	.03	459.5	-5.56	< .001	.55	544.0	-5.90	< .001	.56
IL-6*	1291.5	-0.65	.516	.06	648.0	-4.26	< .001	.42	790.5	-4.42	< .001	.42
IL-8	1347.0	-0.29	.774	.03	1065.0	-1.41	.158	.14	1264.0	-1.62	.105	.15
IP-10	1355.5	-0.23	.817	.02	1195.0	-0.52	.601	.05	1414.5	-0.72	.470	.07
MCP-1م	1233.5	-1.01	.314	.10	679.0	-4.03	< .001	.40	1089.5	-2.64	.008	.25
TNF-α*	1212.5	-1.14	.255	.11	842.5	-2.92	< .001	.29	774.5	-4.50	< .001	.43
TGF-β1*	1329.0	-0.40	.689	.04	684.0	-4.00	< .001	.40	709.0	-4.89	< .001	.46
TGF-β2*	1315.0	-0.49	.625	.05	493.0	-5.30	< .001	.53	716.5	-4.84	< .001	.46
TGF-β3*	1348.0	-0.28	.780	.03	702.0	-3.88	< .001	.39	815.5	-4.26	< .001	.40
IL-10‡	1240.0	-0.96	.335	.09	1012.0	-1.77	.077	.18	919.0	-3.65	< .001	.35
IL-17A*	1278.0	-0.72	.469	.07	965.5	-2.08	.037	.21	920.0	-3.64	< .001	.35

U = Mann-Whitney *U*. *z* = Standardised score of standard deviation. *p* = Two-tailed significance level, not corrected for multiple testing.
*Controls significantly higher than both CFS and FM. † Controls and CFS significantly higher than FM. م CFS and FM significantly higher than controls. ‡ Controls significantly higher than FM (CFS not different from controls nor FM).

Diagnostic group

Table 3.12 shows the results of the Mann-Whitney *U* test between the groups.[xiii]

Differences between the groups were as follows:

- CFS and FM were not different from each other but had significantly lower ranks than controls for: IL-1ra, IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β and IL-17A.
- FM had significantly lower ranks than both CFS and controls for IFN-γ. FM also had significantly lower ranks than controls for IL-10. CFS could not be distinguished from FM nor controls for this cytokine (IL-10).
- CFS and FM were not different from each other but had significantly higher ranks than controls for MCP-1.
- There were no differences between any of the three groups (CFS, FM and controls) for IL-8 and IP-10.

This is alternatively illustrated in Figure 3.6.

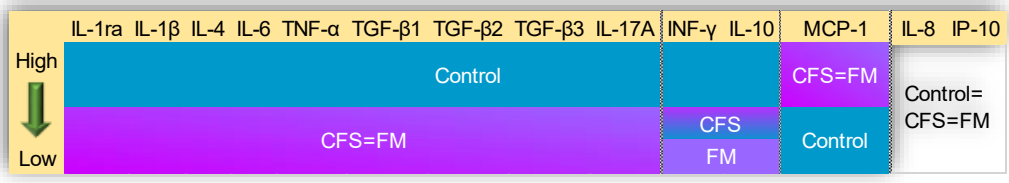


Figure 3.6. *Highest to Lowest Ranks of Cytokines and Chemokines*
A graphic representation of the findings in this study. Same colour = no difference.

Control
FM
CFS

(Groven 2022.)

[xiii] In Article 2 "Patients with Fibromyalgia and Chronic Fatigue Syndrome Show Increased hsCRP Compared to Healthy Controls" the *Dunn's test* was used for pairwise comparison between groups, and thus the results vary slightly from the Mann-Whitney *U* test presented here.

Confounding factors

The confounding factors associated with the following cytokines/chemokines:

- Age:
  - IFN-γ (FM)
  - MCP-1 (CFS, FM and controls)

- BMI:
  - IL-4 (FM)
  - IL-6 (FM)
  - IFN-γ (controls)
  - IP- 10 (controls)
  - MCP-1 (CFS)
  - TGF-β3 (FM)
- Physical activity:
  - MCP-1 (CFS)
- Medication (N06A):
  - IL-4 in FM ($z = -2.22$, $p = .027$)
- Medication (H02Am M01A and N02B E01):
  - IL-6 in FM ($z = -2.12$, $p = .034$)
  - IL-17A in FM ($z = -2.28$, $p = .023$)
- Depression
  - IP-10 (CFS)
- Fatigue
  - IL-17A (FM)
  - MCP-1 (fm)
- FS scores:
  - TGF-β1, TGF-β2 and TGF-β3 (FM)

The results for the confounding factors from the structured interview (except hormonal status and medication) and questionnaires that reached statistical significance levels of $p \leq .05$ with the correlational analyses of Spearman's ρ are found in Supplementary Table 2.

3.6.3 The kynurenine pathway

Metabolites

Non-transformed concentrations and descriptive data of the Try-Kyn-pathway are found in Table 3.13. For all further analyses and group comparisons, the ln-transformed data of the kynurenine pathway metabolites are given.

Table 3.13. *Descriptives of the Kynurenine Pathway Metabolites*

Parameter	CFS (*n* = 48)			FM (*n* = 58)			Control (*n* = 52)				
	M (SD)	*Mdn*	*Range (min–max)*	*M (SD)*	*Mdn*	*Range (min–max)*	*M (SD)*	*Mdn*	*Range (min–max)*	*H*	*p*[a]
Tryptophan	60.15 (10.79)	58.50	41.10–85.40	59.06 (13.30)	55.55	22.20–88.70	65.91 (12.52)	64.60	43.60–112.00	9.256	.010
Kynurenine	1.23 (0.27)	1.28	0.75–2.12	1.46 (0.38)	1.41	0.82–2.62	1.42 (0.25)	1.44	0.93–2.06	5.198	.074
Kynurenic acid	41.41 (17.56)	37.35	18.20–91.40	43.43 (13.84)	39.65	18.60–89.10	46.92 (11.99)	43.75	24.00–75.70	7.566	.023
Anthranilic acid	13.18 (5.63)	11.35	7.58–42.40	14.07 (3.92)	13.85	6.91–25.20	15.36 (5.70)	14.15	7.51–38.00	7.001	.030
3-Hydroxykynurenine	43.02 (12.84)	41.60	21.60–80.90	52.08 (28.42)	46.60	23.00–216.00	43.84 (10.83)	42.90	24.80–71.30	3.270	.195
Xanthurenic acid	17.89 (8.04)	15.30	4.15–39.30	17.37 (10.21)	14.80	3.02–52.00	19.68 (10.61)	16.85	8.34–72.90	2.066	.356
3-Hydroxyanthranilic acid	38.19 (12.30)	35.95	15.20–65.90	41.85 (17.43)	37.60	17.10 –91.40	42.39 (16.62)	38.95	19.00–106.00	1.330	.514
Quinolinic acid	353.69 (84.48)	353.00	189.00–683.00	352.29 (115.39)	333.00	180.00–759	335.02 (78.81)	319.00	217.00–571.00	2.116	.347
Picolinic acid	41.89 (16.19)	38.00	11.10–97.60	45.11 (24.36)	39.40	12.70–156.00	47.34 (19.77)	44.20	21.50–122.00	2.215	.330

[a]Statistical difference (Kruskall-Wallis test: *H*).

Diagnostic group

Pairwise comparisons between the CFS patients, FM patients and controls were run along with the regression models (Box 5). When no confounding factors were added (Model 0), group differences were found for tryptophan (Try), kynurenine (Kyn), kynurenic acid (KA) and anthranilic acid (AA).

When applying Model 1, this group effect only remained for AA and disappeared for all the above-mentioned metabolites. The group effects also disappeared for AA when applying Model 2.

When applying Model 2, significant group differences appeared for QA. In this model, CFS patients had higher levels of QA (M = 5.934) compared to controls (M = 5.682, mean difference = 0.252, SE = 0.083, p =.003). Significant differences were not found between CFS patients and FM (M = 5.829), nor between FM and controls.

The group differences for each model are presented in Table 3.14 and Table 3.15.

Figure 3.7 illustrates the group differences for the kynurenines and how they change when applying more factors (Model 0 through to Model 2; Box 5).

Regression models for the tryptophan-kynurenine pathway

- Model 0 (Baseline):
  - Fixed factors: Group (CFS, FM, controls)
- Model 1 (Hypothesis):
  - Fixed factors: Group (CFS, FM, controls)
  - Co-factors: age, BMI, HADS anxiety, HADS depression
- Model 2 (Explorative):
  - Fixed factors: Group (CFS, FM, controls)
  - Co-factors: age, BMI, HADS anxiety, HADS depression, fatigue, pain, FS scores, nicotine, IgE

Box 5

Table 3.14. *Group Differences for Metabolites of the Kynurenine Pathway — Model Comparisons*

Dependent variables	Model	lnTry			lnKyn			lnKA			lnAA			lnHK		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Group overall			.059	**.009**		.036	.057		.041	**.037**		.041	**.040**		.035	.064
CFS vs. control	0	-.088	.029	**.032**	-.085	.025	**.048**	-.167	.041	.011	-.159	.040	**.012**	-.033	.002	.618
FM vs. control		-.118	.056	**.003**	.008	.000	.841	-.086	.012	.163	-.070	.009	.238	.109	.019	.083
Group overall			.026	.148		.013	.390		.024	.172		.027	.140		.003	.804
CFS vs. control	1	-.066	.013	.168	-.065	.012	.186	-.119	.017	.112	-.145	.026	**.049**	.025	.001	.735
FM vs. control		-.093	.025	**.056**	-.050	.007	.307	-.128	.020	.088	-.093	.011	.207	.041	.002	.583
Group overall			.010	.500		.022	.210		.028	.140		.025	.183		.003	.804
CFS vs. control	2	.013	.000	.865	.116	.019	.107	.194	.022	.079	.155	.015	.152	.077	.003	.513
FM vs. Control		-.050	.003	.526	.129	.021	.091	.225	.027	**.054**	.209	.024	.066	.055	.001	.657

	Model	lnXA			lnHAA			lnQA			lnPic		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Group overall			.023	.165		.010	.462		.009	.546		.013	.355
CFS vs. control	0	-.085	.393	.393	-.090	.010	.222	.055	.008	.275	-.112	.012	.171
FM vs. control		-.181	.058	.058	-.030	.001	.672	.031	.003	.516	-.083	.007	.285
Group overall			.023	.182		.002	.880		.033	.087		.000	.994
CFS vs. control	1	-.007	.000	.950	-.043	.002	.621	.092	.018	.106	-.007	.000	.944
FM vs. control		-.187	.017	.114	-.031	.001	.724	-.022	.001	.700	.003	.000	.977
Group overall			.020	.256		.007	.604		.068	**.007**		.021	.236
CFS vs. control	2	.135	.004	.461	.128	.006	.347	.252	.063	**.003**	.185	.012	.205
FM vs. Control		-.080	.001	.680	.133	.006	.347	.146	.020	.098	.262	.021	.090

ΔR^2 adj. = Model adjusted R^2.
Model 0: No confounding factors. Model 1 confounding factors: Age; BMI (Body mass index); and anxiety and depression scores (from the hospital anxiety and depression scale [HADS]). Model 2 confounding factors: Same as Model 1 with additional: NRS pain (Numeric rating scale [0–10] of pain severity experienced at present); Fibromyalgia severity score (from the fibromyalgia survey diagnostic criteria [FSDC]); Fatigue scores (from the Chalder fatigue questionnaire ([CFQ]); Nicotine (Use of nicotine by smoking or snuff [binominal score]); and IgE from serum.
Red, bold font = $p < .05$. **Bold font** = trend level.

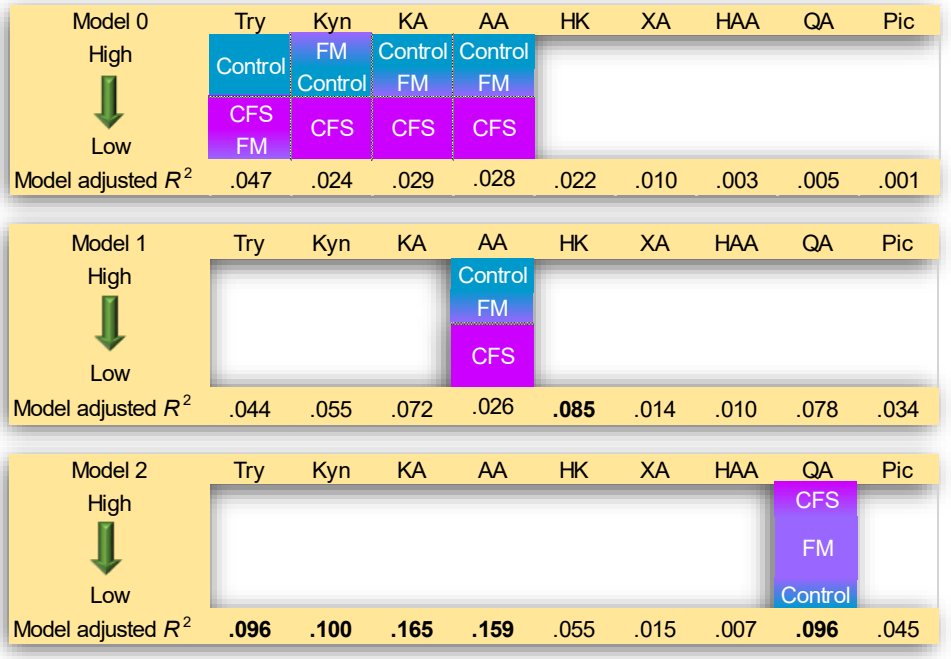


Figure 3.7. *Differences in the Kynurenines Between CFS, FM and Controls*
Illustration of the differences as they change across Model 0–Model 2. When not marked: CFS = FM = controls. **Bold font** = the strongest model R^2.

Ratios

Diagnostic group

When no confounding factors were added (Model 0), group differences were found for [lnKyn]/[lnTry], [lnXA]/[lnHK], [lnHK]/[lnKyn], [lnHAA]/[lnHK], [lnKA]/[lnQA] and [lnKA]/[lnHK] (Table 3.15 and Figure 3.8).

Table 3.15. *Group Differences for Ratios of the Kynurenine Pathway Metabolites — Model Comparisons*

Dependent variables	Model	[lnKyn]/[lnTry]			[lnKA]/[lnKyn]			[lnXA]/[lnHK]			[lnHK]/[lnKyn]		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Group overall			.069	**.004**		.018	.240		.100	**< .001**		.026	.134
CFS vs. control	0	.003	.000	.950	-.079	.009	.247	-.063	.004	.432	.054	.007	.309
FM vs. control		.126	.054	**.003**	.032	.002	.622	-.300	.090	**< .001**	.101	.026	**.045**
Group overall			.007	.610		.003	.785		.049	**.026**		.019	.242
CFS vs. control	1	.001	.000	.979	-.053	.003	.492	-.043	.001	.647	.091	.015	.140
FM vs. control		.042	.005	.398	-.036	.001	.645	-.236	.041	**.013**	.092	.015	.137
Group overall			.036	.082		.035	.086		.024	.187		.005	.714
CFS vs. control	2	.104	.013	.178	.181	.017	.123	.026	.000	.857	-.044	.002	.639
FM vs. control		.179	.034	**.029**	.275	.035	**.028**	-.158	.008	.296	-.078	.005	.428

		[lnAA]/[lnKyn]			[lnHAA]/[lnHK]			[lnKA]/[lnQA]			[lnKA]/[lnHK]		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Group overall			.015	.302		.027	.116		.055	**.012**		.049	**.020**
CFS vs. control	0	-.072	.010	.210	-.014	.005	.400	-.222	.055	**.003**	-.040	.026	.042
FM vs. control		-.078	.013	.158	-.033	.027	**.039**	-.117	.018	.096	-.051	.046	**.007**
Group overall			.009	.505		.004	.720		.039	.054		.032	.091
CFS vs. control	1	-.079	.009	.243	-.014	.003	.478	-.211	.039	**.016**	-.043	.024	.061
FM vs. control		-.042	.003	.537	-.014	.004	.474	-.106	.010	.223	-.046	.027	**.048**
Group overall			.004	.739		.004	.747		.014	.369		.010	.519
CFS vs. control	2	.033	.001	.746	.014	.002	.633	-.058	.001	.665	.020	.002	.565
FM vs. control		.075	.004	.482	.023	.004	.454	.078	.002	.580	.039	.008	.281

Model 0: No confounding factors. Model 1 confounding factors: Age; BMI (Body mass index); and anxiety and depression scores (from the hospital anxiety and depression scale [HADS]). Model 2 confounding factors: Same as Model 1 with additional: NRS pain (Numeric rating scale [0–10] of pain severity experienced at present); Fibromyalgia severity score (from the fibromyalgia survey diagnostic criteria [FSDC]); Fatigue scores (from the Chalder fatigue questionnaire ([CFQ]); Nicotine (Use of nicotine by smoking or snuff [binominal score]); and IgE from serum.

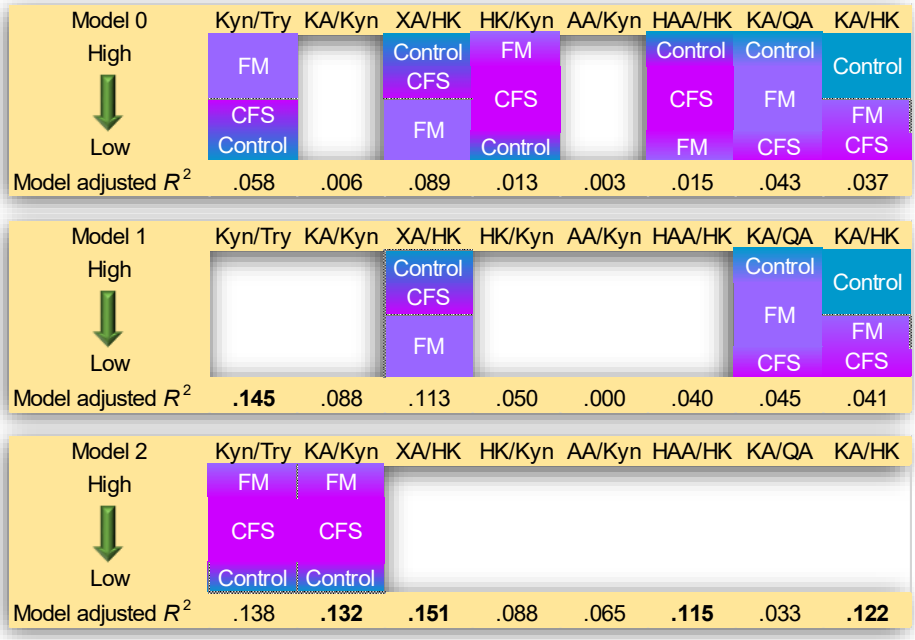


Figure 3.8. *Differences in the Kynurenines Ratios Between CFS, FM and Controls*
Illustration of the differences as they change across Model 0–Model 2. When not marked: CFS = FM = controls. **Bold font** = the strongest model R^2.

Control
FM
CFS

Confounding factors

The confounding factors associated with the metabolites and/or ratios from the kynurenine pathway:

Model 1:

- Age
- BMI

Model 2:

- Age
- BMI
- Pain
- Fatigue

HADS anxiety and depression scores, FS scores and IgE were not associated with any of the metabolites nor ratios from the kynurenine pathway.

The significant findings of group differences for the metabolites and ratios of the kynurenine pathway and the relative size in combination to other associated factors are represented in Supplementary Tables 3 and 4.

Confounding factors not included in the model:

- Medication (N06A)
    - HAA in the FM group ($z = 2.46$, $p = .014$)

Table 3.16. *Confounding Factor Significant Associations with the Metabolites of the Kynurenine Pathway*

Confounder	Model	lnKyn			lnKA			lnAA			lnHK			lnQA		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Age	1	.004	.042	**.013**	.008	.058	**.003**	.005	.025	**.053**	.003	.012	.193	.002	.007	.294
	2	.005	.060	**.004**	.008	.064	**.002**	.004	.015	.149	.003	.010	.247	.003	.013	.176
BMI	1	.006	.016	.122	.009	.015	.135	-.004	.004	.472	.019	.073	**.001**	.017	.094	**<.001**
	2	.003	.005	.409	.008	.014	.168	-.004	.004	.456	.017	.052	**.007**	.014	.069	**.002**
Pain	2	-.005	.002	.605	-.034	.030	**.039**	.005	.001	.762	.022	.012	.191	-.010	.005	.406
Fatigue	2	-.008	.026	**.059**	-.012	.025	.063	-.014	.036	**.025**	-.005	.005	.421	-.006	.013	.181
Nicotine	2	-.005	.000	.903	-.056	.006	.355	-.185	.070	**.002**	.043	.003	.496	-.093	.029	**.043**

BMI = Body mass index. HADS = Hospital anxiety and depression scale. FS score= Fibromyalgia severity from the fibromyalgia survey diagnostic criteria (FSDC). NRS pain = Numeric rating scale (0–10) of pain severity experienced at present. Fatigue = Score from the Chalder fatigue questionnaire. Nicotine: Use of nicotine by smoking or snuff (binominal score). IgE from serum.
Red, bold font = $p < .05$. **Bold font** = trend level.

Table 3.17. *Confounding Factor Significant Associations with the Ratios of the Kynurenine Pathway*

Confounder	Model	[lnKyn]/[lnTry]			[lnKA]/[lnKyn]			[lnXA]/[lnHK]			[lnHK]/[lnKyn]			[lnAA]/[lnKyn]			[lnHAA]/[lnHK]			[lnKA]/[lnHK]		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Age	1	.005	.062	**.002**	.009	.069	**.001**	-.004	.008	.274	-.001	.001	.662	.001	.001	.784	-.001	.028	**.042**	.001	.016	.126
	2	.006	.073	**.001**	.009	.071	**.002**	-.004	.010	.241	-.002	.005	.406	-.001	.003	.557	-.001	.023	.076	.001	.020	.093
BMI	1	.010	.044	**.010**	.013	.031	**.033**	-.015	.029	**.039**	.013	.053	**.005**	-.010	.024	**.059**	-.002	.016	.120	-.003	.015	.143
	2	.009	.036	**.024**	.014	.037	**.023**	-.013	.022	.084	.014	.054	**.006**	-.007	.014	.165	-.002	.013	.188	-.002	.009	.259
Pain	2	.000	.000	.996	-.028	.019	.105	-.059	.055	**.006**	.028	.029	**.044**	.010	.003	.502	-.012	.060	**.004**	-.015	.066	**.002**
Nicotine	2	.013	.001	.758	-.038	.003	.555	-.170	.034	.030	.048	.007	.344	-.181	.073	**.001**	-.040	.045	**.012**	-.028	.017	.130

BMI = Body mass index. HADS = Hospital anxiety and depression scale. FS score= Fibromyalgia severity from the fibromyalgia survey diagnostic criteria (FSDC). NRS pain = Numeric rating scale (0–10) of pain severity experienced at present. Fatigue = Score from the Chalder fatigue questionnaire. Nicotine: Use of nicotine by smoking or snuff (binominal score). IgE from serum.
Red, bold font = $p < .05$. **Bold font** = trend level.

4 Discussion

In this thesis I have presented the CFS and FM disorders, some history and relevant topics related to the immune system. It was hypothesised that an imbalance in the immune expression might be associated with either of these disorders.

The primary aim was to compare the immunological biomarkers hsCRP, cytokines and chemokines, and metabolites of the kynurenine pathway in CFS and FM outpatients, in relation to healthy control subjects. These results are discussed first, before discussing the findings and interactions of items from the psychometric data that were collected.

But before discussing the main findings of the immunological markers, first a brief note of the general blood samples:

4.1 Blood Samples – General

The main rationale for collecting additional blood samples, was quality assessment and disclosure of any disease of diagnosis that might otherwise have gone unnoticed. All blood samples were evaluated by a medical physician (SKR). Two participants had values outside the reference levels, and both were excluded from the study (Figure 2.1. Flowchart). When assessing immune markers in the study participants, it is important to keep in mind that these are all persons with normal white blood cell count and without known ongoing inflammation.

4.2 Blood samples – Immune markers explored in this study

4.2.1 hsCRP

CFS and FM groups showed significantly higher levels of hsCRP than the healthy-control group but could not be distinguished from each other. This may indicate that inflammatory systems are activated in CFS and FM patients.

hsCRP was correlated to BMI but not to age. The increased hsCRP in both patient groups compared to healthy controls was still significant when controlling for BMI.

CFS

In line with our finding on CFS, a report comparing patients with CFS and healthy controls[99] found a slight, but not significant, higher level of hsCRP in the patient group. Another study on CFS patients recruited from gastroenterology and rheumatology departments found significantly increased hsCRP levels among CFS patients compared to controls.[100] Both studies included both genders and a broader age span and did not adjust for BMI. Raison et al[101] found that an increased hsCRP in patients with CFS was no longer significant after

adjusting for age, sex, race, location of residence, BMI, depressive status, and immune-modulating medications. In our study, we had only one gender, none of the participants were taking immune-modulating medications, there was no inflammatory comorbidity, and we did not find any effect of age in our groups. We did not record for race and location of residence, but all our participants were recruited from rather homogenous areas in and around Trondheim, Norway.

FM

A large-population-based study complementing our findings reported increased CRP among participants with self-reported diagnosis of FM,[102] suggesting the increased CRP in this patient group was partially explained by BMI and comorbidity. Furthermore, a review of studies reporting the effect of non-pharmacological interventions in FM patients found higher baseline levels of CRP in three studies included, though they did not find a consistent effect on CRP.[103] A subgroup of FM patients with inflammatory changes including altered CRP has also been suggested.[104] However, there are other studies contradicting our findings. Ataoglu et al[105] found no differences between FM patients and controls regarding hsCRP.

4.2.2 Cytokines and Chemokines

In our study, the differences for cytokines and chemokines between the groups were as follows:

- CFS and FM were not different from each other, but both had significantly lower ranks than controls for: IL-1ra, IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β and IL-17A.
- FM had significantly lower ranks than both CFS and controls for IFN-γ. FM also had significantly lower ranks than controls for IL-10. CFS could not be distinguished from FM nor controls for this cytokine (IL-10).
- CFS and FM were not different from each other, but both had significantly higher ranks than controls for MCP-1.
- There were no differences between any of the three groups (CFS, FM and controls) for IL-8 and IP-10.

This is better illustrated in Figure 3.6.

Our data did not show equal distribution, due to outliers and that the data from nine of the 14 immune markers had concentrations below the detection limits of the analysing kit. Therefore, it was decided that non-parametric tests should be used when comparing levels across groups. The search of any bias due to confounding factors was conducted post-hoc with a simple correlation analysis (Spearman's ρ).

There was considerable overlap between CFS, FM and controls in the concentration levels of all cytokines. Still, our findings suggest that both CFS and FM patients have statistically higher levels of the pro-inflammatory chemokine MCP-1 compared to controls, and lower levels of nine other cytokines compared to controls: INF-γ, IL-1ra, IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL-17A.

There are not many studies comparing both CFS and FM patients on cytokines. Therefore, I will mainly discuss these patient groups separately under each cytokine/chemokine. Starting with the one chemokine that showed increased levels in both patient groups (MCP-1), followed by the pro-inflammatory cytokines and chemokines (IFN-γ, IL-1β, IL-6, and TNF-α; and IL-8 and IP-10), anti-inflammatory cytokines (IL-1ra and IL-10) and lastly the regulative cytokines (IL-4, IL-17A, TGF-β1, TGF-β2 and TGF-β3). Then the kynurenines with their possible effects on the nervous system is presented. At the end a brief discussion follows on how the cytokines might interact, showing the complexity of studying these messengers of the immune – and proposed nervous system.

MCP-1

Only MCP-1 was significantly increased in both patient groups, CFS and FM, compared to healthy controls. MCP-1 is a potent pro-inflammatory chemokine increasing inflammation by migration and infiltration of monocytes/macrophages at site of activity.[106] MCP-1 is also central in neuroinflammation[107] and is, in addition to several other chemokines, involved in pain processing and pain sensitivity in the central nervous system.[108] Also, it is central in mitochondrial metabolism, thus relevant for lack of energy / fatigue.[37] Still, there are few studies on the role of circulating MCP-1 in FM and CFS.

In cancer-related fatigue, MCP-1 and neurocognitive performance were inversely associated with fatigue *before* chemotherapy, but showed a positive correlation between MCP-1 and fatigue *after* chemotherapy.[109] Our MCP-1 finding is in contrast to Wyller et al[110] who did not find any difference for MCP-1 when comparing adolescent CFS patients to controls. Another study on MCP-1 in cerebrospinal fluid did not show any significant difference in 18 CFS patients compared to five healthy controls.[111] There were no cancer patients in our study; the study from Wyller is based on a different population (adolescents); and the study by Peterson has few participants, thus the results may not be comparable to our findings.

In a study by Bote et al[112] MCP-1 was increased along with the pro-inflammatory marker CRP in 25 patients with FM. This is in line with the increased hsCRP in found in our patient groups. The positive finding of increased MCP-1 levels for FM patients in our study is also in accordance with a study by Zhang et al. [113] where plasma MCP-1 levels were increased in 92 FM patients compared to 48 healthy controls. Similarly, *ex vivo* MCP-1 release by blood monocytes from 25 FM patients was increased compared to release from monocytes from 20 controls.[112] MCP-1 was also found in plasma, CSF and synovial fluid in osteoarthritis patients.[114] Yet, another study found that FM patients had lower expression of MCP-1 in skin biopsies than healthy controls.[115] Some of these findings might not be comparable, however, concentrations of cytokines in plasma or serum do not necessarily reflect concentrations in other tissues.[116]

Pro-inflammatory cytokines and chemokines – IFN-γ, IL-1β, IL-6 and TNF-α; IL-8 and IP-10

Pro-inflammatory cytokines and chemokines up-regulate the inflammatory response in fighting infections, healing wounds, and eliminating both external and internal triggers considered harmful to the body.

IFN-γ

CFS and controls did not differ significantly on IFN-γ in our study. In line with our finding, most studies done on CFS patients found no differences between patients and control groups for IFN-γ.[78] Montoya et al[117] found that IFN-γ correlated with the severity of CFS. In our study we did not measure severity of illness, but the patients had to be able to get into the hospital for samples and may therefore not be representative for the most severely ill and bedridden patients.

IFN-γ was lower in FM than for CFS and controls. To our knowledge, this is not previously reported. On the contrary, an increase of IFN-γ has been reported in FM patients.[118]

IL-1β, IL-6 and TNF-α

In accordance with our findings, lower plasma IL-6 levels in CFS patients have been reported.[119] Opposite and conflicting reports exist however – including one of our previous studies – where IL-1β, IL-6 and TNF-α were either increased or showed no differences in CFS compared to healthy controls.[78,120-123]

Lower plasma IL-6 and IL-1β,[124] and serum TNF-α[125] have also been reported in FM patients. However, IL-6 in FM has been reported both to be increased[112,124] and decreased,[118] and in addition to IL-6, conflicting results exist for IL-1β and TNF-α in FM patients compared to healthy controls.[79,126]

IL-8 and IP-10

The other pro-inflammatory chemokines measured, IL-8 and IP-10, did not show any difference between groups. Both chemokines are produced in response to mainly local inflammation to promote immune cells to the site of inflammation.[127]

Our findings on IL-8 are in line with the finding that adolescents diagnosed with CFS were similar to healthy controls for this cytokine.[110] IL-8 has been reported both to be both higher[128] and lower[120] in CFS compared to controls.

Similar to our findings, Ernberg et al[124] and Ranzolin et al[126] showed that FM patients did not differ from controls. However, reports of IL-8 in FM are deviating. Studies showing this chemokine in FM to be higher[112,129] or lower.[118,130]

A recent study measuring plasma levels and the cytokine content of extracellular vesicles in plasma did also not detect any differences in IP-10 in CFS patients compared to controls.[131]

This is, to our knowledge, the first study that measure IP-10 in FM patients, and we could only find one pilot study that included IP-10 measures for CFS patients.[131]

IL-8 and IP-10 may be more related to acute inflammation to recruit cells which may explain the deviating results.

Anti-inflammatory cytokines – IL-1ra and IL-10

IL-1ra and IL-10

Anti-inflammatory cytokines oppose and down-regulate the effects of inflammation, and eventually silence this response, promoting homeostasis. IL-1ra is considered anti-inflammatory. IL-1ra is a soluble receptor that binds to the pro-inflammatory cytokine IL-1, thus neutralising its effects on cells by preventing IL-1 from binding to its cellular receptors. IL-1ra, is cytokine that binds to the soluble receptor for IL-1. Therefore it reduces the effect of IL-1 by neutralising its receptor. IL-10 downregulates the production of several pro-inflammatory cytokines on a cellular level.

Similarly to our findings, IL-10 was reduced in samples of CSF in CFS patients when compared to controls,[111] and in a sub-group of CFS patients with shorter duration of illness.[119]

The less conservative approach of our study showed differences for IL-1ra for CFS and FM patients compared to controls, which is in line with Ernberg et al.[124] However, taking the conservative approach, we did not detect any differences for IL-1ra between our study groups.

IL-10 levels were reduced in FM patients compared to healthy controls in our study. This also has been reported by others.[118] IL-10 in FM has also been reported both increased[112] and unaltered.[124]

Regulatory cytokines – IL-4, IL-17A, TGF-β1, TGF-β2 and TGF-β3

Cytokines often regarded as regulatory (IL-17A, IL-4, TGF-β1, TGF-β2 and TGF-β3) were lower in both CFS and FM patients compared to the healthy control group in our study. A main function of these cytokines is to regulate the immune response, targeting specific pathologies, and orchestrating the different stages of inflammation, as it attacks, subsides and heals the wounded site. To our knowledge, studies on this are scarce and results are conflicting.[78]

IL-4

Previous reports on IL-4 in CFS have shown both lack of deviation from controls[132] and increased levels in patients compared to controls.[120]

IL-17A

In CFS patients, IL-17A levels did not deviate from healthy controls as reported by Fletcher et al.[120] IL-17A was decreased or increased for CFS cases compared to controls depending on comparing CFS as one group or subgrouping the patients according to duration of illness.[119]

TGF-β

For TGF-β there are deviating reports for CFS patients, with the majority of studies reporting no difference between CFS patients and healthy controls.[78] One study found increased TGF-β levels in serum of 192 CFS patients compared to 392 healthy controls.[117]

Not many studies have been conducted on peripheral blood TGF-β in FM.

Three isotypes of TGF-β are known in humans (TGF-β1, TGF-β2 and TGF-β3) which serve a regulatory function of the immune response.[133] Lower TGF-β found in plasma of CFS and FM patients could suggest that this regulatory influence is diminished, leading to further imbalances in the immune system of these patients.

Not many studies have been conducted on peripheral blood of these cytokines in FM patients. Conflicting results in cytokine measurements of FM patients are summarized in a meta-analysis done by Uceyler et al.[79]

4.2.3 The kynurenine pathway

As cytokines are studied extensively, the metabolites of the kynurenine pathway are less widely studied. To better understand what role these metabolites may play in CFS and FM a simple comparison of each metabolite and the ratios between the CFS and FM groups could be useful, but a more explorative approach may be in order. Also presuming that the controls represented the "standard" concentrations of the metabolites and their ratios, the control group was set as the reference group, i.e. both the CFS group and FM group would be compared to the control group in three regression models (Box 5) for the kynurenines in this study. This approach is slightly different from that presented in Article 3,[3] where the focus was mainly on Model 1:

Fatigue scores, pain scores and FS scores (*fibromyalgianess*) are higher in CFS and FM patients, as they should be due to the symptoms of these disorders. Hence, in our original hypothesis, these factors were left out.

Estimate sizes, if standardised, are comparative in nature, and as such the models can be compared. The goodness-of-fit measure (R^2) for each model indicates the percentage of the dependent variable (here: metabolites and ratios of the kynurenine pathway) that is collectively explained by the independent (predictor) variables. When comparing models, we used the adjusted (R^2), which is adjusted for the number of predictors. This can be used to evaluate the model when adding (or removing) co-factors to see whether the model improved or got worse. Figure 4.1 illustrates the strongest evidence of our findings based on effect size and significance ($p < .05$) for the strongest models (highest adjusted R^2).

Following the discussion of the metabolites and ratios of the kynurenine pathway, the possible confounders are discussed together with the other immune markers (hsCRP, cytokines/chemokines).

The model build-up rationale

Model 0

This is a simple regression model with one dependent factor (the metabolite or ratio of the kynurenine pathway) and three independent factors: the CFS group, the FM group and the control group. Choosing a regression model over a one-way ANOVA was simply based on preference. This model would serve as a comparative "baseline" for the two following models: Model 1 and Model 2.

Model 1

To better understand what role these metabolites may play in CFS and FM we built a statistical model based on the original hypothesis in Article 3[3]; that anxiety and depression might be contributing to the (possible) organic findings in this study. Age and BMI was also included in the model because the groups differed in both of these factors, thus controlling for this bias, but also because age and BMI are related to general changes in metabolic rates.

Model 2

The last model was more of an explorative model. This model included typical symptoms of CFS and FM, such as fatigue, pain and FS scores. Additional items were included that are easily overlooked: congestion of nicotinic products, and active allergies. One could have included a whole range of covariates in this model, but adding items also weakens the model. The items included in Model 2 are not necessarily independent from each other.

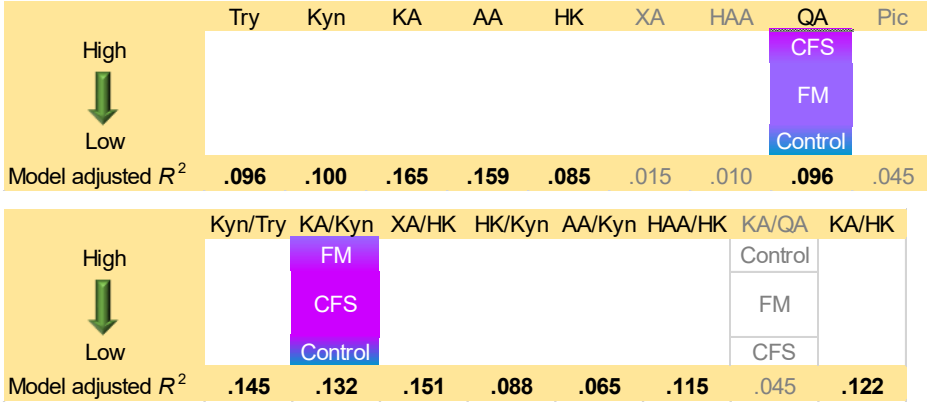


Figure 4.1. *Differences Between CFS, FM and Controls for the Metabolites and Ratios of the Kynurenine Pathway*
Representation of the best model fit (adjusted R^2) and how the CFS, FM and control groups may differ for the metabolites and ratios used in this study. The grey shaded font indicates a lack of contribution of either models as the effect sizes are very small ($R^2 < .05$) and the statistical value miniscule.

Metabolites

Tryptophan (Try)

At first sight, it may seem like Try levels were higher for controls than FM and CFS. However, the best model indicating no differences in Try levels between the groups (Figure 4.1).

Try is an essential amino acid, i.e. the body does not produce it and it must be provided from a protein source. Protein is made up from several amino acids and eating foods rich in protein (that includes Try) will increase the supply of this amino acid. Lower levels of Try could indicate insufficient Try ingestion or digestion. Since food consumption was never recorded in this study, we do not know if there are any true dietary differences between our study groups.

The role of the breakdown products of Try is not well known, but some can be neuroprotective or neurotoxic.

Kynurenine (Kyn)

The initial finding that CFS patients showed lower levels of Kyn compared to controls, was not replicated in the better models (Figure 4.1). As I will discuss later in Section 4.3, Kyn is strongly influenced by age as a confounding factor.

Kyn is the first breakdown metabolite from Try in the kynurenine pathway (Figure 1.**0**). Diminished Kyn production could be due to Try being shifted towards the other Try metabolic pathways, and shunted away from the kynurenine pathways. Lower Kyn levels could also mirror lower Try levels due to dietary deficiencies as previously explained.

Kynurenic Acid (KA)

The preliminary finding was that the CFS group had higher KA concentration levels than controls (Table 3.14; Figure 3.7), yet the best model indicated that the FM group had higher KA concentration level than controls, that almost reached significance (p = .052; Figure 4.1). KA is also strongly influenced by age as the confounding factor and this will be discussed further in Section 4.3.

Normally, aging is associated with increased neurotoxicity and inflammation, see Section 4.3.2 Age and sex. KA is considered neuroprotective.[134] Increased levels of KA may be a way of compensating for this neurotoxicity, but this is just speculation on our behalf. Since this is not relevant to this study, it will not be discussed any further.

Anthranilic Acid (AA)

CFS patients showed lower concentrations of AA compared to controls initially, which was also replicated (Table 3.14; Figure 3.7), yet Model 2 showed that other factors may contribute to changes in AA levels (such as fatigue scores which will be discussed further in Section 4.3), obliviating the differences between CFS and controls. Although not reaching significance in Model 2, higher AA concentrations in the FM group compared to controls could be observed (p = .066;Table 3.14).

Xanthurenic acid (XA)

Lower XA levels in the FM group compared to controls could initially be observed, yet the results were not significant (p = .058; Table 3.14). However, none of the models were a good fit for explaining levels of XA in our study population, and this finding is most likely a Type I error.

Quinolinic acid (QA)

The best model for explaining differences in QA levels indicated that the CFS group had higher QA levels than controls with the FM concentrations falling somewhere in between (lower than CFS and higher than controls; Table 3.14). QA was also strongly associated with BMI and this effect will be discussed in Section 4.3.

CFS and FM patients often complain about poor cognition and memory.[39,40] This is also reported by FM patients.[39] QA itself is considered a neurotoxic component, by damaging neuronal cells, and may hypothetically lead to poor cognition. The memory impairment reported by CFS patients are mainly subjective, however, and may be related to reduced processing speed and problems retrieving memories.[40] More evidence is needed before concluding on the neurotoxic role of QA in CFS and FM patients.

Ratios – Enzyme activity

In this study, the ratios were always calculated as break-down indexes, e.g. Try breaks down to Kyn, and the ratio is made from how much Kyn is produced from Try, i.e. Kyn/Try. Another way of seeing it, is that the breakdown of one metabolite is only possible by the enzymes involved in the breakdown process. If the ratio represents how much of a metabolite is converted into its breakdown metabolite, this would also represent the enzyme activity of the enzyme involved. For example, both increased activity of kynurenine amino transferases (KAT) would shunt the Kyn pathway away from HAA and QA, and ultimately energy production (Figure 1.3).

The enzymes involved in the kynurenine pathway are listed in Table 2.3 and illustrated in Figure 1.3.

Table 2.3. *The Enzymes of the Kynurenine Pathway and the Kynurenine Ratios Representing Those Enzymes*

Name / Enzyme	Abbreviation	Ratio	Concentration ratio
Indoleamine 2,3-dioxygenase	IDO	$\frac{\text{Kyn}}{\text{Try}}$	$\frac{[\text{lnKyn}]}{[\text{lnTry}]}$
Kynurenine aminotransferase	KAT I	$\frac{\text{KA}}{\text{Kyn}}$	$\frac{[\text{lnKA}]}{[\text{lnKyn}]}$
Kynurenine aminotransferase II	KAT II	$\frac{\text{XA}}{\text{HK}}$	$\frac{[\text{lnXA}]}{[\text{lnHK}]}$
Kynurenine 3-monooxygenase	KMO	$\frac{\text{HK}}{\text{Kyn}}$	$\frac{[\text{lnHK}]}{[\text{lnKyn}]}$
Kynureninase	-	$\frac{\text{AA}}{\text{Kyn}}$	$\frac{[\text{lnAA}]}{[\text{lnKyn}]}$
Kynureninase	-	$\frac{\text{HAA}}{\text{HK}}$	$\frac{[\text{lnHAA}]}{[\text{lnHK}]}$
Neuroprotective ratio 1	NPR-1	$\frac{\text{KA}}{\text{QA}}$	$\frac{[\text{lnKA}]}{[\text{lnQA}]}$
Neuroprotective ratio 2	NPR-2	$\frac{\text{KA}}{\text{HK}}$	$\frac{[\text{lnKA}]}{[\text{lnHK}]}$
The enzymes are not actually included in this study. KAT has several isoforms, and KAT I and KAT II are used here to differentiate between the KA/Kyn and XA/HK ratios. The metabolic turnover (given as ratio) is used as an indicator of these enzymes.			

Kynurenine-Tryptophan ratio (Kyn/Try) – TDO/IDO activity

FM patients initially showed higher Kyn/Try ratio compared to controls, and this finding was replicated in one of the models (Model 2 that included FS scores) but no differences between the groups were found in the strongest model (Model 1; Table 3.14). The latter two models were almost equally good at explaining differences in the Kyn/Try ratio where age and BMI are strongly associated with this factor. This will be discussed further in Section 4.3.

It is possible that the lower Try levels associated with FM patients discussed above might influence the finding of the Kyn/Try ratio in Model 0 but this does not explain the finding of higher Kyn/Try ratio in the FM group in Model 2 (Table 3.14 and Supplementary Tables 3 and 4).

Kyn/Try ratio is also indicative of enhanced activity of the enzymes TDO and IDO. Our findings suggest that this enzyme / these enzymes might have increased activity in FM

patients. IDO is enhanced by inflammatory cytokines, especially IFN-γ. Surprisingly, we found decreased levels of IFN-γ in our material. This does not fit with the potentially increased IDO. However, knowledge on the total function of the Kyn/Try system is limited and no conclusions should be drawn so far. Altered function of IDO in CFS patients have been hypothesised,[135] but we did not find any similar studies for FM patients.

Kynurenic Acid-Kynurenine ratio (KA/Kyn) – KAT I activity

The best model fit predicted higher KA/Kyn levels in FM patients compared to controls (Table 3.14), with the CFS group levels falling somewhere in between (lower than FM and higher than controls; Table 3.15). In addition to the group effect of FM on the KA/Kyn ratio, other cofactors such as age and BMI did contribute to changes in KA/Kyn levels and will be discussed in Section 4.3.

As discussed above, KA might be higher and in FM patients compared to the other groups, which makes the KA/Kyn ratio higher as well. However, ratios are still of relevance because they indirectly represent enzyme activity.

Try is converted into KA by the enzyme Kynurenine Aminotransferase (KAT). Our findings suggest that KAT II activity is enhanced in FM patients, but not in CFS and controls. We did not find any studies regarding KAT activity in CFS nor FM patients.

Xanthurenic Acid – 3-hydroxykynurenine ratio (XA/HK) – KATII activity

The enzyme converting HK into XA is the Kynurenine Aminotransferase II.

At the first glance it may appear that the XA/HK ratio was lower in the FM group compared to both CFS and controls as two of the models showed (Model 0 and Model 1; Table 3.15). This effect however, disappeared when cofactors associated with the FM group was included (i.e. pain and nicotine) and this will be discussed in Section 4.3, which reduced the likelihood of FM being associated with changes in the XA/HK ratio. As stated above, we did not find any studies regarding KAT activity to compare our results.

3-hydroxykynurenine – kynurenine ratio (HK/Kyn) – KMO activity

The enzyme converting Kyn into HK is the Kynurenine 3-monooxygenase.

FM patients seemingly had higher levels of the HK/Kyn ratio compared to controls, but this effect disappeared and could rather be explained by other cofactors (BMI and pain) and will thus be discussed in 4.3 Confounding factors in relation to hsCRP, cytokines/chemokines and kynurenines. We could not find any studies concerning this ratio / enzyme activity.

3-hydroxyanthranilic acid – 3-hydroxykynurenine ratio (HAA/HK) – Kynureninase activity

The enzyme converting HAA into HK is the Kynureninase.

FM patients seemingly had higher levels of the HAA/HK ratio compared to controls, but similar to the XA/HK ratio, this effect disappeared and could rather be explained by the cofactors pain and nicotine (see 4.3). We could not find any studies concerning this ratio / enzyme activity.

Neuroprotective ratios

Kynurenic Acid – Quinolinic Acid ratio (KA/QA) – neuroprotective ratio 1

CFS patients had significantly lower KA/QA ratio compared to controls with the FM group concentration falling somewhere in between (higher than CFS and lower than controls; .

Adding any confounding factors did not reveal any other associations with the KA/QA ratio.

The KA/QA ratio is considered a neuroprotective ratio. Since KA is neuroprotective and QA neurotoxic, the relative difference between the neuroprotective agent and the neurotoxic agent indicates the balance of neurotoxicity and neuroprotection. Hence, an increase of KA and/or a decrease in QA results in higher neuroprotection, and vice versa: decrease in KA and/or increase in QA results in lower neuroprotection, i.e. higher neurotoxicity.

Our results indicate that CFS patients have lower neuroprotective ratio (KA/QA), i.e. higher neurotoxicity, compared to controls. The FM patients did not differ from CFS nor controls in this study, indicating that they are in some way in between the two groups. Higher neurotoxicity would be an appealing approach to understanding the symptoms of CFS. Neurotoxicity equals inflammation in that cells in the CNS are destroyed, and optimal functioning decreased. Brain fog accompany inflammation in the brain and may explain the cognitive difficulties experienced by CFS patients.[136] Brain inflammation will also lead to both mental and physical fatigue, in line with sickness behaviour. However, none of the models were exceptionally good at explaining any variation in this neuroprotective ratio and our finding should therefore be taken with precaution.

Kynurenic Acid-3-hydroxykynurenine ratio (KA/HK) – neuroprotective ratio 2

Other researchers have argued that the KA/HK ratio is neuroprotective due to the neuroprotective properties of KA and presumably neurotoxic properties of HK,[134] and therefore we decided to include this ratio in our calculations. Our findings suggested that any initial group differences in KA/HK ratio disappeared completely and could rather be explained by one of the confounding factors (pain) that were associated with CFS and FM diagnosis.

Summary of the kynurenines

- CFS and FM share similar profiles for:
  - QA
  - KAT I activity (KA/Kyn)
  - Neuroprotective ratio (KA/QA)
- More specific for CFS:
  - Higher QA
  - Lower neuroprotective ratio (KA/QA)
- More Specific for FM:
  - Higher TDO/IDO activity (Kyn/Try)
  - Higher KAT I activity (KA/Kyn)
  - Lower KAT II activity (XA/HK)

Before discussing any of these confounders further and how they may influence the results 4.3), we dive into how the inflammatory markers hsCRP, cytokines, chemokines and the kynurenine pathway findings are integrated.

4.2.4 Interactions and conclusions of inflammatory markers

A general marker for inflammation is CRP. hsCRP was measured in this study, and the results showed that both CFS and FM patients had higher levels of hsCRP than the control group. Because of this, we expected to find increased levels of certain pro-inflammatory cytokines. However, among cytokine and chemokines only MCP-1 was elevated in the patient groups compared to controls in this study. Interestingly, and in accordance with our findings, Bote et al[112] found that MCP-1 was increased along with the pro-inflammatory marker CRP in 25 patients with FM. All the other pro-inflammatory, anti-inflammatory and regulatory cytokines and chemokines were lower in the patient groups or showed no difference between the groups.

Monocyte/macrophage production of MCP-1 and hepatocyte CRP production are both stimulated by the same pro-inflammatory cytokines IFN-γ, IL-1β, IL-6 and TNF-α. These four cytokines were significantly reduced in our patient population, and not higher, as might be expected given the increased CRP and MCP-1. Similarly, lower IL-10 and other anti-inflammatory cytokine production could result in a pro-inflammatory state. We did not see any such relationships. This may appear a paradox and the explanation for this is not obvious. Still, cytokines are not mutually exclusive, for example IFN-γ upregulated MCP-1 production increases production of IL-4 (Murphy 2008).

Lower TGF-β and other regulatory cytokines found in plasma of CFS and FM patients could suggest that this regulatory influence is diminished, leading to further imbalances in the immune system of these patients. This may show up in irregular cytokine profiles, such as a low pro-inflammatory profile as described in our study.

The conflicting results with both increased and/or decreased pro- and anti-inflammatory cytokines in different studies could be due to methodological differences in sampling and laboratory procedures, or they may reflect the heterogeneity of these disorders and how they manifest over time. The mechanisms are complex and may be related to other factors than

diagnoses alone and that these molecules are indirectly or secondary associated to the pathogenesis of the disorder.

It is not clear how cytokine profiles are related to the kynurenines in this study. Pro-inflammatory cytokines, e.g., IFN-γ and IL-1β, have shown to increase the activation of IDO (ref), i.e., tryptophan breakdown into kynurenines, and thus shunting tryptophan (Try) conversion away from serotonin (5-HT) (ref). We did not find any increase in the breakdown index for tryptophan into kynurenine for the CFS and FM patients in this study, yet we also did not find increased pro-inflammatory cytokines known to increase IDO activation. IDO is also not the only enzyme converting Try into Kyn, and the mechanisms, if any, of interactions between cytokines, chemokines and the kynurenines and all enzymes involved are not well known. It could be that some kynurenines work as signaling molecules for cytokine production or inhibition or vice versa. This highlights the complexity and novicey in this field, and how each sub-component interacts in a myriad of inter- and intracellular processes.

4.3 Confounding factors in relation to hsCRP, cytokines/chemokines and kynurenines

The second objective of this study was to evaluate symptoms of anxiety and depression and how they may be related to the inflammatory markers explored. HADS is a recognised method for measuring symptoms of anxiety and depression. Other questionnaires used in this study, were used to evaluate some of the core symptoms of CFS and FM: Pain (NRS pain scores); Fatigue (CFQ scale); and fibromyalgia severity (FS scores from the FSDC). The CFQ scale and FSDC are questionnaires that we also used to evaluate the overlap between the diagnoses CFS and FM and this will be discussed in Section 4.4 Overlap of the diagnoses CFS and FM.

When constructing plausible models for analysing the outcome variable, it is not possible to get a clear-cut answer for which confounding factors may be related to the immunological markers. It has to be a plausible reason for including these variables, either to support/question previous research, or have some logical explanation linking different research topics together.

The questions in the structured interview (Supplementary Table 1) were based on our best assumptions of items that may interfere with the immune system at some level or were related to the diagnoses studied. The items will be discussed in the following section and are presented in the same order as they appear in the method section / Supplementary Table 1.

The confounders that were not included in the hsCRP main analysis (2.4.1 Statistical analysis Article 1) are discussed in this section. The confounding factors for the cytokines/chemokines are more explorative in nature due to the method chosen for comparing these immune markers (2.4.2 Statistical analysis Article 2). The Explorative approach to Article 3 (Section 2.4.4) in this thesis are also found here and in the Supplementary Tables 3 and 4.

One of the other pit-falls including any of the symptoms or symptomology that are part of, or related to, the diagnoses of CFS or FM, is that they are so heavily intercorrelated. Analysing the whole study population as one group mainly confirmed the findings for group differences already discussed (data not shown) and does not add any additional information to this study. Therefore a non-parametric correlation analysis (Spearman's ρ) with hsCRP and the cytokines/chemokines was conducted for each group separately (CFS, FM and controls). In addition, a Mann-Whitney *U* test was performed on the binominal scales (medication, hormones, activity level, nicotine and duration of illness). Only the significant ($p < .05$) correlation coefficients (ρ) are presented in Supplementary Table 2 (except for medication and hormonal status).

4.3.1 Time and Date – Seasonal and diurnal effects

Patients were recruited consecutively, and the data collection was distributed throughout the year, with most patients and controls were included during the spring. Although there are claims that seasons may have an effect on the immune response,[137] we could not find any references supporting this for cytokines in venous blood, and this was not supported by our findings either (data not shown).

Getting a concurrent time of the day for data sampling was challenging, and to ensure enough patients were recruited, the schedule was adjusted to match the participants needs. Most testing were done during normal working hours. Time of day did not seem to influence any of the results (data not shown).

4.3.2 Age and sex

All participants in this study were female. Our findings may thus mainly apply for adult females.

In this study, there were differences in age distribution with CFS group being younger (M = 34 years) than both the FM group (M = 42 years) and controls (M = 40 years). To avoid any bias of the results, we included age as a confounding factor when conducting further analyses.

The aim of the study was to study an adult patient population with the age span between 18 and 60 years. This was to avoid possible bias from adolescents still in growth and hormonal development. Similarly, an elderly population was avoided because of the accumulation of disease, general pain conditions and fatigue that often occurs after living a longer life.

As adult females have hormones controlling their menstrual cycle, and the hormonal status is altered after menopause, these data were recorded, and the results are discussed in Section 4.3.7 Hormones and menstrual cycle found below.

Higher age is also associated with higher inflammation.[138,139]

hsCRP

Age did not have any effect on hsCRP in our study. This is in line with Xiao et al.[130] This was a bit surprising, as age is often associated with higher inflammation, leading to the term *inflammaging*.[140] However, when the association of age and other risk factors of cardiovascular disease is found in men, this is only found in women after menopause.[138] Our study population were only female, and only a small proportion (approximately 10–15%) had reached menopause. This can explain why age does not seem to influence our results regarding hsCRP.

Cytokines/Chemokines

A positive correlation was found between age and MCP-1 in each diagnostic group (CFS, FM and control) separately (Supplementary Table 2). The CFS group were younger than both FM and controls, and since age was positively correlated to MCP-1 in all the groups in this study, age cannot alone explain our findings of higher levels of MCP-1 for both the patient groups (CFS and FM) compared to controls. Age was also negatively associated with IFN-γ in the FM group (Supplementary Table 2), possibly serving as a strong confounding factor for the lower IFN-γ results for FM patients in our study.

Kynurenines

The kynurenines Kyn and KA, and the ratios Kyn/Try and KA/Kyn were all positively associated with age (Figure 4.2 and Supplementary Table 3). For the ratios, this may be a mere reflection of the positive Kyn and KA associations. However, higher Kyn/Try ratio is indicative of higher TDO and IDO activity, and IDO is stimulated by certain pro-inflammatory cytokines. As already discussed, the lower cytokine levels in our patient groups cannot explain such an enhanced activity. It is, however, possible that other inflammatory interactions with IDO are at play that our methods did not detect.

4.3.3 BMI

BMI was higher in FM patients compared to controls and CFS, and over half of the FM cases (55%) had BMI > 25 (which is considered overweight by WHO[97]), compared to 40% of the controls and 31% of the CFS cases. CFS patients had the lowest BMI of all three groups, but this was not statistically different from controls. FM is often associated with obesity.[141] BMI and adipose tissue could influence the results, as high BMI are associated with higher inflammation,[138,139] and thus BMI was corrected for. However, BMI influence on symptoms is also of clinical relevance.

hsCRP

In this study BMI was associated with hsCRP in the FM group and control group (Table 3.8, Figure 4.2, and Supplementary Table 2). This has also been shown by others.[130] This is expected as high BMI are associated with higher inflammation.[138,139]

BMI is also indicative, though not exclusively so, of the amount of adipose tissue in the body. Adipose tissue has well known effects on the production of CRP.[142] The reason for this association not showing in the CFS group could be that this group also had the lowest BMI of all groups in this study. However, after adjusting for BMI, there was still a significantly higher level of hsCRP among patients compared to controls. This is in line with findings by others.[102,130,143] Raison[101] found that people with CFS and a group with CFS-like illness could not be distinguished from each other based on hsCRP levels. Considering the great overlap between CFS and FM (see Section 4.4 Overlap of the diagnoses CFS and FM), these diagnostic groups cannot be distinguished based on the hsCRP findings in our study, and the relevance of the diagnoses regarding hsCRP-levels are not entirely clear.

Cytokines/Chemokines

BMI was also positively correlated with some of the cytokines and chemokines in this study (Supplementary Table 2). Apart from the positive association for IFN-γ and IP-10 in the control group, and MCP-1 in the CFS group, only weaker associations ($\rho < .3$) were found for IL-4, IL-6 and TGF-β3 in the FM group.

Kynurenines

QA: BMI was positively associated with QA. CFS patients had lower BMI than the other two groups, and since BMI also was positively associated with QA, these effects are opposite of each other. Still, the effect of BMI did not level out the higher levels of QA in CFS (Supplementary Table 3). The effect of BMI on QA was greater than any effect FM group might have on QA. It is therefore most likely that BMI is associated with higher levels of QA. FM did not have any effect on QA, but instead increased QA is found in CFS patients.

This is not to say that there are any other factors that might contribute to increased production of QA. As shown, increased BMI and individuals being overweight, might lead to increased QA. Our models were not very good at explaining variations in QA (up to approximately 10% at the best), and there may be many other factors that we did not includ in our analyses. Our hypothesis, however, was to look for the confounding factors often associated with CFS and FM (e.g. fatigue and pain), of which none came out significant.

XA/HK: Higher BMI was associated with lower XA/HK ratio (Figure 4.2; Supplementary Table 3), and although the effect sizes remained similar in both Model 1 and Model 2, this did not reach significance levels in Model 2. Also, higher BMI was associated with higher levels of the metabolite HK (Figure 4.2), which may explain the negative XA/HK association. Instead pain scores seemed to have the strongest influence on the XA/HK ratio (see below).

Summary and conclusion of age and BMI interaction

Kyn/Try: By adding age and BMI to the model made the model stronger, and hence age and BMI was better at explaining the differences in the Kyn/Try ratio. Since the FM patients were both older and had higher BMI compared to the other two groups (CFS and controls), the

initial finding of higher Kyn/Try ratio in the FM group could be attributed to the higher age and BMI in this group.

KA/Kyn: Our finding suggests that FM patients had higher KA/Kyn ratio. However, as our data shows, the FM patients were both older and had higher BMI than the other two groups (CFS and controls). For the KA/Kyn ratio, higher age and BMI were also associated with higher KA/Kyn ratios. In the best model (Model 2) age had almost twice the effect on KA/Kyn ratio compared to BMI. The effect of the differences of the KA/Kyn ratio found in the FM group, was also half of the effect that age had on this ratio.

This would indicate that when controlling for age and BMI, FM patients still have higher KA/Kyn ratio, and it may be concluded that FM patients do differ from CFS and controls in regard to this ratio, but that age is the strongest factor.

4.3.4 Subjective symptoms of infection and feverishness

Subjective feeling of malaise is not necessary for a positive CFS or FM diagnosis. Still a great proportion of the patients in this study reported subjective signs of being constantly ill or of feverishness (data not shown), which is consistent with the symptoms often reported in these patient groups. Exclusion of participants with objective signs of infection due to abnormal leukocyte count and/or elevated hsCRP, did not change any of the results in the analyses of the immune markers (data not shown). There was also no obvious relationship between subjective and objective reports of inflammation (data not shown).

4.3.5 Comorbid disorders/diagnoses and allergies

Comorbid disease seemed very common in the patient groups, with approximately 50–80% comorbidity.

By means of serology, leukocyte count and hsCRP inspection, we did our best to eliminate any active inflammation in our study population by excluding participants with signs of active infections.

Asthma was commonly reported in the patient population (data not shown). Asthma may be due to an immunological vulnerability or shift in the immunological response in these patients. Also its activity/severeness may be related to mental stress in both directions. The occurrence of asthma did not affect any of the results in the analyses of the immune markers (data not shown).

A lot of patients reported all sorts of allergies, and these were not necessarily confirmed by a medical doctor. The parameter of allergies and allergic reaction was therefore objectively measured by serum IgE (see Section 3.5 – IgE). Total IgE in serum is of course not a marker for all sorts of allergies. Our analyses showed that there were no differences between the groups on this parameter, and IgE did not influence any of the results (data not shown).

4.3.6 Medication

The highest frequency of the use of medication was found in the FM group, followed by the CFS group. Most of the medication were analgetic and allergy respiratory prescribed medication. It is not surprising that FM patients and many CFS patients use more medication in general since they seek symptom relief, and one obvious candidate is medication for pain relief. This ought not need any further explanation since pain is a very common symptom in both patient groups.

Since any use of immunosuppressive medication led to exclusion of the study, this did not influence any of the results. The medications thought to have an effect on the results (immunomodulatory medication ACT-codes: H02A, M01A, and N02B E01; and antidepressants ACT-code: N06A) are found in Table 3.2). There was a relative high number of participants, especially FM-patients (24%), using anti-depressive medication. SSRIs and NRSIs are often prescribed as analgesics, and thus does not necessarily reflect treatment for depression in this patient group. The same should be the case for the CFS patients in this study (n = 5) since depressive disorder led to exclusion from this study.

Comparing Medication did not influence the levels of hsCRP in this study (results not shown).

Cytokines/Chemokines

In our material, the effects of anti-depressants and immunomodulatory medication was only observed in a relatively small number of participants in the FM group (n = 14 and n = 9, respectively). The number of participants in the CFS and control groups was too small (n < 5) to conclude on any effect of these medications on any of the explored blood markers used in this study.

Higher levels of the cytokine IL-4 were found in FM patients who were on anti-depressive medication (ACT-code N06A) compared to the FM patients not using anti-depressants. IL-4 is considered aTh2, opposing inflammatory Th1 responses. There are indications that anti-depressive medications are restoring the pro-inflammatory state of MDD.[144]

Higher ranks of IL-6 and IL-17A were found in FM patients who were using immunomodulatory medication (ACT-codes H02Am M01A and N02B E01) on the day of sampling compared to those who were not. Immunomodulatory medication will surely affect level of immune signals – whether the effects are a result of the immunomodulatory medication or a primary effect of the condition causing use of immunomodulatory medication is not possible to clarify.

Kynurenines

In the FM group, anti-depressive medication was positively associated with the kynurenine metabolite HAA.

4.3.7 Hormones and menstrual cycle

Of the CFS patients, 9% reported having reached menopause, in the FM group this was 16%, and for the control group 8%. The proportion still in fertile age was 85% for the CFS patients, 71% for FM, and 83% for controls. The rest were still undergoing menopause or did not know. This larger proportion of FM patients in menopause, reflects the higher age in this group compared to the other groups.

Comparing pre- and post-menopause participants did not result in any difference for hsCRP, and this analysis was never conducted for the kynurenines.

Cytokines/Chemokines

These results are not presented in the supplementary material (Appendix B) but the findings are still briefly discussed in Article 2.[145] For cytokines and chemokines, pre-menopause participants had significantly higher levels of IP-10 and MCP-1. This was also found for IP-10 in CFS and FM and MCP-1 in CFS, FM and controls when splitting the population into the respective diagnostic groups (CFS, FM and controls). This is similar to the age effects on the same cytokines, and age is more likely to be the true confounding factor in this regard, since the other hormones (contraceptives and menstrual cycle) did not associate with any of the immunological markers studied.

It is suggested that the different phases of the menstrual cycle influence the production of cytokines, but this seems to be locally (in the cervicovaginal mucosa)[146] rather than in peripheral blood.[147] We controlled for different phases in the menstrual cycle, the use of different contraceptives, and menopause. None of the factors influenced our results.

4.3.8 Physical activity

Only one patient reported being bedridden. The number of participants conducting regular exercise were lower in the patient groups than in controls. The lower activity levels of both CFS and FM patients is not surprising, as symptoms of fatigue will make physical exercise harder. People reporting chronic pain are also less physically active.[148] A sedentary life style will lead to deconditioning, possible enhancing the symptoms and increasing the risk of developing FM.[149,150] Movements may be experienced as somewhat slower and less vigorous in patients, and posture is affected in CFS and FM patients.[151] Considering that the activity level in this study was not measured by any standardised measurement tools, we cannot conclude whether physical activity lead to fatigue and pain or the other way around.

No associations were found between levels of activity and hsCRP in our study. This comparison was never conducted for the kynurenines.

Cytokines/Chemokines

When analysing the groups separately, an association was only seen in the CFS patients where the most inactive CFS patients (only move/walk to conduct core tasks) also had the highest levels of MCP-1 compared to the active patients.

Maes et al[121] emphasize that there is a chronic fatigue spectrum, suggesting a model with three categories with a continuum of increasing severity of illness. This indicates that the severity of illness should be taken into account in studies on CFS and related conditions.

We did not record severity as a separate variable. However, one could argue that our measure of activity (the intensity and frequency of movement) is related to the severity of the illness

MCP-1 might be related to the degree of severity of disorder, for which activity may be an indicator. The control group still had significantly lower levels of MCP-1 than both active and inactive patients (data not shown). This indicates that the severity of illness cannot by itself explain the group effects of MCP-1 reported in our study.

4.3.9 Nicotine

In the control group 15% reported using nicotine regularly. This was less than in the two patient groups CFS (33%) and FM (38%).

Nicotine may be a factor in energy balance.[152] Nicotine is typically consumed in a form of tobacco, either by smoking cigarettes or, in Scandinavian countries, snuff. In addition, it is possible to use the e-cigarette, nicotine patches, and chewing gum. It was presumed that smoking and chewing tobacco was the most common way of consumption, and the questions did not specify any of the other forms of nicotine use. It is therefore possible that the use of nicotine is underestimated in our population sample.

hsCRP

An effect was observed in the control group where those who used nicotine regularly ($n = 8$) had lower levels of hsCRP than those who did not use any nicotinic substances ($n = 45$). The effect was small ($\rho < -.3$) and the number of nicotine users was very low, and we consider this finding a type 1 error.

Cytokines/Chemokines

There were no differences in cytokine or chemokine concentrations between participants that used nicotine compared to those that did not; not in the total study population nor in each separate diagnostic group (CFS, FM and controls).

Kynurenines

QA: The higher nicotine consumption in the FM group could also even out any possible effect that FM might have had in the model.

Although FM patients showed a trend towards higher levels of AA compared to controls in Model 2 (Supplementary Table 3), they were also the group that used nicotine regularly. Therefore, when the model was adjusted, nicotine was the strongest factor and most likely to have any effect on AA.

XA/HK: Nicotine had an effect on the XA/HK ratio. Since there were more participants using nicotine regularly in the FM group, this might explain the initial association between FM and

the XA/HK activity. We could not identify any studies in the literature which report interaction between nicotine and the aforementioned pathways.

4.3.10 Duration of illness

The patients were asked about how long they have experienced their symptoms similar to their illness as a measurement for how many years since the onset of the disorder. One patient reported the disorder starting just under one year ago, but more than six months. Having symptoms longer than six months is the requirement for CFS diagnosis.[5] For FM the requirement is 3 months.[11] The majority of patients (≈ 70% of the CFS patients, and ≈ 80% of the FM patients) reported the disorder starting more than three years ago. For some patients it was difficult to remember exactly, so the estimates had to be adjusted somewhat. Also, because Horning et al[119] reported possible effect of duration on cytokine profile in CFS patients shorter than three years, we made a similar division of two categories when analysing the results of cytokine profiles:

- < 3 years
- > 3 years

hsCRP

After a prolonged time of suffering from CFS (longer than 3 years), the hsCRP levels dropped slightly (not significant) in the CFS patients. This was opposite for the FM group (not significant); higher CRP levels were associated with prolonged duration of the disorder in FM patients (data not shown).

Cytokines/Chemokines

CFS patients with illness < 3 years (n = 13) had significantly lower levels compared to CFS patients with illness > 3 years (n = 33) for TNF-α, IL-10, TGF-β1, TGF-β2 and TGF-β3 (Supplementary Table 2).

The differences in cytokines and chemokine between all the CFS patients (n = 48) and controls (n = 53) originally observed (Table 3.12), remained the same between CFS patients with shorter duration of illness (< 3 years; n = 13) and the control group (n = 53).

In Article 2[145] we showed that the lower levels of TGF-β1, TGF-β2, and TGF-β3 for the CFS patients with longer duration of illness (> 3 years; n = 34) compared to the control group (n = 53) also remained. But these patients could no longer be distinguished from controls on TNF-α and IL-10.

Interestingly, in the CFS group, only CFS patients with short duration of illness differed significantly from controls in these cytokines regarded as regulatory (IL-17A, IL-4, TGF-β1, TGF-β2 and TGF-β3). This could indicate that there is a broad dysregulation in immune activity as suggested by Broderick et al[153] and Hornig et al[119] These studies suggest an initial increase in immune activity followed by a reduction after three years in CFS patients. In this case the deviation in most immune markers seem to be secondary to an etiological factor.

In contrast to our findings, Horning and colleagues[119] reported significantly increased IL-1ra, IL-1β, IL-4, IL-6, IL17A, TNFα, IFN-γ in those with CFS illness lasting shorter than three years compared to those with longer duration and/or healthy controls. Also in the same study, when comparing all CFS cases with controls, the CFS cases showed lower cytokine levels for IL-6, IL-10 and IL-17A, which is in line with our general findings.

We did not find any differences in any cytokine concentrations for FM patients according to reported duration of illness (Supplementary Table 2). Wang et al[154] reported a reduction of IL-8 and TNF-α in FM patients after six months of multidisciplinary pain treatment. In our study, we did not record treatment procedures other than medication taken at the time of sampling.

An initial, prolonged, enhanced inflammatory state may possibly lead to chronification of the symptoms associated with inflammation (e.g. sickness behaviour) – but may eventually also lead to a disrupted immune response over time. For the CFS patients in our study, this may either reflect a change over time as the disorder continues, or it may reflect the heterogenicity of the diagnostic group CFS.

4.3.11 Anxiety and depression – The hospital anxiety and depression scale

In this study, we decided to use HADS for evaluating symptoms of anxiety and depression for the last week. HADS is suitable for scoring anxiety and depressive symptoms in somatically ill patients. The emphasis of this questionnaire is on the non-somatic aspects of the psychiatric symptoms, and since many of the symptoms of CFS and FM have somatic characteristics, this will lead to less false positive cases of anxiety and depression in these patient groups. When using the recommended cut-off score for positive cases of anxiety and depression of 11 out of 21,[85] 16% of CFS patients scored above this cut-off for both anxiety and depression, and 33% and 14% of FM patients scored above this cut-off for anxiety and depression, respectively. Anxiety and depression scores were higher in both patient groups compared to controls. Yet, HADS is not a primary diagnostic tool for anxiety and depression; Rather, it is a tool for evaluating the *symptoms* of anxiety and depression. The patients in this study were screened for psychiatric disorders, including depression, and any patients with such diagnoses were never enrolled in the study.

No associations were found between HADS anxiety and depression scores and hsCRP nor the kynurenines in our study.

Cytokines/Chemokines

IP-10 was associated with depression scores in CFS patients when analysing the groups separately.

IP-10 has been reported to be elevated in a population with current episodes of major depressive disorder (MDD)[155] and in patients with bipolar disorder (BD),[156] a disorder characterised by depressive episodes. Still, there were no participants in this study with

MDD, and as discussed previously, higher scores of HADS does not qualify for a diagnosis of MDD. The positive association between HADS depression scores and IP-10 may thus be due to the reflection of higher HADS depression scores found in the CFS group in combination with this group also having higher levels (although not significant) of IP-10 compared to controls.

Severe depression is associated with altered immune activity. As the recruitment procedure in the clinical practice *excluded* clinical depression in our patient groups (CFS and FM), this does not explain our findings. To my knowledge there are no other research supporting the same immunological associations and anxiety and depression in CFS and FM patients.

4.3.12 Pain – Numeric rating scale

Pain scores were taken from NRS 1, which was the subject's evaluation of general pain at the time of sampling. The BPI was used to evaluate the pain experienced during the last week. By observing the scores of the average pain experienced during the last week (BPI 1) and current pain (NRS 1) did not deviate. The FM patients had significantly higher pain scores than CFS patients, and CFS patients significantly higher scores than controls. This is to be expected, as FM patients were included according to the ACR 1990 criteria, which focuses on pain-related symptoms, and that five out of the eight additional criteria for CFS diagnosis are pain related (sore throat, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, and headaches).

The pain scores, however, were not equally quantified elsewhere. Pain scores are also found in one question in the FSDC (quantifying the severity of pain). This item has fewer points on its scale (scores 0–4) compared to the NRS (0–10, and thus NRS scores are more "precise" in catching the variety of pain intensity. The decision to use the current pain status (NRS 1) instead of any of the BPI items (measuring the average for last week) was based on the idea that pain intensity at any moment may be more representative to any biological outcome in this study. Thus, the NRS 1 scores were used for further analyses when studying confounding factors.

Cytokines/Chemokines

Although we did not see any correlation for pain hsCRP, cytokines nor chemokines within each group in this study, both higher pain scores and MCP-1 levels were found in both patient groups compared to controls. Relevant to the clinical findings of pain in FM, MCP-1 also has a known function in pain as it is reported to enhance excitability of nociceptive neurons.[157] Pain is a central symptom in FM, and MCP-1 is involved in pain processing and pain sensitivity in the CNS[158] and MCP-1 is reported to enhance excitability of nociceptive neurons.[157]

In addition, MCP-1 measurements at two time points were correlated to the worsening of pain in 16 FM patients.[159]

Kynurenines

KA: Pain scores were negatively associated with pain. This negative association may have levelled out the positive association between FM and KA in the same model.

KA/HK: Similar to KA, when pain was added to the model, the initial finding of group differences disappeared. The model also improved, and higher pain scores led to a lower KA/HK ratio.

XA/HK: In addition to the effect of nicotine on the XA/HK ratio, adding the confounding factor pain (strongly associated with FM) did not only improve the model; it also showed that higher pain scores were associated with lower XA/HK ratio. Therefore, it may be that pain, rather than having an FM diagnosis, is associated with lower KATII activity.

Numeric rating scale – Other items

In addition to the BPI and NRS 1, we asked about the current pain experience and current fatigue and perceived stress levels. The last two items were used as reference to spot any deviance between the pain items and fatigue or stress, in the case of other possible confounding factors at the time of sampling. The scores on current fatigue and stress were similar to those of average pain scores, with the exception of the two patient groups CFS and FM scoring similar on both items. It is possible that fatigue or perceived stress may be explanatory for any correlation found between the pain scores and immunological markers discussed later. Still, we find it more likely that pain as a core symptom may be the rightful candidate to take into consideration when studying the patient groups, since fatigue is also measured in the CFQ, and bringing in two measures of the same thing when analysing the results would add complexity, yet without altering the results, and ought to be omitted.

4.3.13 Fatigue – Chalder fatigue questionnaire scale

Fatigue was measured by the total score from the CFQ.

Fatigue scores were highest for the CFS group and lowest for the control group. In the FM group, the fatigue scores were slightly lower than for the CFS group, which is in accordance with the trait of this patient group where high scores of fatigue are not necessary for diagnosis. The high scores of fatigue in the CFS group are self-explanatory, as fatigue is the core symptom in the diagnosis of CFS.

We used a cut-off score of ≥ 6 for fatigued cases after recommendations from White et al.[92] The fatigue scale showed high sensitivity and specificity in accordance to the actual CFS diagnosis acquired from the clinical evaluation. Alternatively, a cut-off score of ≥ 4 is suggested.[90] This would lead to a lower sensitivity (.92 in our material) but higher specificity (.96 in our material). But comparing the 49 true CFS patients enrolled in this study to the 53 healthy controls, would yield such scores. By using the numbers given, one can assume that the 48 FM cases falling into the category of "fatigued cases" could indeed be true CFS cases, that is approximately 80% of the FM patients in this study. This will be discussed further in Section 4.4 Overlap of the diagnoses CFS and FM.

The CFQ is a validated questionnaire, and the scores were used as a continuous scale when conducting further analyses of possible confounding factors in our biological measurements.

hsCRP

In the CFS group, lower fatigue scores were associated with higher hsCRP. This finding caused some initial confusion and is most likely a type 2 error since the association between fatigue and hsCRP was weak ($\rho < 3$).

Cytokines/Chemokines

Higher fatigue scores in the FM group showed lower levels of MCP-1 and IL-17A. There were no other associations between fatigue scores and any of the cytokines or chemokines in either the CFS group or the controls. These associations were weak ($\rho < .3$), and it is not clear why such associations were found. However, in cancer-related fatigue, Yang et al[109] indicated that although MCP-1 and neurocognitive performance are inversely associated with fatigue before chemotherapy, there is a positive correlation between MCP-1 and fatigue after chemotherapy.

Kynurenines

AA: From the initial finding of lower AA levels in CFS patients we can assume that this was not the actual factor influencing low AA; instead, it was the higher fatigue levels in these patients that was associated with lower AA levels. We could not find any studies to compare our results of AA.

4.3.14 Fibromyalgia severity score – Fibromyalgia survey diagnostic criteria

FS scores were highest for the FM group, followed by the CFS group, and lowest for the control group. No healthy controls were classified into FM cases by the use of FSDC, which was expected. 15% of FM patients included in this study according to the ACR 1990-criteria did not fulfil a FM diagnosis according to the FSDC. This lower sensitivity of 88% could be due to the ACR 1990 criteria to misclassify FM patients with less specific test methods, e.g. the use of tenderpoints. The art of pressing with a force of 4 kg “by palpation with the pulp of the thumb or the first 2 or 3 fingers”[11] would require immense training and constant calibration for stable intra-rater stability. There are instruments (algometers) that could solve this bias, but this is not something that is found in most clinics. The clinician must also gain enough experience to differentiate between grading the severity tenderpoints by observing different reactions (i.e. grimace, flinch or withdrawal) to correctly quantify tenderpoints. Similarly, the inter-rater stability for measuring tenderpoints can vary greatly.[93] In the clinic where the patients in this study were diagnosed, there were several clinicians, most will long experience in conducting tenderpoint examination. Still, this was not tested at any point during the study, and the intra- and interrater stability is uncertain. A recent study validating the Norwegian translation of the FSDC using the ACR 1990 criteria as reference, found a

94% sensitivity and 68% specificity for the new 2016 FM criteria[160] compared to our 88% and 87%, respectively.

One of the main arguments for introducing a new diagnostic tool for recognising FM cases is that it abolishes the use of tenderpoint examination because of the uncertainty described above. This is not to say that FM patients do not suffer from hyperalgesia or might have specific locations that this can be measured more frequently, it just takes away the inaccuracy of tenderpoint examination. Rather, it emphasises that there is no strict cut-off case-definition of FM (with the > 10 tenderpoints in the ACR 1990 classification. And although there is a cut-off score of fibromyalgia severity of 12 or more that correlates to having a FM diagnosis, the newer 2016 criteria also emphasise the severity or *fibromyalgianess* in each case.[13]

No associations were found between FS scores and hsCRP.

Cytokines/Chemokines

The only associations between FS scores and the cytokines/chemokines in this study, were found in the FM group for TGF-β1, TGF-β2 and TGF-β3 and not in the other groups (CFS and controls). Perhaps these regulatory cytokines that there is a dysregulation in immune activity related to the *fibromyalgianess* (equivalent to the FS scores).

Kynurenines

Kyn/Try: In Model 2, that included symptoms of CFS and FM, could indicate some opposite effect (although not significant) of *fibromyalgianess*. The effect of the FM group did not disappear completely, however, and it is worth exploring further.

4.3.15 Summary of confounding factors

No associations were found between the cytokines/chemokines and smoking, use of birth control and stage of menstrual cycle (data not shown).

None of the associations for any of the confounding factors discussed above were particularly strong (ranging from ρ = .16–.40), and the group effects for CFS, FM or controls on the same confounders were mainly higher (ranging from .14–.86; Supplementary Table 2). However, some of the confounders for the immunological markers hsCRP and the cytokines/chemokines are worth emphasising: Age, BMI, and duration of illness.

For hsCRP, BMI was a strong confounder (Figure 4.2). For MCP-1 the effect of age was similar to that of the group effects of CFS, FM and controls (ρ = .32–.42). For IFN-γ, age might have contributed to the lower levels of IFN-γ found in the FM group.

Because of the great immunological overlap between CFS and FM patients, our findings indicate that duration of illness should be considered valuable information when looking at immune deviations for CFS and FM disorders as there may be some immunological development as the disorders are progressing.

The confounders that may have influenced the kynurenines were age, BMI, nicotine, pain, and fatigue, and are found in Figure 4.2.

Effect	Try	Kyn	KA	AA	HK	XA	HAA	QA	Pic
High		Age (6.2%)	Age (6.4%)	Nicotine (7.0%)	BMI (7.3%)			BMI (6.9%)	
				Fatigue (3.6%)				CFS (6.3%)	
Low								Nicotine (2.9%)	
Model adjusted R^2	**.096**	**.100**	**.165**	**.159**	**.085**	.015	.010	**.096**	.045

Effect	Kyn/Try	KA/Kyn	XA/HK	HK/Kyn	AA/Kyn	HAA/HK	KA/QA	KA/HK
High	Age (6.2%)	Age (7.1%)	Pain (5.5%)	BMI (5.4%)	Nicotine (7.3%)	Pain (6.0%)	CFS (3.9%)	Pain (6.6%)
	BMI (4.4%)	BMI (3.7%)	Nicotine (3.4%)	Pain (2.9%)		Nicotine (4.5%)		
Low	FM (3.6%)	FM (3.5%)						
Model adjusted R^2	**.145**	**.132**	**.151**	**.088**	**.065**	**.115**	.045	**.122**

Figure 4.2. *The Relative Contribution of Confounders on the Metabolites and Ratios of the Kynurenine Pathway*
Representation of the best model fit (adjusted R^2) for the metabolites and ratios used in this study with the relative contribution of the diagnostic groups: CFS and FM; and the confounding factors: age, BMI, nicotine use, pain and fatigue. Grayscale for results when R^2 considered small (arbitrary value chosen: $R^2 < .05$).

This last section has explored the field beyond the original study and, with no hypothesis in mind, may contribute to better research questions in the future. We do not draw any conclusions from this.

4.3.16 Risk assessment

The majority (75%) of the CFS subjects in this study had hsCRP concentrations lower than 3 mg/L. For the FM group, the majority (75%) had hsCRP concentrations below 3.91 mg/L. According to the American Heart Association guidelines (Pearson, Mensah et al. 2003), hsCRP levels above 3 mg/L is considered a high risk of cardio-vascular disease. I.e., approximately 12.5% of the patients in our study population have increased risk of this comorbidity. For the control group, the majority (75%) had hsCRP concentrations below 1.27 mg/L, thus making the majority in the low risk of cardiovascular disease (33 of 51 valid cases; 65%), with fewer cases in the average risk category. The distribution of the low, medium and high risks for the hsCRP for each group in this study are illustrated in Figure 4.3.. Only three patients had hsCRP levels > 10 mg/L. The cut-off of 10 mg/L is used for indicating inflammation or infection. By scrutinizing the blood samples, none of these cases otherwise showed any signs of infection and were thus included in all further analysis. Also, excluding these cases did not alter any of the findings (data not shown).

Three patients had hsCRP levels > 10 mg/L. The cut-off of 10 mg/L was chosen for indicating inflammation or infection if other signs of infection was found in the sample material. None of these cases otherwise showed any signs of infection and were thus included in all further analysis. Also, excluding these cases did not alter any of the findings (data not shown).

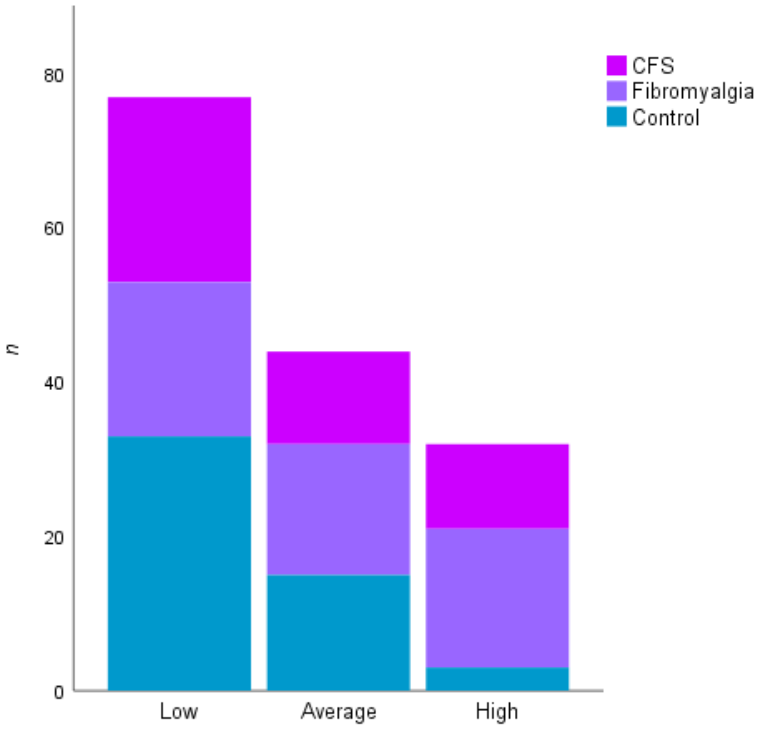


Figure 4.3.
Risk of assessment for CFS, FM and controls when following the American Heart Association guidelines of cardio-vascular disease. Low (< 1.0 mg/L); Average (1.0–3.0 mg/L); and High (> 3.0 mg/L).

When 1/5 to a quarter of the CFS patients and a little less than 1/3 of FM patients in this study met the criteria of increased risk of heart related disorders, this in itself is a health risk, and whatever the underlying issue, higher levels of CRP over time should be addressed.

However, the cytokines did not show a clear-cut pro-inflammatory state for any of the patient groups in any time frame recorded in this study. Increased CRP could also reflect the body's prolonged stress response to living with a chronic disorder. The number of patients in this study with a shorter duration of illness was low (10 in FM and 13 in CFS), and longitudinal studies are needed to explore any true causality of the possibilities discussed above.

There seems to be a general health risk due to the higher hsCRP levels in both patient groups. Increased exercise and lower BMI reduce the risk of developing FM,[150] and exercise have shown to decrease the symptoms in FM patients.[161] Therefore, life-style changes resulting in lower BMI may be recommended to reverse the effects of prolonged exposure to increased CRP.

4.4 Overlap of the diagnoses CFS and FM

When conducting this study, we used the Fukuda criteria[5] as the diagnostic criteria for CFS and the ACR 1990 criteria for diagnosing FM patients, which were the common criteria when the data were collected. In our study population we found that 28% of CFS patients also qualified for FM ACR 1990[11] diagnosis, and 43% of the CFS patients fulfilled a FM diagnosis according to the newer FM 2016 criteria.[13] We also found that 81% of FM patients suffered from fatigue equal to CFS (based on the definition of fatigue and CFS from the CFQ[90]).

The higher 80% overlap between FM and CFS when using the CFQ as reference for CFS, could be due to false positive cases because of the lack of additional symptom evaluation that needs to be met before a proper diagnosis is given. The great discrepancy between the 28% and 43% overlap between originally CFS diagnosed patients with FM comorbidity in this study could partially be explained by the sensitivity, and possible mis-classification when following the ACR 1990 criteria. Following this logic, the higher sensitivity of the CFQ to correctly classify true cases of diagnosis, and the slightly lower sensitivity of the FSDC, the prevalence of CFS and FM comorbidity varies between 28% and 80% depending on which classification system used. Indeed, there are reports of 35% to 70% of CFS patients have FM.[162] Our findings may well reflect this overlap, making it difficult to distinguish between "pure" CFS and FM patients. Comparing these two specific subsets of patients also did not reveal any specificity of either disorder (data not shown), making it unlikely that our findings can be explained by one or the other patient group. This may rather reflect the uncertainty of the diagnoses, which vary greatly in symptoms and severity.

Both Calder et al[90] and Wolfe et al[94] recommend using the fatigue scores of CFQ and FS scores in FSDC as measures of the severity of fatigue and *fibromyalgianess*, respectively. This would for research purposes make sense, because in lack of consensus of the diagnostic criteria (especially for CFS), research should emphasise on symptoms in the search for knowledge about the disorders. The heterogenicity of CFS (especially) but also for the additional symptoms following the more recent FM diagnostic criteria (e.g. fatigue, unrefreshed sleep, cognitive symptoms, headache, abdominal pain and depression) may well be the expression of disorders that are related.

We are not the only researchers facing this challenge of overlapping symptomatology and diagnosis.[163] The criteria we used in this research do not necessarily reflect the reality in clinical settings. Finding objective measures that are useful both in research and the clinic has proven difficult. To complicate things further, there is no real consensus on the diagnostic criteria for CFS or FM, neither among researchers nor amongst clinicians. Since the beginning of this project, the Fukuda criteria has been disputed the Canada criteria seemingly being more popular and considered "precise" for adding PEM (Post Exercise Malaise) as a critical symptom for CFS. Just recently, another set of criteria has been introduced: European Network on Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (EUROMENE).[164] According to these criteria, CFS patients are recommended to rest. GAT (gradual exercise

therapy), which appears to be the only therapy that ever led to significant improvement in CFS symptoms,[92] are abolished by EUROMENE.

The original idea by development of diagnostic systems for diseases was to make it easier for health professionals to give the correct treatment. Lately, the debate seems to have shifted and the newer diagnostic criteria (*ICD-11*) have been extensively revised emphasising on symptoms and severity of disorders rather than clear cut-off diagnoses. This thesis shows that there is both overlap and differences when it comes to comparing the symptoms of CFS to FM. This may reflect some shared underlying mechanisms in both disorders. Or possibly, a common aetiology in a sub-group of these patients. It may be that CFS and FM are syndromes and not disorders – we simply do not know.

Regarding our immunological findings, there seem to be considerable overlap between the hsCRP and cytokine profiles of CFS and FM patients. For kynurenines, there might be some differences between CFS and FM patients, although this may be attributed to symptoms, e.g. pain, rather than diagnose-specific. The clinical relevance of our findings is not known.

4.5 Limitations

This is a large and comprehensive study, and there are several limitations that need to be addressed.

This study contains multiple factors thus running the risk of any positive findings by chance. When several markers are analysed, correction or multiple testing should be taken into account. However, the different cytokines as well as kynurenines do not occur independently of each other. Thus, a less conservative approach may be taken. Quoting Rothman[165]: "When scientists are studying biological relation rather than random numbers, the premise that Type I errors are the major concern may be wrong;" and offers a more moderate approach in research studying biological systems.

In this study, we collected blood samples, and we did not collect any other biological samples. It is not possible to conclude exactly on the origin of the cytokines or kynurenines in our study. Cytokines do not readily cross the blood-brain-barrier, yet, in times of inflammation, there may be fluctuations between the peripheral blood and CNS. Peripherally produced cytokines may thus not reflect the levels found in CSF. Peripherally produced cytokines may influence the production of cytokines in the CNS, however, and some of these may be detectable in CSF. Yet, cytokines are often produced locally and may not be found when measuring the levels in plasma. The kynurenines in this study were also measured in blood and are most likely produced in the periphery (e.g. in the gut) and does not reflect the true Try conversion into either 5-HT nor kynurenines in the CNS.

In this study, the other main metabolite of Try, namely serotonin (5-HT), was never measured. Because of the, at least in part, involvement of 5-HT in depression, and the anti-depressive effects of SSRIs and NSRIs, comparing 5-HT relative to the activation of IDO or Kyn/Try ratio would have been beneficial. The simple reason for not including 5-HT in this

study, was that the package deal delivered by the company that conducted the analyses of the kynurenines did not include 5-HT.

The only inflammatory factor included to predict the kynurenines in our study was serum IgE concentrations, indicative of allergies. IgE did not show any relationship with any of the kynurenines. We did not include any of the pro-inflammatory markers (cytokines nor CRP) when analysing the kynurenines. Yet there was no specific pro-inflammatory cytokine profile that could indicate an enhanced expression of IDO in our sample.

None of the FM patients were evaluated according to the Fukuda 1994 criteria for a possible CFS diagnosis in the clinic. This was common practice in the clinic, as the primary objective of patients with primary pain-related disorders, were referred to the pain clinic and not the CFS outpatient clinic and evaluated as such. This is a weakness of the study and enlightens the difficulty of running a research study in a busy clinic.

We did not compare our patients to patients with other chronic disorders, and our results may not solely be related to CFS or FM. Neither did we compare our patients to MDD patients, and any conclusions can be drawn regarding inflammation and depression in our study.

Body weight and height were self-reported for the majority of the participants in this study. This was due to the lack of availability of standard equipment for these measures in the facilities used. All participants met with the researcher (NG) who could grossly evaluate the participants' body composition.

There may be many other factors that could be included in exploring all of the dependent variables in this study, yet, educated choices should be made when including confounding factors. These results did confirm that none of the variables were solely explanatory for the outcome of the results. Some confounding factors may, in addition to BMI, be included in future research, such as: fatigue scores, FSDC scores and pain scores. These are not independent from each other, however, and caution should be made in conducting and designing similar studies. In this study we were mainly focusing on differences or similarities between CFS and FM groups as there are not many studies comparing immunological parameters in these two groups.

4.6 Conclusions

The aim of this thesis was to shed light upon the dissimilarities or similarities between CFS and FM from an immunological perspective.

Our findings suggest that there are some immunological aberrations in these disorders compared to healthy controls. In particular, we found that CFS and FM patients have higher plasma hsCRP levels and that a variety of cytokines are reduced in these patient's plasma compared to the controls. The chemokine MCP-1, however, was increased in both patient groups. To address and highlight the complexity of this kind of research, we extended the research further to include the tryptophan-kynurenine pathway – additional markers that may be related to inflammation.

Overall, the CFS and FM groups in our study could not be distinguished from one another when analysing the immunological markers and additional markers related to inflammation. In other words, there was no obvious distinction between the CFS and FM groups when examining our main hypotheses, but they both differed from the control group.

When exploring our data, we found that some factors were interfering with the outcome. In particular, BMI was highly associated with hsCRP, yet it did not fully explain the observed differences between the groups. For some of the data (fatigue scores, FS scores and pain scores) showed the same direction of association with the cytokines as did the patient groups. This indicates that the symptoms may be just as relevant as the actual diagnosis when evaluating the results and there may be different aetiological factors behind different subgroups (e.g. short and long duration of illness).

The different physiological and biochemical mechanisms in humans and other species are intertwined. The exact mechanisms between the messengers of the immune system (i.e. cytokines) and physiological responses are not fully understood. The cytokines can act as neurotransmitters and neuromodulators,[166] and relatable to our study, inflammatory cytokines can interfere with and change the properties of nociceptors both peripherally and in the spine.[167] In observational studies like this, however, we only catch a snapshot of the otherwise dynamic human mind and biological mechanisms.

Interviewing these patients gave the impression that chronic stress or trauma were preceding events before the onset of FM. CFS patients often spoke of prolonged viral infections, often in combination with stressful life events or trauma, prior to the onset of the symptoms associated with the disorder. This raises the question that there may be an infective agent, trauma or stressful life events that separately or together are related to the aetiology of CFS or FM. Both infections and traumas/stress will cause an inflammatory state. Both the CFS and FM patients in this study showed enhanced hsCRP levels, indicating a low chronic pro-inflammatory state.

The role and type of inflammation in both CFS and FM need to be clarified for prevention and improvement of treatment of these conditions. Our study is a contribution to this field because it explores the two patient groups in which there are few immunological and clinical differences.

4.7 Future implications – What lies ahead?

The cause of a rise in hsCRP in CFS and FM patients found in this study is not known. We know that stress, short-term and long-term may lead to a pro-inflammatory state. Living with a cronic disorder may have the same effects on the stress- and immune system. If chronic inflammation by means of increased hsCRP is a primary force for developing CFS and FM or cause the maintinance of symptoms, anti-inflammatory treatment could be prioritised. There are different approaches for restoring a more balanced inflammatory state: In addition to pharmacological intervention, non-pharmalogical intervention, such as stress management could be recommended. As treatment (such as immunomodulatory medication) can interfere

with the outcome of cytokines and other immunological measures, this should be controlled for in future, longitudinal studies.

To strive for objective measures of the subjective symptoms fatigue and pain, and finding clear definitions of the two is without a doubt a difficult task, still should be a point of focus for future studies.

Investigating biogical mechanisms, and energy production with respect to for instance fatigue may be benefitial when studying inflammation and its role in both CFS and FM. This may lead to better understanding of the true aetiology of the symptoms of these conditions and hopefully reveal wether they are distinct or phases of a spectrum disorder.

Forthcoming studies indicating interactions and causative effects, or restoration of the inflammatory status, may place cytokines and kynurenine metabolites more in the center of treatment. This has a potential of improving prevention and future treatment strategies.

> *We will use our regiments which will benefit our patients according to our greatest ability and judgement, and we will do no harm or injustice to them.*
>
> [Freely adopted from the Hippocratic Oath.]

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Appendix A

Original Articles 1–3

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Patients with Fibromyalgia and Chronic Fatigue Syndrome show increased hsCRP compared to healthy controls

Nina Groven[a,c,*], Egil A. Fors[b], Solveig Klæbo Reitan[a,c]

[a] Department of Mental Health, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[b] Department of Public Health and Nursing, General Practice Research Unit, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[c] Department of Mental Health, St. Olav's University Hospital, Trondheim, Norway

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ABSTRACT

Chronic Fatigue Syndrome (CFS) and Fibromyalgia (FM) are both chronic disorders that have a devastating effect on the lives of the affected patients and their families. Both conditions have overlapping clinical features that partly resemble those of inflammatory disorders. The etiology is still not understood, and it is suggested that the immune system might be a contributing factor. So far, the results are inconclusive. The purpose of this study was to compare the two conditions and investigate the level of the inflammatory marker high-sensitivity CRP (hsCRP) in CFS and FM patients compared to healthy controls.

Female participants aged 18–60 years were enrolled in this study. The group consisted of 49 CFS patients, 57 FM patients, and 54 healthy controls. hsCRP levels were significantly higher for both the CFS and the FM groups compared to healthy controls when adjusting for age, smoking, and BMI ($p < .001$). There was no difference between the two patient groups. The level of hsCRP was affected by BMI but not by age and smoking.

Patients with CFS and FM have higher concentrations of hsCRP compared to healthy controls. This remains significant even after adjusting for BMI. CFS and FM cannot be distinguished from each other on the basis of hsCRP in our study.

1. Introduction

The disorders Chronic Fatigue Syndrome (CFS) and Fibromyalgia (FM) are two distinct diagnostic groups; however, they show overlapping symptoms (Clauw, 2010). CFS is characterized by severe fatigue with distinct onset, lasting more than 6 months, not necessarily connected to ongoing exertion, not affected by rest, and causing reduced function. In addition, the occurrence of at least four of the following eight symptoms is observed: impairment in short-term memory or concentration, sore throat, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, headaches of a new type, pattern, or severity, unrefreshing sleep, and post-exertional malaise lasting more than 24 h (Fukuda et al., 1994). Both CFS and FM diagnoses are based on specific inclusion criteria and exclusion of other diagnoses causing the same symptoms, although symptoms from both somatic and psychiatric origin seem to be present (Carruthers et al., 2011; Fukuda et al., 1994; Wolfe et al., 2016, 1990). These two conditions cause distress for the patients and potentially increase expenses for the health-care system. Thus, more knowledge is needed to alleviate the issues caused by CFS and FM.

Pain and fatigue are common traits in several inflammatory disorders. Inflammation directly activates pain systems (Sommer and Kress, 2004) and causes fatigue (Norheim et al., 2011; Sluka and Clauw, 2016). Another trait of several inflammatory disorders is "sickness behavior" referring to non-specific symptoms such as anorexia, depressive activity, loss of interest, and disappearance of body-care activities (Kent et al., 1992). Sickness behavior may be caused by immune mediators (e.g., IL-1; Kent et al., 1992). Thus, the immune system is an obvious candidate to investigate for its role in CFS and FM. So far, studies are inconclusive (Feinberg et al., 2017; Lyall et al., 2003; Raison et al., 2009; Sotzny et al., 2018; Wyller et al., 2017). We previously have shown a tendency to increased inflammation measured as increased TNF-α in CFS patients compared to controls (Groven et al., 2018).

Because the etiology for FM is as vague as that for CFS, we wanted to study similarities and differences between the two conditions. In the present study, the general and widely used immune marker hsCRP is explored in patients with CFS and FM and in healthy controls. hsCRP is a more accurate method of measuring levels of CRP.

* Corresponding author at: Department of Mental Health, Faculty of Medicine and Health Sciences, NTNU, PO Box 8905 MTFS, NO-7491 Trondheim, Norway.
E-mail address: nina.groven@ntnu.no (N. Groven).

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2. Method

2.1. Sample population

2.1.1. Patient groups

Female, non-pregnant patients aged 18–60 years admitted to the Multidisciplinary Pain Centre at St. Olav's University Hospital, Norway, for CFS and FM were eligible for the study. Patients with challenging clinical pictures regarding problems such as CFS and FM are referred to this centre by general practitioners in Mid-Norway.

Each participant went through a comprehensive clinical examination and was thoroughly evaluated by an expert team of medical doctors, physiotherapists, and psychologists. FM patients ($n = 58$) were diagnosed by using the 1990 ACR criteria (Wolfe et al., 1990). CFS patients ($n = 49$) were diagnosed according to the Fukuda criteria (Fukuda et al., 1994). Exclusion criteria were in accordance with diagnostic criteria including known inflammatory disease.

2.1.2. Healthy controls

A healthy group of 53 females aged 18–60 years was consecutively recruited by advertising through websites among the staff of the Norwegian University of Science and Technology and St. Olav's University Hospital. Their health was assessed by conducting a structured medical history and by using questionnaires included in this study measuring the symptoms of CFS and FM (see 2.4 Questionnaires and 2.5 Interview).

2.2. Procedure

The CFS patients were informed about the study by a letter sent by the hospital prior to or shortly after their evaluation or given during their evaluation at the centre. The FM patients were given an information letter by the staff during the examination and evaluation of their FM diagnosis. Both patient groups were then contacted by phone and asked for participation in this study by a member of staff, and an appointment was scheduled for those who accepted to join the study.

2.3. Study design and ethics

The assessment lasted approximately 30–40 min and included an interview, questionnaires, and blood sampling. Data were collected in the period from March 2015 to December 2016. The order of the assessments was random.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK 2014/711). Written informed consent was obtained from all participants.

2.4. Questionnaires

All participants filled out the Hospital Anxiety and Depression Scale (HADS; Zigmond and Snaith, 1983), the FM 2011 and 2016 criteria (Wolfe et al., 2016; Wolfe and Hauser, 2011), the Chalder Fatigue Scale (Chalder et al., 1993), and the Brief Pain Inventory (BPI) (Cleeland, 1991; Klepstad et al., 2002).

2.5. Interview

For each participant, age, height, and weight were recorded. A structured clinical interview was conducted by the first author. History regarding infections, immune disorders, illness in general (somatic as well as psychiatric), comorbid disease, medication, menstrual cycle, use of contraceptives, status of menopause, duration of illness (if applicable), and level of physical activity during the previous two weeks was recorded.

2.6. Blood sampling

There were no restrictions given prior to blood sampling. The blood samples were collected in serum tubes (Vacuette® 5 ml Z Serum Sep Clot Activator) and analyzed for hsCRP at the hospital clinical lab by Siemens Advia Chemistry XPT and Roche Modular P according to the laboratory procedure. Blood samples were also screened for any signs of infection and inflammation (e.g., microbiological serology, white blood cell count, etc.). Signs of any abnormalities led to exclusion from the study.

2.7. Statistical analysis

We used the Statistical SoftWare Package (SPSS) Statistics, version 22. All variables were tested for normality and homogeneity by using the Kormaninov-Smirnov and Shapiro-Wilk tests and the Levene's test.

For comparison of age and BMI between groups, the Kruskall-Wallis test was applied. Mann-Whitney U was used for post-hoc analysis of pair-wise comparison. For the variable CRP, natural log transformed data (lnCRP) were used, and linear regression was applied. For these data, post-hoc pair-wise comparison was conducted by means of *Student's t-test.*

3. Results

The basic descriptives of key variables are summarized in Table 1. The total number of participants was 160, distributed among the three groups as follows: CFS ($n = 49$), FM ($n = 58$), and healthy controls ($n = 53$). The median hsCRP concentration was 0.94 mg/L for CFS, 1.30 mg/L for FM, and 0.60 mg/L for the control group. hsCRP was not normally distributed and hence was transferred into the natural log (lnCRP) for further analyses.

The Kruskall-Wallis test revealed statistically significant differences in age and BMI between the groups (Table 1). Pair-wise analyses for age and BMI are shown in Tables 2 and 3.

BMI made a considerable contribution to the model, accounting for 23.1% of the variance of lnCRP ($p < .001$, $F(1, 145) = 43.62$), whereas the group parameter accounted for 6.9% of the variance ($p = .001$, $F(2, 145) = 5.37$). Age had no effect on the outcome ($p = .200$, $F(1, 145) = 1.66$). There was no relationship between smoking status and hsCRP levels ($p = .925$, $\rho = -0.008$).

There was a strong positive correlation between hsCRP and BMI for the total sample population ($N = 150$, *Spearman's* $\rho = 0.439$, $p < .001$). We also observed a correlation between diagnostic group and hsCRP ($N = 153$, *Spearman's* $\rho = -0.190$, $p = .019$).

The difference in lnCRP was significantly higher in FM and CFS groups compared to the control group ($b = 0.591$, $p = .004$ and $b = 0.563$, $p = .009$, respectively). There was no difference between the two patient groups FM and CFS ($p = .902$; Table 2 and Fig. 1).

4. Discussion

CFS and FM groups showed significantly higher levels of hsCRP than the healthy-control group ($p = .009$ and $p = .004$, respectively) but could not be distinguished between each other ($p = .902$). hsCRP was correlated to BMI but not to age nor smoking. After adjustment for BMI, the increased hsCRP in both patient groups compared to healthy controls was still significant.

Although there are reports finding a lack of association between inflammation and CFS (Wyller et al., 2017), a substantial number of reports indicating an association are published (Patarca-Montero et al., 2001; Patarca, 2001; Raison et al., 2009; Russell et al., 2018). A recent review on CFS and autoimmunity does not mention CRP, although other immune markers are discussed (Sotzny et al., 2018). A recent report (Giloteaux et al., 2016) comparing patients with CFS and healthy controls found a slightly, but not significantly, higher level of hsCRP in

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Table 1
Descriptives of C-reactive protein (CRP), age, and Body Mass Index (BMI).

	CFS				FM				Control				p^a
	n	Missing (*n*)	*M (SD)*	*Mdn*	*Range*	*n*	Missing (*n*)	*M (SD)*	*Mdn*	*Range*	*n*	Missing (*n*)	*M (SD)*	*Mdn*	*Range*	
Age	49	0	33.8 (11.3)	35	[18, 60]	58	0	42.0 (9.1)	42.5	[22, 60]	53	0	39.4 (10.4)	39	[23, 59]	< 0.001
BMI	47	2	24.0 (3.6)	23.1	[18.1, 34.6]	57	1	26.7 (5.6)	25.7	[16.3, 40.4]	53	0	24.7 (4.0)	23.8	[16.3, 41.7]	0.017
hsCRP[b]	47	2	2.34 (3.00)	0.94	[0.10, 13.74]	55	3	2.62 (2.74)	1.30	[0.18, 10.94]	51	2	1.13 (1.39)	0.60	[0.10, 7.11]	0.002
lnCRP[c]	47	2	0.117 (1.283)	−0.062	[−2.303, 2.620]	55	3	0.387 (1.152)	0.262	[−1.715, 2.392]	51	2	−0.413 (1.019)	−0.511	[−2.303, 1.962]	0.002

[a] Statistical difference (Kruskall-Wallis test).
[b] Raw scores of hsCRP in mg/L, unadjusted.
[c] Log transformed (natural log) of hsCRP.

Table 2
Pair-wise analysis of age between groups.

Comparison groups		U^a	*z*	*p*	p^b
CFS	Control	916.0	−2.56	0.010	0.029
FM	Control	1314.5	−1.32	0.189	0.545
CFS	FM	786.5	−3.97	< 0.001	< 0.001

[a] Mann-Whitney *U*.
[b] Adjusted by the Bonferroni correction for multiple tests.

Table 3
Pair-wise analysis of BMI between groups.

Comparison groups		U^a	*z*	*p*	p^b
CFS	Control	1095	−1.04	0.299	1
FM	Control	1182	−1.97	0.049	0.179
CFS	FM	928	−2.69	0.007	0.016

[a] Mann-Whitney *U*.
[b] Adjusted by the Bonferroni correction for multiple tests.

patients with CFS, whereas a previous study on CFS patients recruited from gastroenterology and rheumatology departments found significantly increased hsCRP levels among CFS patients compared to controls (Groeger et al., 2013). Both studies included both genders and a broader age span and did not adjust for BMI. Also, genetic studies in adolescents have indicated a link between immune activity and CFS (Nguyen et al., 2018). Raison et al. (2009) found that an increased hsCRP in patients with CFS was no longer significant after adjusting for age, sex, race, location of residence, BMI, depressive status, and immune-modulating medications. We had only one gender, none of the participants were taking immune-modulating medications, there was no comorbidity, and we did not find any effect of age in our groups. Regarding for race and location of residence, we did not record these data, but all our participants were recruited from rather homogenous areas in and around Trondheim, Middle Norway. The role and type of inflammation in CFS needs to be clarified to improve prevention and treatment of the condition. Our study is a contribution to this field because it explores the phenomenon between groups in which there are few differences apart from the presence of CFS (i.e., otherwise healthy, only one gender, socio-economically homogenous group, and narrow age span), and BMI is adjusted for.

A recent report measuring CRP in FM patients did not find any differences between patients and controls regarding hsCRP, although an effect was seen for leptin (Ataoglu et al., 2018). A large-population-based study found increased CRP among participants with self-reported diagnosis of FM and suggested that it was partially explained by BMI and comorbidity (Feinberg et al., 2017). Furthermore, a review of studies reporting the effect of non-pharmacological interventions in FM patients did not find a consistent effect on CRP. Still, baseline CRP levels were higher than the reference value in three of the included studies (Sanada et al., 2015). A subgroup of FM patients with inflammatory changes including altered CRP has also been suggested (Metyas et al., 2015). As shown by others (Xiao et al., 2013), we found that hsCRP was associated with BMI among all FM patients as well as healthy controls. However, after adjusting for BMI, there was still a significantly higher level of hsCRP among patients compared to controls. To our knowledge, this has not been consistently reported previously, and the phenomenon should be further explored.

In our study, both patient groups show significantly higher hsCRP than healthy controls. However, the patient groups do not deviate from each other. CFS and FM are defined as two distinct disorders although there is a high comorbidity, and there are several overlapping symptoms and findings between the two disorders (Clauw, 2010). Raison et al. (2009) found that people with CFS and a group with CFS-like illness could not be distinguished from each other on the basis of hsCRP

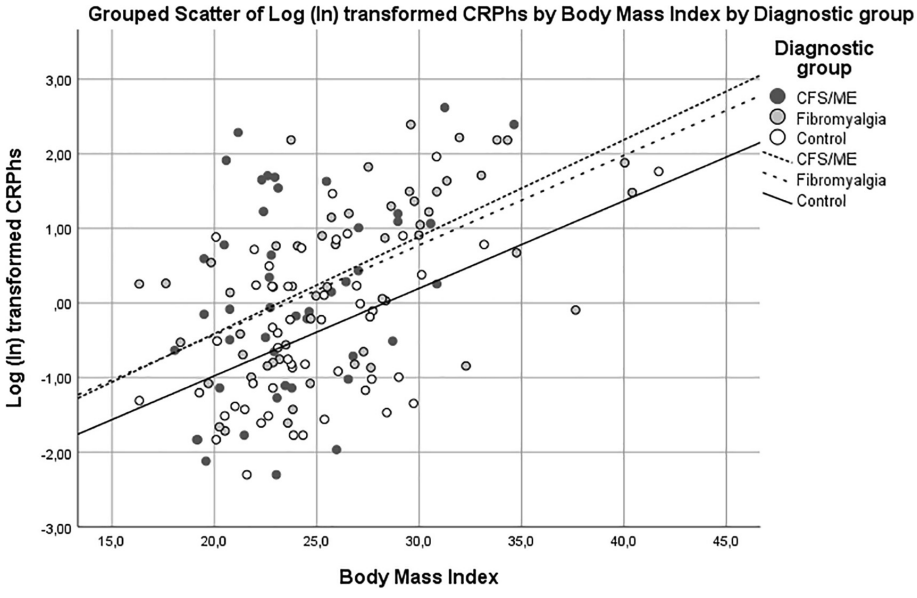

Fig. 1. *Linear regression for BMI and lnCRP.* Scatterplot showing the relationship between BMI and lnCRP for each of the diagnostic groups: CFS, ── tight dashed line; FM, – – – lose dashed line; and control group, — solid line.

levels. It is important to keep in mind these biological similarities because there are deviating reports on the differences between CFS and FM regarding clinical symptoms such as personality (Ablin et al., 2016; Balbaloglu et al., 2018; Sirois and Molnar, 2014), cognition (Rasouli et al., 2019; Schmaling and Betterton, 2016), and balance (Rasouli et al., 2018).

BMI had a clear effect on hsCRP. This is in line with findings in several other studies and with the known effect of adipose tissue on the production of CRP (Lau et al., 2005). Our study confirms that studies on CRP as well as inflammation in general should be corrected for BMI.

We did not find an effect of age on CRP. This is in line with Xiao et al. (2013). This might be surprising because it is generally assumed that inflammation increases with age. However, our population overall may be too young to reveal this effect. CRP only seems to be increased with age in men but is manifested in women only after menopause (Poledne et al., 2009). Also, no effect of smoking was seen.

There might be differences in inflammatory markers between short- and long-term duration of CFS cases (Hornig et al., 2015). In a study of FM, weather sensitivity, and pain, duration also seemed to matter; it was concluded that FM patients with shorter duration of their illness were more sensitive to weather (Fors and Sexton, 2002). However, duration of illness did not affect the findings in the present material (data not shown).

4.1. Weaknesses and strengths

The study only gives information on a limited population, that is, female individuals aged 18–60 living in a homogenous area with well-developed social and health services. For other groups (males, children and adolescents, elderly, and somatically as well as psychiatrically very ill people), the mechanisms revealed may not be important for fatigue and pain. None of the patients were clinically depressed. In our study, we also recorded symptoms of anxiety and depression by using the HADS. Adjusting for these scores did not have any effect on hsCRP (data not shown).

Presumed low activity levels for the patients and high activity levels for the healthy controls could be a confounding factor influencing the results (Fedewa et al., 2017). CFS patients reported the lowest activity levels; FM patients' activity levels were higher; and the healthy control group reported the highest activity levels (data not shown). It is not surprising that patients with CFS report higher levels of inactivity because this is part of the characteristics of the disorder. Still, over half of the CFS patients were indeed active (data not shown), and we do not believe that this is a contributing factor to the higher inflammatory finding in our study. We also included BMI, thus controlling for the indirect link between low activity levels and BMI.

The study population is rather homogenous regarding age, gender, and socio-economical status and otherwise healthy and not on medications. This enables us to reveal differences independently of many confounding factors. Also, the study is well powered with a rather large clinical material. Patients were diagnosed according to the Fukuda and Canada criteria (Carruthers et al., 2011; Fukuda et al., 1994) at a specialized multidisciplinary unit in a university hospital, in addition to registering the new FM criteria (Wolfe et al., 2016; Wolfe and Hauser, 2011), making clinical diagnoses valid compared to what can be seen in larger population-based studies.

5. Conclusions

CFS and FM patients have higher concentrations of hsCRP compared to healthy controls. This remains significant after adjusting for age and BMI. CFS and FM cannot be distinguished between each other on the basis of hsCRP in our study.

Overall, our study gives an important contribution to the knowledge on CFS and FM. There seems to be a biological inflammatory activity in patients with CFS and FM that is not found in healthy controls of the same age and gender. The inflammatory changes, whether they are primary or secondary to other symptoms, may be perturbing symptoms. Inflammation is a well-known cause of fatigue (Norheim et al., 2011) and pain (Louati and Berenbaum, 2015; Sluka and Clauw, 2016) and may be a target for attack by medications (Zhang et al., 2016) as well as a marker for monitoring any treatment of these conditions. In accordance with the Centers for Disease Control and Prevention and the American Heart Association recommendations of hsCRP and risk-factor

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assessment (Pearson et al., 2003), the hsCRP levels in our patient groups were mainly within the moderate-to-high-risk concentrations. As such, this has clinical relevance beyond defining the cause of CFS and FM.

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Conflict of interest

All the authors declare no conflict of interest.

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Full Length Article

MCP-1 is increased in patients with CFS and FM, whilst several other immune markers are significantly lower than healthy controls

Nina Groven [a,b,*], Egil Andreas Fors [c], Astrid Kamilla Stunes [d,e], Solveig Klæbo Reitan [a,b]

[a] Department of Mental Health, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[b] Department of Psychiatry and Clinical Neurophysiology, St. Olav's University Hospital, Trondheim, Norway
[c] Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[d] Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[e] Medical Clinic, St. Olav's University Hospital, Trondheim, Norway

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ABSTRACT

The role of the immune system in the pathogenesis of Fibromyalgia (FM) and Chronic fatigue syndrome (CFS) is not clear. We have previously reported increased levels of C-reactive protein (CRP) in these patient groups compared to healthy controls and wanted to further explore the levels of circulating immune markers in these populations.

The population consisted of three groups, 58 patients with FM, 49 with CFS and 54 healthy controls. All participants were females aged 18–60. Patients were recruited from a specialised university hospital clinic and controls were recruited by advertisement among the staff and students at the hospital and university. Plasma levels of Interferon (IFN)-γ, Interleukin (IL)-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17, Interferon gamma-induced protein (IP)-10, Monocyte Chemoattractant Protein (MCP)-1, Transforming Growth Factor (TGF)-β1, TGF-β2, TGF-β3 and Tumour Necrosis Factor (TNF)-α were analysed by multiplex. Differences between the three groups CFS, FM and controls, were analysed by Kruskal Wallis tests.

MCP-1 was significantly increased in both patient groups compared to healthy controls. IL-1β, Il-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL17 all were significantly lower in the patient groups than healthy controls. IFN-γ was significantly lower in the FM group. For IL-8, IL-10 and IL-1ra there were no significant difference when controlled for multiple testing.

In conclusion, in our material MCP-1 seems to be increased in patients both with CFS and with FM, while several other immune markers are significantly lower in patients than controls.

1. Introduction

Chronic fatigue syndrome (CFS) and fibromyalgia (FM) are challenging conditions affecting 0.5–8% of the population, with both socio-economic and personal burdens. Aetiologies of these conditions are not well understood (Singh et al., 2019; Yang et al., 2019b). The two syndromes are classified distinctly in ICD-10 and ICD-11. Diagnostic criteria for CFS in the majority of previous research is based on the 1994 Fukuda Criteria (Fukuda et al., 1994) and diagnosis of FM has until recently been based on the 1990 ACR criteria (Wolfe et al., 1990). Fatigue and widespread pain are common symptoms for most CFS and FM patients, where the emphasis on one of the symptoms, fatigue or pain, has been typical differentiating characteristic for CFS or FM, respectively, even though this paradigm has changed somewhat with the new SEID criteria (Institute of Medicine [IOM], 2015) for CFS and 2016 Fibromyalgia criteria (Wolfe et al., 2016).

Involvement of immunological mechanisms have been postulated in the pathology of CFS and FM patients (Coskun Benlidayi, 2019; Morris et al., 2019). The symptoms of CFS and FM mimic those seen in different inflammatory disorders (Jonsjo et al., 2020). Inflammation is related to cytokines and chemokines (immune markers) regulating the immune response. However, cytokines and chemokines can also influence behaviour and mental state (*e.g.* by inducing fatigue, depression, and hyperalgesia) (Hestad et al., 2009). Thus, comparing levels of immune markers in patients may indicate immune activity and even reveal pathological mechanisms in patients.

Although a few studies (Iacob et al., 2016; Light et al., 2012; Nakamura et al., 2010; Scully et al., 2010) have compared immunological

* Corresponding author. St. Olav's Hospital, AFFU, Postboks 3250 Torgarden, NO-7006, Trondheim, Norway.
E-mail address: nina.groven@ntnu.no (N. Groven).

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markers between CFS and FM, most studies focus on each disorder separately, and do not consider the heterogenicity in and overlap between the two conditions (Backryd et al., 2017; Yang et al., 2019b). Importantly, studies including levels of several immunological factors in larger samples are lacking.

We previously have reported a tendency ($p = .056$) towards increased plasma levels of TNF-α in a group of 20 CFS patients compared to 20 healthy controls (Groven et al., 2018). Furthermore, we have reported increased hsCRP in both FM and CFS in a larger group of patients compared to controls (Groven et al., 2019). Based on these findings, we therefore hypothesized a pro-inflammatory pattern in CFS and FM patients with elevated pro-inflammatory immune markers (like IFN-γ, IL-1β, IL-6, IL-8, Interferon gamma-induced protein (IP)-10, TNF-α, and MCP-1); lower levels of anti-inflammatory (like IL-1ra and IL-10), and regulatory (like IL-4, TGF-β1, TGF-β2, TGF-β3, and IL-17) immune markers.

2. Method

2.1. Sample population

2.1.1. Patient groups

As previously reported (Groven et al., 2019), patients were female, non-pregnant patients aged 18–60 years admitted to the Multidisciplinary Pain Centre at St. Olav's University Hospital, Norway. Patients were referred to this centre by general practitioners in Mid-Norway.

Each participant went through a comprehensive clinical examination and was thoroughly evaluated by an expert team of medical doctors, physiotherapists and psychologists. All patients were assessed by using the 1990 ACR (Wolfe et al., 1990) and the 1994 Fukuda criteria (Fukuda et al., 1994) as both were still used as diagnostic tools in the clinic during the recruitment period. FM patients ($n = 58$) were eligible if they fulfilled the 1990 ACR criteria (Wolfe et al., 1990). CFS patients ($n = 49$) were eligible if they fulfilled the Fukuda diagnostic criteria (Fukuda et al., 1994). Exclusion criteria were in accordance with diagnostic criteria including known inflammatory diseases.

2.1.2. Healthy controls

A group of 53 healthy females aged 18–60 years was consecutively recruited by advertising through websites among the staff of the Norwegian University of Science and Technology (NTNU) and St. Olav's University Hospital. Their health was assessed by conducting a structured medical history and by using questionnaires included in this study measuring the symptoms of CFS and FM (see 2.4 Questionnaires and 2.5 Interview).

2.2. Procedure, study design and ethics

The CFS patients were informed about the study by a letter sent by the hospital prior to or shortly after their clinical evaluation or given during their evaluation at the centre. The FM patients were given an information letter by the staff during the clinical examination. Both patient groups were then contacted by phone and asked for participation in this study by a member of staff, and an appointment was scheduled for those who accepted to join the study.

The study assessment lasted approximately 30–40 min and included an interview, questionnaires, and blood sampling. All data were collected by NG in the period from March 2015 to December 2016. The order of the assessments was random.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK 2014/711). Written informed consent was obtained from all participants.

2.3. Questionnaires

2.3.1. Hospital Anxiety and Depression Scale

The Hospital Anxiety and Depression Scale (HADS) is a validated, self-complete scale (Bjelland et al., 2002; Zigmond and Snaith, 1983) for monitoring depressive and anxiety symptoms. It is summarised as HADS-D (depression) and HADS-A (anxiety). The potential HADS sub-scores range from 0 to 21 with high scores being suggestive of more symptoms.

2.3.2. Chalder fatigue scale

The Chalder Fatigue Scale (Chalder et al., 1993; Loge et al., 1998) is used to evaluate (the severity of) fatigue in CFS patients. The total sum of each of the 11 items, scored on a 0–3 Likert scale, total score ranging from 0 to 33, is applied; higher scores imply more severe fatigue.

2.3.3. Pain – Numeric Rating Scale

A Numeric Rating Scale (NRS) was used to evaluate the subjective feeling of experienced pain on average (for the last week and is taken from the Brief Pain Inventory (Cleeland, 1991; Klepstad et al., 2002) which is a Likert scale ranging from 0 ("no pain") to 10 ("maximal possible pain").

2.3.4. Fibromyalgia Survey Diagnostic Criteria

Fibromyalgia Survey Diagnostic Criteria (FSDC) was used in this study for quality assessment of the ACR 1990 criteria used for inclusion. The FSDC is based on the Fibromyalgia Survey Questionnaire developed in 2010/2011 (Wolfe et al., 2010, 2011) and later revised in 2016 (Wolfe et al., 2016). FSDC is a self-report questionnaire used for diagnostics and classification in epidemiological studies. The FSDC consists of two sub-scales: Widespread Pain Index, scores 0–9; and Symptom Severity Scale, scores 0–12. Widespread Pain Index and Symptom Severity Scale are summarised into a third, score, *i.e.* the Fibromyalgia Severity (FS) score, ranging from 0 *(no symptoms)* to 31 (*most severe symptoms*) and indicate the severity of symptoms.

2.4. Interview and anthropometrics

For each participant, age, height, and weight as well as a structured clinical interview were recorded. History regarding infections, immune disorders, illness in general (somatic as well as psychiatric), medication, menstrual cycle, use of contraceptives, status of menopause, duration of illness (if applicable), and level of physical activity during the previous two weeks were recorded. The latter was scored on a scale from 1 (bedridden) to 4 (conducting regular exercise more than two times per week).

2.5. Blood sampling and analyses

There were no restrictions, such as fasting, dietary restrictions or use of medication, given prior to blood sampling. Samples were analysed at the clinical laboratory at St. Olav's Hospital, Trondheim. Samples were screened for deviating levels of white blood cells (WBC), CRP, and serology against mycoplasma pneumonia, borrelia burgdorferi, cytomegalo-, Epstein-Barr -, hepatitis B - and hepatitis C virus. Any sign of infection led to exclusion from the study.

Blood samples for immune markers Interferon (IFN)-γ, Interleukin (IL)-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17, Interferon gamma-induced protein (IP)-10, Monocyte Chemoattractant Protein (MCP)-1, Transforming Growth Factor (TGF)-β1, TGF-β2, TGF-β3 and Tumor Necrosis Factor (TNF)-α were collected in EDTA plasma tubes, immediately put on ice, centrifuged (1500g, 15 min, 4 °C), aliquoted into cryovials and frozen at −80 °C until further analyses.

Immune markers were analysed using multianalyte profiling Milliplex MAP assay (Millipore, Billerica, MA) with a Bio-plex 2000 and the Bio-plex manager software (Biorad, Hercules, CA). The percentages of samples that were below the detection limits ranged from 0 to 29%. All samples analysed had levels of IP-10, MCP-1, TNF-α, TGF-β1 and TGF-β2 above the manufacturer's detection limits (8.6 pg/mL, 1.9 pg/mL, 0.7 pg/mL, 3.9 pg/mL and 1.9 pg/mL, respectively). Detection limits for IFN-

Table 1
Descriptive of age, Body Mass Index (BMI), depression, anxiety, fatigue - and fibromyalgia scores in CFS, FM and controls.

Parameter	CFS				FM				Control				p^a
	(n = 49)				(n = 58)				(n = 53)				
	Missing (n)	*M (SD)*	*Mdn*	*Range (min– max)*	Missing (n)	*M (SD)*	*Mdn*	*Range (min– max)*	Missing (n)	*M (SD)*	*Mdn*	*Range (min– max)*	
Age	0	33.8 (11.3)	35.0	18–60	0	42.0 (9.1)	42.5	22–60	0	39.4 (10.4)	39.0	23–59	<.001
BMI	2	24.0 (3.6)	23.1	18.1–34.6	1	26.7 (5.6)	25.7	16.3–40.4	0	24.7 (4.0)	23.8	16.3–41.7	.017
HADS depression	0	6.0 (4.2)	5.0	0–17	1	6.4 (3.9)	6.0	0–16	1	1.3 (1.8)	1.0	0–8	<.001
HADS anxiety	0	5.9 (4.6)	5.0	0–19	1	8.4 (4.1)	8.0	0–17	1	3.2 (2.6)	3.0	0–10	<.001
Fatigue score	0	36.5 (5.3)	37.0	23–44	1	33.5 (5.3)	34.0	18–44	1	21.3 (3.2)	21.0	14–33	<.001
Pain NRS	0	3.9 (2.0)	4.0	0–9	0	6.0 (1.8)	6.0	0–10	2	0.9 (1.2)	1.0	0–5	<.001
FS score	0	15.2 (5.5)	13.0	7–29	1	20.1 (5.2)	20.0	3–30	1	3.1 (2.5)	3.0	0–11	<.001

Note: BMI = body mass index. HADS = Hospital Anxiety and Depression Scale. FS = Fibromyalgia Severity.
[a] Kruskall-Wallis test.

γ, IL-1β, IL-1ra, IL-4, IL-6, IL-8, TGF-β3, IL-10 and IL-17A were based on the lowest detected concentrations in our samples: 0.43 pg/mL, 0.05 pg/mL, 1.6 pg/mL, 0.31 pg/mL, 0.40 pg/mL, 0.05 pg/mL, 0.91 pg/mL, 1.15 pg/mL, 6.36 pg/mL, respectively. Samples below the detection limits for these nine cytokines were set to half of the detection limits (0.215 pg/mL, 0.025 pg/mL, 0.8 pg/mL, 0.155 pg/mL, 0.20 pg/mL, 0.025 pg/mL, 0.46 pg/mL, 0.58 pg/mL and 3.18 pg/mL, respectively). All analyses were performed according to the manufacturer's protocol.

2.6. Statistical analysis

The statistical analyses were performed using the Statistical SoftWare Package (SPSS) Statistics for Windows, version 22. All variables were tested for normality and homogeneity by using the Shapiro-Wilk tests and visual inspection of histograms and Q-Q-plots.

The data consisted of a considerable number of samples below the detection limit. Transformation of the data did not improve this bias, and the Kruskal-Wallis ranks test was applied for comparison between groups. Dunn's test was used for post-hoc analysis of pair-wise group comparisons. Associations between variables were analysed by Spearman's Rank-Order Correlation analyses (ρ). Confounding factors were defined as variables with significant associations of $p < .05$. A conservative approach was taken to account for multiple comparisons between groups, and these results were considered significant at $p < .01$. Results reaching levels of $p < .05$ were added for comparison purposes.

3. Results

3.1. Population

A total of 160 participants were included in this study, consisting of 49 CFS patients, 58 FM patients, and 53 healthy controls. The CFS patients in this study were younger than the FM and control group ($p < .001$ and $p = .010$, respectively). The FM group had higher BMI than the CFS and control groups ($p = .007$ and $p = .049$, respectively). Both patient groups had significantly higher HADS depression and anxiety scores, fatigue scores, pain scores (NRS) and FS scores compared to controls. The demographic and clinical characteristics of the sample population is summarised in Table 1.

3.2. Immune markers

Comparisons of cytokine levels between the three groups are shown in Table 2. The Kruskal-Wallis test showed significant group differences for 12 of the 14 cytokines: INF-γ, IL-1ra, IL-1β, IL-4, IL-6, MCP-1, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL-17A. Two cytokines did not show any significant group differences, *i.e.* IL-8 and IP-10.

Both patient groups had significantly lower plasma levels than controls for the following seven cytokines: IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, and TGF-β3. CFS and FM patients could not be distinguished between each other for these cytokines. Post-hoc Dunn's tests showed that for INF-γ, FM patients had significantly lower ranks than CFS patients and controls ($p < .001$), and CFS patients and controls could not be distinguished from each other ($p < .05$). FM patients also had significantly lower plasma levels compared to controls for IL-10 and IL-17A ($p < .001$), but the FM group was not different from CFS patients ($p = .220$; and $p = .339$, respectively) and CFS patients did not show any differences compared to controls ($p < .05$).

For MCP-1, both CFS and FM patients had significantly higher levels than the control group ($p < .001$). However, the patient groups could not be distinguished from each other ($p = .235$).

3.3. Confounding factors

3.3.1. Age

Age ranged from 18 to 60 years for the total study population. In the total study population, age was positively associated to levels of IP-10 and MCP-1 ($p = .002$, $\rho = 0.249$; and $p < .001$, $\rho = 0.301$).[1]

3.3.2. BMI

BMI was positively correlated with IL-6 ($p = .021$, $\rho = 0.158$), IL-8 ($p = .039$, $\rho = 0.165$), and MCP-1 ($p = .055$, $\rho = 0.154$) in the total population.[1]

Age and BMI were not associated with other immune markers for the total population sample.

3.3.3. Questionnaires

3.3.3.1. Anxiety and depression. For the whole study population, the HADS depression score showed a positive correlation with IP-10 and MCP-1 ($p = .008$, $\rho = 0.211$; and $p = .008$, $\rho = 0.212$), and negative correlation with IL-1β, IL-4, IL-6, TGF-β1, TGF-β2, TGF-β3, and TNF-α ($p = .010$, $\rho = -0.204$; $p < .001$, $\rho = -0.252$; $p = .010$, $\rho = -0.206$; $p = .001$, $\rho = -0.264$; $p < .001$, $\rho = -0.295$; $p = .001$, $\rho = -0.264$; and $p < .012$, $\rho = -0.199$ respectively). For the total study population, HADS anxiety scores showed a negative correlation with IL-4 and IL-6 ($p = .021$, $\rho = -0.184$; and $p = .023$, $\rho = -0.182$). When examining the groups of participants separately, only a positive correlation between HADS depression and IP-10 in the CFS group remained ($p = .048$, $\rho = 0.287$).[1]

3.3.3.2. Chalder fatigue scale. For the whole study population, levels of fatigue did not correlate with any of the immune markers. When studying the groups separately, the only significant finding was a negative correlation between fatigue levels and MCP-1 in the FM group ($p = .029$, $\rho =$

[1] Sub-groups analyses for age, BMI, HADS, FDSC, fatigue, pain and activity level are found in supplementary Table 1.

Table 2
Plasma levels of cytokines (pg/mL) in CFS, FM and healthy controls.

Parameter	CFS			FM			Control			p^b
	(n = 48)			(n = 58)			(n = 53)			
	M (SD)	*Mdn*	*IQR*[a]	*M (SD)*	*Mdn*	*IQR*[a]	*M (SD)*	*Mdn*	*IQR*	
INF-γ[c]	175.01 (879.68)	29.51	11.58–48.67	35.33 (90.19)	17.59	10.44–26.39	92.00 (318.18)	30.31	17.13–54.77	<.001*
IL-1ra	226.92 (1052.08)	20.40	0.80–125.46	113.74 (255.37)	25.39	0.80–83.88	135.73 (243.92)	61.10	11.30–123.75	.031
IL-1β[d]	28.08 (143.74)	4.23	0.56–6.68	3.86 (3.68)	2.75	0.93–6.16	10.53 (16.26)	7.07	4.25–11.66	<.001*
IL-4[d]	43.07 (219.89)	5.31	0.16–19.26	24.66 (96.43)	1.57	0.16–12.05	47.72 (48.56)	34.51	21.16–61.90	<.001*
IL-6[d]	13.69 (49.4)	2.28	0.20–7.05	5.22 (6.27)	3.45	0.20–7.14	13.35 (23.17)	8.04	4.90–15.16	<.001*
IL-8[e]	16.75 (61.55)	1.74	0.20–11.20	10.49 (23.27)	1.55	0.03–9.17	14.27 (28.33)	3.41	1.00–12.62	.205
IP-10[e]	382.69 (204.40)	334.80	275.63–438.63	381.84 (152.68)	326.63	284.48–443.32	374.76 (191.74)	310.43	264.84–437.66	.750
MCP-1[f]	221.13 (62.08)	210.78	187.83–241.11	209.97 (71.69)	202.62	169.89–235.42	190.17 (75.71)	183.25	160.36–198.26	<.001*
TNF-α[d]	37.28 (155.64)	14.81	10.20–19.32	13.37 (5.24)	12.66	9.79–17.47	22.95 (26.03)	18.59	13.87–24.95	<.001*
TGF-β1[d]	3806.11 (3007.46)	2783.11	1476.36–5632.41	3334.18 (2174.34)	2400.00	1491.95–5166.31	5436.44 (1899.64)	5650.65	4261.62–6675.55	<.001*
TGF-β2[d]	390.72 (156.38)	414.70	252.69–531.69	404.48 (146.21)	341.07	267.33–539.59	559.94 (116.38)	545.91	499.86–611.57	<.001*
TGF-β[d]	32.20 (32.68)	18.42	4.40–59.80	30.91 (27.85)	17.70	7.48–57.10	57.90 (30.46)	55.78	39.02–74.06	<.001*
IL-10[c]	19.50 (27.75)	15.32	7.28–22.98	14.50 (9.03)	13.56	6.85–20.08	19.20 (6.14)	18.56	15.65–22.55	.003*
IL-17A[c]	114.58 (92.66)	115.56	60.53–142.48	98.51 (43.06)	104.66	66.44–130.38	130.61 (40.12)	128.44	109.21–145.34	.002*

Note: *IQR* = inter-quartile range. *Significance $p < .01$.
[a] Values below the detection limit were set to half detection limit.
[b] Kruskall-Wallis test.
[c] FM has significant lower value compared to both CFS and controls.
[d] Both FM and CFS have significantly lower values than controls.
[e] No significant differences between any groups.
[f] Both FM and CFS have significantly higher values than controls.

−0.290).[1]

3.3.3.3. Pain NRS. There were negative associations between the subjective feeling of the average experienced pain for the past week (NRS) and, in addition to IL-8, the same immune markers that also were lower in both patient groups compared to controls (INF-γ, IL-1ra, IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL-17A; see section 3.), and a positive correlation between this NRS score and MCP-1. There were no associations between the subjective feeling of the average experienced pain for the past week (NRS) and any of the immune markers measured when analysing CFS, FM and controls separately.[1]

3.3.3.4. Fibromyalgia: ACR 1990 and FSDC. In the CFS group, 13 (26.5%) patients fulfilled the ACR 1990 criteria (Wolfe et al., 1990) while 21 (38.8%) patients fulfilled the new 2016 FSDC criteria (Wolfe et al., 2016). For the FM group, all patients fulfilled the ACR 1990 criteria by inclusion. Forty-nine (84.5%) of the FM patients also fulfilled the FSDC 2016 criteria. The sensitivity and specificity of how well the ACR 1990 criteria predicted the new FSDC 2016 criteria were both 0.88 in our population sample.

There were negative associations between the FS score and the same immune markers that also were lower in both patient groups compared to controls (INF-γ, IL-1ra, IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL-17A; see section 3.2), and a positive correlation between the FS score MCP-1.[1]

3.3.4. Interview

3.3.4.1. Menopause. Differences in IP-10 (p = .001, U = 487.0) and MCP-1 ($p < .001$, U = 379.0) were found before versus after menopause, with higher levels of both cytokines after menopause. Only 4 in the CFS group, 7 in the FM group and 5 control participants reported having reached menopause. No associations were seen for other cytokines.

3.3.4.2. Duration of illness. The time that had elapsed from onset of illness to the day of the interview was recorded in years. Patients were divided into those reporting having their illness lasting less than three years (short duration) (22%), and the those reporting having their illness lasting longer than three years (long duration) (78%). Shorter duration of illness for CFS patients had significantly lower levels compared to CFS patients with longer duration of illness for TNF-α, TGF-β1, TGF-β2, TGF-β3 and IL-10 ($p \leq .001$, for these cytokines). Comparing CFS patients with longer duration of illness to the control group, showed that CFS patients had lower levels compared to controls for TGF-β1, TGF-β2, and TGF-β3 (p = .005, $p < .001$, and p = .004, respectively), but these patients could no longer be distinguished from controls on TNF-α and IL-10 (p = .073 and p = .673, respectively).

There were no differences between FM patients with short or long duration of illness for any immune markers measured in this study.

3.3.4.3. Activity level. A negative association was found between higher levels of activity and MCP-1 (p = .005, ρ = −0.223) for the total study population.[1]

3.3.4.4. Other. No associations were found between immune markers and smoking, type and use of medication, use of birth control and stage of menstrual cycle.

4. Discussion

The two patient groups (CFS and FM) had significantly lower circulating levels compared to healthy controls for the following nine cytokines: IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL-17A, and the two patient groups could not be distinguished from each other. INF-γ was significantly lower in the FM group compared to both CFS and controls. No significant group differences were observed for the circulating levels of IL-8 and IP-10.

4.1. MCP-1

In the current study of several plasma immune markers, only pro-

inflammatory MCP-1 was significantly increased in both patient groups, CFS and FM, compared to healthy controls.

MCP-1 (CCL2) is a potent pro-inflammatory chemokine increasing inflammation by directing migration and infiltration of monocytes/macrophages to the site of activity (Deshmane et al., 2009). Though MCP-1 is central in inflammation, including neuroinflammation (Conductier et al., 2010) there are few studies on the role of circulating MCP-1 in FM and CFS.

The increased MCP-1 levels for FM patients in our study is in accordance with a study by Zhang et al. (2008) where plasma MCP-1 levels were increased in 92 FM patients compared to 48 healthy controls. Similarly, *ex vivo* MCP-1 release by blood monocytes from 25 FM patients was increased compared to release from monocytes from 20 controls (Bote et al., 2012). Pain is a central symptom in FM, and MCP-1 is involved in pain processing and pain sensitivity in the central nervous system (Rodriguez-Pinto et al., 2014) and MCP-1 is reported to enhance excitability of nociceptive neurons (Sun et al., 2006). In a study by Bote et al. (2012) MCP-1 was increased along with the pro-inflammatory marker CRP in 25 patients with FM. In line with this, we have previously described increased hsCRP in the same patient sample population (Groven et al., 2019).

The finding of increased levels of MCP-1 in CFS patients in our study is in contrast to Wyller et al. (2015) not finding any difference in MCP-1 when comparing adolescent CFS patients to controls. However, adolescent patients with CFS may be different from the adult population we examined and to our knowledge there are few studies on MCP-1 in CFS, the field needs further exploration.

Monocyte/macrophage production of MCP-1 and hepatocyte CRP production are both stimulated by the same pro-inflammatory cytokines IFN-γ, IL-1β, IL-6 and TNF-α. As both CRP and MCP-1 are increased in patients in our study, one might expect IFN-γ, IL-1β, IL-6 and TNF-α to be increased too. However, they were not, and the explanation for this is not obvious.

4.2. Other immune markers

IFN-γ was lower in FM than for CFS and controls, CFS and controls no differing significantly. In line with this, most studies done on CFS patients found no differences between patients and control groups for IFN-γ (Blundell et al., 2015). Contradicting our findings, an increase of IFN-γ has been reported in FM patients (Behm et al., 2012).

CFS and FM patients had lower levels compared to controls for pro-inflammatory IL-1β, IL-6 and TNF-α. Some studies support our findings of lower plasma IL-1β in FM patients (Ernberg et al., 2018), lower plasma IL-6 levels in CFS patients (Horrnig et al., 2015) and FM patients (Ernberg et al., 2018), and decreased serum TNF-α in FM patients (Hernandez et al., 2010). However, conflicting reports exist for IL-1β, IL-6 and TNF-α in both FM patients (Uceyler et al., 2011) and CFS patients (Blundell et al., 2015; Lyall et al., 2003). Also, we found a tendency towards increased plasma TNF-α in CFS patients (Groven et al., 2018).

Anti-inflammatory IL-10 levels were reduced in FM patients compared to healthy controls in our study. This also has been reported by others (Behm et al., 2012).

Cytokines often regarded as regulatory (IL-17A, IL-4, TGF-β1, TGF-β2 and TGF-β3) were lower in both CFS and FM patients compared to the healthy control group in our study. Interestingly, in the CFS group, only CFS patients with short duration of illness differed significantly from controls in these cytokines. To our knowledge studies on this are scarce and results are conflicting (Blundell et al., 2015; Uceyler and Hauser, 2011).

We hypothesized an increased inflammatory state in CFS and FM patients compared to controls. We have reported increased pro-inflammatory MCP-1 but the four pro-inflammatory cytokines IFN-γ, IL-1β, IL-6 and TNF-α were significantly reduced in our patient population, and not higher, as might be expected given the increased CRP and MCP-1. This may appear a paradox. However, it indicates that the mechanisms are complex and may be related to other factors than diagnoses alone and that these molecules are indirectly or secondarily associated to the pathogenesis of the disorder. Also, these cytokines are not mutually exclusive, for example will upregulated production of MCP-1 by IFN-γ also increase the production of IL-4 (Murphy, 2008). We also found reduced levels in CFS and FM compared to controls for the anti-inflammatory cytokine IL-10, which is in line with a pro-inflammatory state in these disorders. The lower levels of regulatory cytokines (IL-17A, IL-4, TGF-β1, TGF-β2 and TGF-β3) found in plasma of patients with FM and short duration of illness in CFS, could suggest that this regulatory influence is diminished, leading to further imbalances in the immune system of these patients. However, subscribing a strict role of these cytokines in CFS and FM should be taken cautiously, and more studies are needed.

We attempted to discriminate CFS from FM to explores similarities and differences in immune activity in the two groups. There is an overlap between FM and CFS. Only five FM patients (9%) could be considered fibromyalgia cases without fatigue. Yet, the screening of FM patients as part of the diagnostic evaluation in the clinic concluded that these patients did not fulfil the Fukuda et al., (1994) CFS criteria for diagnosis. Similarly, 28% of the CFS patients in our study also were diagnosed with fibromyalgia according to the ACR (1990) criteria.

Sub-dividing the patient group further and comparing the groups pure FM ($n = 58$), CFS without FM ($n = 36$) and those with a combination of FM and CFS ($n = 13$), did not show any difference between the three groups for any of the cytokines apart from IFN-γ. For IFN-γ the patients with FM in either group differed from patients without FM. This supports our finding that IFN-γ is different for FM symptomology.

The main aim and finding in the present study was to compare suffering patients from healthy controls. The field is constantly being explored and some features of CFS and FM are not strictly distinct, and both categories may well be heterogeneous and include other related disorders with unknown aetiology. Thus, the findings still are interesting.

4.3. Confounding factors

Several potential confounding factors were tested. Increased MCP-1 was correlated with increased age, BMI, and score on HADS depression scale in subgroups as well as total population and decreased activity in CFS. Levels of regulatory cytokines like TGF and IL-4 were negatively associated with HADS depression score and with short duration of illness in CFS. Increased IP-10 was seen after menopause. No associations were seen between levels of cytokines and fatigue, pain, smoking, type and use of medication, use of birth control and stage of menstrual cycle.

Age and BMI could influence the results, as both high age and BMI are associated with higher inflammation (Poledne et al., 2009; Rea et al., 2018). In our study the CFS group was younger than both FM and controls; and CFS patients and controls had lower BMI than the FM group. This pattern does not fit with age and BMI being responsible for group differences. Also, the patient groups with high MCP-1 had lower levels of other proinflammatory cytokines making the hypotheses general inflammation less likely.

MCP-1 might be related to degree of severity of disorder, for which activity may be an indicator. In line with this the most inactive CFS patients (only move/walk to conduct core tasks) also had the highest levels of MCP-1 compared to the active patients. The control group still having significantly lower levels of MCP-1 than both active and inactive patients (data not shown). Maes et al. (2012) emphasize that there is a chronic fatigue spectrum, suggesting a model with three categories with a continuum of increasing severity of illness. This indicates that the severity of illness should be taken into account in studies on CFS and related conditions.

Like others (Hornig et al., 2015) we found certain differences in expression of cytokines in CFS patients with short and long duration of CFS. CFS patients with an illness duration less than three years had significantly lower levels of TNF-α and IL-10 in our study, while CFS

lasting more than 3 years could not be distinguished from controls regarding these cytokines. This difference in short and long lasting disorder is described by others (Broderick et al., 2010; Hornig et al., 2015). Thus, duration of illness and treatment should be taken into account when looking at immune deviations for these disorders.

4.4. Limitations

The lack of validated standard measures for cytokines and chemokines is a constant challenge in studies like ours. However, though kits of reagents vary in sensitivity, the relative concentrations/patterns between individuals tested in the same kit is valid. All immune marker samples in this study were analysed by one experienced person in the same lab, using the same assay, and run at the same time. Hence, the cytokine and chemokine pattern for each sample should not be affected.

The groups were not age-matched, and associations between immune markers and age were found. Due to the distribution of our data (samples below the detection limits), the possible confounding factors influencing immune marker levels could not be controlled for, and were only reported as significant associations, thus leading to possible bias in our results.

The diagnostic groups FM and CFS are purely clinically based and with no objective paraclinical measures, and the recruitment of patients from a university specialist clinic only may not be representative for a patient population cohort. However, the patients were referred to this clinic from general practitioners in the primary health care, and we have used strict diagnostic criteria by a specially trained group of specialists in a specialised chronic fatigue and pain centre and this reduces this possible limitation as much as possible. The control group consisted of mainly hospital and university staff, which may not in all aspects represent the general population.

The size of the groups might be larger – especially in the field of deviating findings. However, our groups are large compared to most other studies with these strict inclusion criteria and were based on power calculations.

Another objection is the selection of cytokines and chemokines. It was based on previous reports, available tests and an attempt to cover a broad array of "immune arms". The role of inflammation and cytokines/chemokines in the immune system as well as other systems like nerve systems is not at all fully understood, thus the relevance of all markers is not absolute. However, this goes for all studies in this field at the moment.

Our study, based on 107 patient samples and 53 healthy control test samples, is a relatively large study in comparison to similar research in this field. The size of the groups, the strict diagnostic criteria and the high competency at the lab where all samples are run at the same time with the same equipment and the same person are all strengths of the study, as well as the competency of the group in the clinic and laboratory. Also, the Caucasian population in this study is rather homogenous with a rather good and homogenous health, status of living etc.

4.5. Further research

Clinical overlap of the two diagnoses CFS and FM is well established (Clauw, 2010) and both diagnostic groups probably are heterogenous. Our findings support the hypothesis that CFS and FM share some overlapping immunological similarities. The inconclusive findings in other studies is in line with this. However, so far it cannot be ruled out that immune activity is related to aetiology or pathogenesis of the conditions. If there is an aetiological or pathological function of immune markers in CFS and FM prevention and treatment (*e.g.* blocking MCP-1 activity) could be beneficial to these patients. Substances blocking MCP-1 are under testing for treatment of neuroinflammation, cardiovascular inflammation (Franca et al., 2017), inflammatory disorders (Zoja et al., 2015) and might thus even be of interest in FM and CFS if our findings of increased MCP-1 are confirmed and found to have a function in pathogeneses. Thus, the field should be further explored.

5. Conclusion

There were increased levels of MCP-1 in both patient groups, *i.e.* findings in line with previously reported increase in hsCRP in our study population. However, it was unexpected that several other immune markers measured were significantly lower for the same patients. The CFS and FM patient groups were significantly lower than controls in plasma levels for IL-1β, IL-4, IL-6, TNF-α, TGF-β1, TGF-β2, TGF-β3, IL-10 and IL-17A. However, solely based on plasma samples for these immune markers, there were no differences between the CFS and FM patient groups, all together supporting the assumption that these two disorders show overlapping features.

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Declaration of competing interest

All the authors declare no conflict of interest.

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Appendix A. Supplementary data

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Kynurenine metabolites and ratios differ between Chronic Fatigue Syndrome, Fibromyalgia, and healthy controls

Nina Groven [a,b,*], Solveig Klæbo Reitan [a,b], Egil Andreas Fors [c], Ismail Cuneyt Guzey [a,b]

[a] Department of Mental Health, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[b] Department of Mental Health, St. Olav's University Hospital, AFFU, Trondheim, Norway
[c] Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

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ABSTRACT

Background: There is growing evidence that the kynurenine pathway is involved in the pathology of diseases related to the central nervous system (CNS), because of the neuroprotective or neurotoxic properties of certain metabolites, yet the role of each metabolite is not clear. The pathology of Chronic Fatigue Syndrome (CFS) and Fibromyalgia (FM) is currently under investigation, and the overlapping symptoms such as depression suggest that the CNS may be involved. These symptoms may be driven by enhanced neurotoxicity and/or diminished neuroprotection. However, the kynurenine metabolite status has not been well studied in these two possible related disorders of CFS and FM.

The objective of this study was to investigate the metabolites and ratios of the kynurenine pathway in CFS and FM compared to healthy controls and examine the possible correlations with symptoms of anxiety and depression.

Method: In this study, females aged 18–60 were included: 49 CFS patients; 57 FM patients; and 54 healthy controls. Blood plasma was analysed for the following metabolites involved in the kynurenine pathway: Tryptophan, kynurenine, kynurenic acid (KA), 3-hydroxykykynurenine (HK), anthranilic acid, xanthurenic acid (XA), 3-hydroxyanthranilic acid, quinolinic acid (QA) and picolinic acid. The concentrations of these metabolites, as well as the ratios of different metabolites indicating enzymatic activity, were compared between the groups. Findings were controlled for age, body mass index (BMI), and symptoms of anxiety and depression.

Results: QA differed between CFS and FM patients ($\beta = .144$, $p = .036$) and was related to higher levels of BMI ($\beta = .017$, $p = .002$). The neuroprotective ratio given by KA/QA was lower for CFS patients compared to healthy controls ($\beta = -.211$, $p = .016$). The neuroprotective ratio given by KA/HK was lower for FM patients compared to healthy controls, and this lower neuroprotective ratio was associated with increased symptoms of pain. The kynurenine aminotransferase II (KAT II) enzymatic activity given by XA/HK was lower for FM patients compared to healthy controls ($\beta = -.236$, $p = .013$). In addition, BMI was negatively associated with enhanced KAT II enzymatic activity ($\beta = -.015$, $p = .039$). Symptoms of anxiety and depression were not associated with the metabolites or ratios studied.

Conclusion: Our study indicates associations between kynurenine metabolism and CFS and FM as well as characteristic symptoms like fatigue and pain. Forthcoming studies indicating a causative effect may place kynurenine metabolites as a target for treatment as well as prevention of these conditions in the future.

1. Introduction

Kynurenines are suggested to play a central role in psychiatric diseases such as depression (Branchi et al., 2020). Symptoms of depression are frequently accompanying Chronic Fatigue Syndrome (CFS) and Fibromyalgia (FM) (Groven et al., 2019). Thus, exploring kynurenines also in CFS and FM is of interest. CFS and FM are two related disorders with unknown pathology (Clauw and Chrousos, 1997; Rasouli et al., 2019). Both disorders are common, with prevalence ranging from 0.5% to 2.5% for CFS (Estévez-López et al., 2020) and up to 5% for FM (Heidari et al., 2017). The personal burden of individuals struggling to maintain daily tasks and activities added to the high cost on society, through its strain

* Correspondence to: Department of Mental Health, St. Olav's University Hospital, AFFU, Postboks 3250 Torgarden, 7006 Trondheim, Norway.
E-mail address: nina.groven@ntnu.no (N. Groven).

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on the work force (Global Burden of Disease Study (GBD) 2013, 2015), increase the significance of these conditions. Although their aetiology is unclear, there are several indications of disturbed immunological responses in both CFS and FM (Anderson et al., 2014; Coskun Benlidayi, 2019; Groven et al., 2019, 2020).

Kynurenines are the metabolites from the kynurenine pathway, following the breakdown of tryptophan (Try), through a cascade involving several enzymes. Immune activity is known to affect the kynurenine metabolic pathway and has been suggested to play a role in the pathophysiological mechanisms of both CFS and FM (Blankfield, 2012; Anderson et al., 2014, 2018). We have previously reported increased C-reactive protein (CRP) in CFS and FM patients (Groven et al., 2019). The upregulation of enzymatic activity in the kynurenine pathway follows increased inflammation (Dantzer et al., 2008). Specific pro-inflammatory cytokines activate the enzyme indoleamine 2–3-dioxygenase (IDO), enhancing Try conversion into Kyn and its metabolites at the cost of serotonin production. Serotonin is involved in many central mechanisms, ranging from sleep regulation to digestion, and is a key neurotransmitter in depression. Some metabolites of the kynurenine pathway are considered either neurotoxic (quinolinic acid [QA]) or neuroprotective (kynurenic acid [KA]), as they act as agonists (neurotoxic) or antagonists (neuroprotective) in glutamate nerve transmission (Colin-Gonzalez et al., 2013, Schwarcz and Stone, 2017). A shunt towards of Try breakdown into the kynurenine pathway and increased neurotoxic metabolites may affect fatigue, pain (Rojewska et al., 2018), and depression (Ogyu et al., 2018, Branchi et al., 2020) and explain mechanisms behind syndromes involving these symptoms, all of which are commonly found in CFS and FM.

In depression the neuroprotective ratios (KA/QA and KA/HK) are decreased compared to healthy controls (Ogyu et al., 2018). This is likely caused by variations in the activity of the enzymes involved, which may tip the balance into more neurotoxic products and effect the neuropsychiatric outcomes.

The importance of the neurotoxic metabolites of kynurenine in psychiatry are conceivable but we know very little about their effects neither directly nor indirectly on CFS and FM. The upregulation of enzymatic activity in the kynurenine pathway and potentially decreased availability of serotonin, coupled with the frequent comorbidity of depression in CFS and FM patients may suggest a shared background and requires a deeper investigation. The kynurenine pathway has been poorly studied in these patient groups, and studies comparing both CFS and FM are lacking. To investigate this, we conducted a study comparing kynurenine metabolites between patients with CFS and FM with healthy controls.

The present study on kynurenine and its metabolites in CFS and FM is part of a larger study on defined subgroups of patients with CFS and FM (Groven et al., 2019, 2020).

In the present paper we explore levels of kynurenines in the three groups CFS, FM and healthy controls. The hypothesis of this study was that patients with CFS and patients with FM have altered levels and ratios of tryptophan and its metabolites in the kynurenine pathway compared to healthy controls. A relation to symptoms of depression and anxiety was suggested. Confounders such as age, BMI anxiety and depression were explored.

[Fig. 1 Illustrates the kynurenine pathway with the metabolites used in this study.].

2. Method

2.1. Sample population

2.1.1. Patient groups

As previously reported (Groven et al., 2019), female patients aged 18–60 years admitted to the Multidisciplinary Pain Centre at St. Olav's University Hospital, Norway, found to qualify for the diagnoses CFS and FM were eligible for the study. Patients with challenging clinical pictures regarding problems such as CFS and FM are referred to this centre by general practitioners in Mid-Norway.

Each participant went through a comprehensive clinical examination and was thoroughly evaluated by an expert team of medical doctors, physiotherapists, and psychologists for inclusion- and exclusion criteria. FM patients ($n = 58$) were diagnosed by using the 1990 ACR criteria (Wolfe et al., 1990). CFS patients ($n = 49$) were diagnosed according to the CDC/Fukuda criteria (Fukuda, Straus et al., 1994). Exclusion criteria were in accordance with diagnostic criteria including known inflammatory diseases.

2.1.2. Healthy controls

A group of 53 healthy females aged 18–60 years was consecutively recruited by advertising through websites among the staff of the Norwegian University of Science and Technology (NTNU) and St. Olav's University Hospital. Their health was assessed by taking a structured medical history and by questionnaires evaluating the symptoms of CFS and FM (see 2.4 Questionnaires and 2.5 Interview).

2.2. Procedure

The CFS patients were informed about the study by a letter sent by the hospital prior to or shortly after their clinical examination or during their evaluation at the centre. The FM patients were given an information letter by the staff during the examination and evaluation of their FM diagnosis. Both patient groups were then contacted by phone by a member of staff and invited to participate in the study, and an appointment was scheduled for those who accepted to join.

2.3. Study design and ethics

The assessment lasted 30–40 min and included an interview, questionnaires, and blood sampling as formerly defined by Groven et al. (2019) in the period from March 2015 to December 2016. The study was

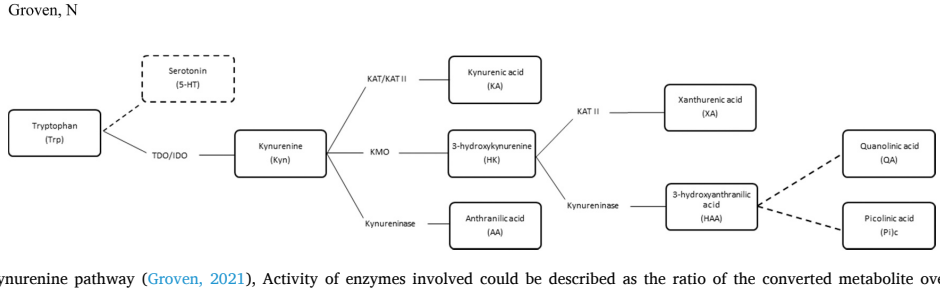

Fig. 1. The Kynurenine pathway (Groven, 2021), Activity of enzymes involved could be described as the ratio of the converted metabolite over the previous metabolite, Enzymes involved are: TDO = Tryptophan 2,3-dioxygenase. IDO = Indoleamine 2,3-dioxygenase. KAT = Kynurenine aminotransferase. KMO = Kynurenine 3-monooxygenase.

approved by the Regional Committee for Medical and Health Research Ethics (REK no. 2014/711). Written informed consent was obtained from all participants.

2.4. Questionnaires

The Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith, 1983; Bjelland et al., 2002) was used for symptoms of anxiety and depression. This scale divided into HADS-D (depression) and HADS-A (anxiety) sub-scores of 0–14, with higher scores indicating more severe symptoms.

The Chalder Fatigue Scale (Chalder et al., 1993; Loge et al., 1998) is used to evaluate (the severity of) fatigue in CFS patients. The total sum of each of the 11 items, scored on a 0–3 Likert scale, total sum ranging from 0 to 33, is applied; higher scores imply more severe fatigue.

A Numeric Rating Scale (NRS) was used to evaluate the subjective feeling of experienced pain on average in the last week. NRS is taken from the Brief Pain Inventory (Cleeland, 1991; Klepstad et al., 2002) which is a Likert scale ranging from 0 ("no pain") to 10 ("maximal possible pain").

The Fibromyalgia Survey Diagnostic Criteria (FSDC) is a self-report questionnaire that is used for diagnostics and classification in clinical and epidemiological studies (Wolfe et al., 2016; Fors et al., 2020). The FSDC consists of two sub-scales: Widespread Pain Index (WPI), scores 0–19; and Symptom Severity Scale (SSS), scores 0–12. WPI and SSS are summarised into a third score, i.e. the Fibromyalgia Severity (FS) score, ranging from 0 (*no symptoms*) to 31 (*most severe symptoms*) and indicate the severity of symptoms.

2.5. Interview

For each participant, age, height, and weight were recorded mainly by self-report, and a structured clinical interview was performed. History regarding infections, immune disorders, illness in general (somatic as well as mental), medication, menstrual cycle, menarche, use of contraceptives, status of menopause, duration of illness (if applicable), smoking or other nicotine use, and level of physical activity during the previous two weeks were recorded. The latter was scored on a scale from 1 (bedridden) to 4 (conducting regular exercise more than two times per week).

2.6. Blood sampling and analyses

There were no restrictions regarding fasting, medication or caffeine intake given prior to blood sampling. Plasma and serum samples for all study participants were collected and sent to the St. Olav's University Hospital clinical laboratories for further analysis. The samples were screened for deviating white blood cells, hsCRP, and serology against mycoplasma pneumonia, borrelia burgdorferi, cytomegalo-, Epstein-Barr -, hepatitis B - and hepatitis C virus, and total plasma IgE. Any sign of infection led to exclusion from the study.

Blood samples for tryptophan (Try) and its metabolites kynurenine (Kyn), kynurenic acid (KA), 3-hydroxykynurenine (HK), anthranilic acid (AA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (HAA), quinolinic acid (QA) and picolinic acid (Pic) (Box 1) were collected in EDTA plasma tubes, immediately put on ice, centrifuged (1500 g, 15 min, 4 °C) and aliquoted into cryovials and frozen at − 80 °C until further analyses. The frozen samples were shipped to Bevital AS, Bergen, Norway, and analysed according to the company's protocols (bevital.no) by the means of liquid chromatography/tandem mass spectrometry (LC-MS/MS). The ratios between the metabolites could expresses the different breakdown-indexes in the kynurenine pathway, or the ratios between neuroprotective and neurotoxic metabolites. The metabolite ratios calculated were: Kyn/Try, KA/Kyn, XA/HK, HK/Kyn, AA/Kyn and HAA/HK; which involved the following neuroprotective indexes: KA/QA and KA/HK (Box 1).

2.7. Statistical analysis

The statistical analyses were performed using the Statistical Software package IBM Statistics (SPSS) for Windows, version 22. The criteria for using parametric statistics were met when all variables were transformed into natural log (ln), i.e. lnTry, lnKyn, lnKA, lnHK, lnAA, lnXA, lnHAA, lnQA, and lnPic, and these ln-values were used throughout this

Box 1
Abbreviations of kynurenine metabolites and ratios.

Metabolites:

Try	Tryptophan.
Kyn	Kynurenine.
KA	Kynurenic acid.
HK	3-hydroxykynurenine.
AA	Anthranilic acid.
XA	Xanthurenic acid.
HAA	3-hydroxyanthranilic acid.
QA	Quinolinic acid.
Pic	Picolinic acid.

Metabolite ratios (corresponding enzyme / ratio)[1]:

Kyn/Try	(TDO/IDO).
KA/Kyn	(KAT / KAT II).
XA/HK	(KAT II).
HK/Kyn	(KMO).
AA/Kyn	(Kynureninase).
HAA/HK	(Kynureninase).
KA/QA	(NPR1).
KA/HK	(NPR2).

[1]TDO = Tryptophan 2,3-dioxygenase. IDO = Indoleamine 2,3-dioxygenase. KAT = Kynurenine aminotransferase. KMO = Kynurenine 3-monooxygenase. NPR = Neuroprotective ratio.

study. The metabolite ratios were the ratios between one log transformed metabolite over another log transformed metabolite: [lnKyn]/[lnTry], [lnKA]/[lnKyn], [lnXA]/[lnHK], [lnHK]/[lnKyn], [lnAA]/[lnKyn] [lnHAA]/[lnHK], [lnKA]/[lnQA], and [lnHK]/[lnKA].

A linear regression model (model 1) with the covariates age, BMI, HADS-A and HADS-D was applied, serving as the basis model in this study (Box 2).

When the group showed significant effects on the kynurenine pathway metabolites or the ratios, an additional model (model 2) was applied. Model 2 included the co-factors of model 1 (age BMI, HADS-A and HADS-D scores), and adding the co-factors: fatigue scores, pain scores, FS scores, use of nicotine/smoking, and allergy (natural log-transformed [ln] total plasma concentration of IgE) (Box 2).

Student's t-test was used for post-hoc pair-wise comparison between the CFS, FM and control groups. Significance levels were set to $p < .05$.

3. Results

3.1. Population

A total of 160 participants were included in this study, consisting of 49 CFS patients, 58 FM patients, and 54 healthy controls. The demographic of the sample population is summarised in Table 1. The CFS patients in this study were significantly younger than the FM and control group ($p < .001$ and $p = .010$, respectively). The FM group had significantly higher BMI than the CFS and control group ($p = .007$ and $p = .049$, respectively). Both patient groups had significantly higher HADS-A scores, HADS-D scores, fatigue scores and FS scores compared to controls. There were less nicotine users (29%) than non-users (71%) in the total population, but more nicotine users in the two patient groups CFS ($n = 16$) and FM ($n = 22$) than in the control group ($n = 8$).

3.2. Tryptophan and the kynurenine pathway

3.2.1. Metabolites

3.2.1.1. Quinolinic acid (QA). Group differences were found for quinolinic acid (QA), where the CFS group had significantly higher levels compared to the FM group ($\Delta R^2 = .029$, $\beta = .114$, SE = 0.054, $t(153) = 2.11$, $p = .036$, overall adjusted $R^2 = .078$). Neither CFS nor the FM differed from controls (Table 2). BMI had an effect on QA ($\Delta R^2 = .094$, $\beta = .017$, SE = 0.004, $t(153) = 3.90$, $p < .001$). In the second model, BMI still had an effect on QA ($\Delta R^2 = .068$, $\beta = .014$, SE = 0.004, $t(149) = 3.18$, $p = .002$, overall adjusted $R^2 = .096$). Nicotine use also had an effect on the differences in QA levels ($\Delta R^2 = .029$, $\beta = -.091$, SE = 0.046, $t(149) = -2.05$, $p = .043$). (Supplementary Table 1.).

3.2.1.2. Anthranilic acid (AA). Group differences were found for anthranilic acid (AA), where the CFS group had lower levels compared to controls ($\Delta R^2 = .049$, $\beta = -.145$, SE = 0.073, $t(152) = -1.98$, $p = .049$, overall adjusted $R^2 = .026$). This effect disappeared in model 2 (Supplementary Table 2), where fatigue and nicotine had effect on AA ($\Delta R^2 = .036$, $\beta = -.014$, SE = 0.006, $t(148) = -2.26$, $p = .026$; and $\Delta R^2 = .070$, $\beta = -.185$, SE = 0.058, $t(148) = -3.20$, $p = .002$; adjusted $R^2 = .159$). The CFS patients could not be distinguished from the FM group, nor could the FM patients be distinguished from the control group (Table 2).

None of the other metabolites in the kynurenine pathway (Try, Kyn,

Box 2
Overview of the two statistical regression models used in this study.

Model 1:

Dependent variable: Metabolite / Metabolite ratio.

Fixed factor: Group variable (CFS, FM, healthy controls).

Co-factors:

1) Age
2) BMI
3) HADS-A
4) HADS-D

Model 2:

Dependent variable: Metabolite / Metabolite ratio.

Fixed factor: Group variable (CFS, FM, healthy controls).

Co-factors:

1) Age
2) BMI
3) HADS-A
4) HADS-D
5) fatigue scores
6) pain scores
7) FS scores (fibromyalgianess)
8) Nicotine
9) IgE (allergy)

[1),2)]Variables included because of differences between the diagnostic groups in this study (and their assumed relationship with inflammation). [3),4)]Variables included based on the hypothesis of this study. [5),6),7)]Variables included based on primary symptoms in CFS and FM. [8),9)]Variables included based on the possibility of their relationship with inflammation.

Table 1
Descriptives of age, Body Mass Index (BMI), depression and anxiety scores in CFS, FM and controls.

	CFS				FM				Control				
	(*n*=49)				(*n*=58)				(*n*=53)				
Parameter	Missing (*n*)	*M (SD)*	*Mdn*	*Range (min– max)*	Missing (*n*)	*M (SD)*	*Mdn*	*Range (min– max)*	Missing (*n*)	*M (SD)*	*Mdn*	*Range (min– max)*	*p*
Age	0	33.8 (11.3)	35.0	18–60	0	42.0 (9.1)	42.5	22–60	0	39.4 (10.4)	39.0	23–59	< .001[a]
BMI	2	24.0 (3.6)	23	18.1–34.6	1	26.7 (5.6)	26	16.3–40.4	0	24.7 (4.0)	24	16.3–41.7	.017[a]
HADS depression	0	6.0 (4.2)	5.0	0–17	1	6.4 (3.9)	6.0	0–16	1	1.3 (1.8)	1.0	0–8	< .001[a]
HADS anxiety	0	5.9 (4.6)	5.0	0–19	1	8.4 (4.1)	8.0	0–17	1	3.2 (2.6)	3.0	0–10	< .001[a]
Fatigue score	0	36.5 (5.3)	37	23–44	1	33.5 (5.3)	34	18–44	1	21.3 (3.2)	21	14–33	< .001[a]
FS score	0	15.2 (5.5)	13	7–29	1	20.1 (5.2)	20	3–30	1	3.1 (2.5)	3	0–11	< .001[a]
lnIgE[b]	0	3.19 (1.58)	3.22	0–7.84	2	3.31 (1.41)	3.37	0.69–7.39	1	3.20 (1.40)	2.94	0.69–7.26	.895[c]

Note: BMI = body mass index. HADS = Hospital Anxiety and Depression Scale (0–21, respectively). FS = (0–31) Fibromyalgia Severity. Fatigue = Chalder Fatigue Score (0–33).

[a] Kruskall-Wallis test.

[b] ln-transformed concentration of total plasma IgE.

[c] One-way ANOVA.

KA, HK, XA, HAA, and Pic) showed any difference between CFS, FM and controls in model 1 (controlling for age, BMI, and HADS scores).

3.2.2. Ratios

3.2.2.1. XA/HK (KAT II enzymatic activity). Group differences were found for the ratio between xanthurenic acid and 3-hydroxyanthranilic acid [lnXA]/[lnHK], where the FM group showed lower value than both the control group ($\Delta R^2 = .041$, $\beta = -.236$, SE = 0.094, $t(152) = -2.508$, $p = .013$; overall model adjusted $R^2 = .113$), and CFS patients ($p = .032$). The CFS group could not be distinguished from the control group (Table 2). Age, HADS-A and HADS-D did not affect the ratio. However, BMI had an effect, although no longer significant, on [lnXA]/[lnHK] ratio ($\Delta R^2 = .029$, $\beta = -.015$, SE = 0.007, $t(152) = -2.08$, $p = .039$). In model 2, the group effect disappeared. BMI still had an effect, although no longer significant, on [lnXA]/[lnHK] ($\beta = -.013$, SE = 0.008, $t(148) = -1.74$, $p = .084$; overall model adjusted $R^2 = .151$). Also in the second model, pain scores and nicotine could explain a significant proportion of the [lnXA]/[lnHK] ratio ($\Delta R^2 = .055$, $\beta = -.059$, SE = 0.021, $t(148) = -2.81$, $p = .006$; and $\Delta R^2 = .034$, $\beta = -.170$, SE = 0.078, $t(148) = -2.19$, $p = .030$). (Supplementary Table 3).

3.2.2.2. KA/QA (neuroprotective ratio 1). Group differences were found for the neuroprotective ratio between kynurenic acid and quinolinic acid [lnKA]/[lnQA], where the CFS group had lower levels compared to the control group ($\Delta R^2 = .039$, $\beta = -.211$, SE = 0.086, $t(153) = -2.44$, $p = .016$; overall model adjusted $R^2 = .045$). The CFS group could not be distinguished from the FM group, nor could FM patients be distinguished from the control group (Supplementary Table 3). Neither age, BMI, HADS-A nor HADS-D had any effects on [lnKA]/[lnQA] in this model. The group effect disappeared in model 2 ($\beta = -.058$, SE = 0.134, $t(149) = -0.43$, $p = .665$; overall model adjusted $R^2 = .033$). (Supplementary Table 4.).

3.2.2.3. KA/HK (neuroprotective ratio 2). Group differences were found for the ratio between kynurenic acid and 3-hydroxyanthranilic acid [lnKA]/[lnHK], where the FM group showed lower value than the control group ($\Delta R^2 = .027$, $\beta = -.046$, SE = 0.023, $t(152) = -2.00$, $p = .048$; overall model adjusted $R^2 = .041$). This group effect disappeared in model 2. CFS could not be distinguished from controls ($\Delta R^2 = .008$, $\beta = .039$, SE = 0.036, $t(148) = 1.083$, $p = .281$; overall model adjusted $R^2 = .122$). The CFS group could not be distinguished from the control group nor the FM group (Table 2). In model 2 only pain scores could explain a significant proportion of the [lnKA]/[lnHK] ratio ($\Delta R^2 = .066$, $\beta = -0.015$, SE = 0.005, $t(148) = -3.10$, $p = .002$).
(Supplementary Table 5.).

The results for all metabolites and ratios (Box 1) are found in Supplementary Tables 1–17.

4. Discussion

In this study we found reduced neuroprotective [lnKA]/[lnQA] ratio in CFS patients compared to healthy controls, and lower ratios of both [lnXA]/[lnHK] and neuroprotective [lnKA]/[lnHK] in FM patients compared to healthy controls. These differences persisted when controlled for age, BMI, and symptoms of anxiety depression. There were no differences between the CFS and FM groups. Anxiety and depression scores did not have any effect on any of the metabolites or ratios of the tryptophan-kynurenine pathway. Age, BMI, fatigue, pain scores and nicotine use affected several of the findings. Furthermore, we observed higher quinolinic acid (QA) concentrations in CFS patients.

4.1. Metabolite concentrations

4.1.1. Quinolinic acid (QA)

The CFS group had significantly higher levels of QA compared to the FM group when controlling for age, BMI, HADS-A, and HADS-D (model 1). In addition, when controlling for more co-factors (model 2), higher BMI indicated higher levels of QA ($\beta = .014$, SE = 0.004).

BMI in the CFS group was lower than the other groups, and yet this did not mask the effect of higher QA levels in the CFS group. The FM group however, had lower QA levels, yet significantly higher BMI than the CFS group. This could result in opposing effects on the model (model 1), and may be the reason why the FM group effect on QA disappeared when additionally controlling for fatigue, pain, FS scores, nicotine use and ln-IgE in model 2 (Supplementary Table 1). Likewise, more nicotine use in FM patients compared to the other groups combined with a slight, negative effect of nicotine on QA may neutralize the same group effects. Correcting for multiple co-factors didn't increase the overall power of the model, and this could indicate that the CFS patients may indeed have higher concentrations of QA compared to controls.

Table 2
Comparisons of the (ln-transformed) kynurenine pathway metabolites and ratios with group effects.

	Control			CFS					FM					
	Mean	SE	95% CI	Mean	SE	95% CI	β	p^a	Mean	SE	95% CI	β	p^a	p^b
lnAA[c]	2.686	0.050	2.588	2.785	2.541	0.050	2.443	2.639	−.145	**.049**	2.593	0.046	2.502	2.685	−.093	.207	.455
lnQA[d]	5.796	0.038	5.721	5.872	5.888	0.038	5.812	5.964	.092	.106	5.775	0.036	5.703	5.846	−.022	.106	**.036**
[lnKA]/[lnQA][e]	−1.985	0.059	−2.097	−1.864	−2.191	0.059	−2.308	−2.075	−.211	**.016**	−2.087	0.055	−2.196	−1.978	−.106	.223	.207
[lnXA]/[lnHK][f]	−0.898	0.064	−1.025	−0.772	−0.941	0.064	−1.067	−0.815	−.043	.647	−1.135	0.060	−1.252	−1.017	−.236	**.013**	**.035**
[lnKA]/[lnHK][f]	0.015	0.014	−0.012	0.042	−0.021	0.015	−0.051	0.008	−.036	.074	−0.032	0.013	−0.059	−0.006	−.047	**.015**	.600

Note: Covariates appearing in the model are evaluated at the following values: Age at test = 38.8; Body Mass Index ≈ 25; HADS-A = 5.9; HADS-D = 4,5. SE = standard error. CI = confidence interval.

[a] The control group is set as the reference group. β and *t* are the estimated from the control group and p^a are the significance values for CFS and FM compared to the control group.

[b] Significance levels for pair-wise comparison between CFS and FM.

[c] CFS lower than the control group.

[d] CFS higher than the FM group.

[e] FM lower than the CFS and control groups.

[f] FM lower than the control group.

4.1.2. Anthranilic acid (AA)

Anthranilic acid (AA) was significantly lower in CFS patients compared to controls when corrected for age, BMI, anxiety, and depression. This group difference disappeared when extending this model to include more co-factors (model 2). The extended model showed that fatigue and nicotine exhibited significant effects on AA (Supplementary Table 2). Since both patient groups (CFS and FM) had higher scores on fatigue and there were more nicotine users compared to controls, this indicates that fatigue and nicotine use are more likely than the diagnostic group variable to explain changes in this metabolite.

If less AA is derived from Kyn, this could mean that more Kyn is converted into KA or HK and eventually into the neurotoxic metabolite QA (which we claim were elevated in the CFS group discussed above). Still, these findings need further exploration, preferably in other study populations with relevant symptoms. It would be useful to specify "fatigue" to see if elements of the kynurenine pathway are related to mere physical and/or mental fatigue.

4.1.3. Metabolite ratios

4.1.3.1. Xanthurenic acid (XA) and 3-hydroxykynurenine (HK) – expression of KAT II activity). HK is converted into XA by the enzyme kynurenine aminotransferase II (KAT II), and the [lnXA]/[lnHK] ratio could be indicative of the activity of this enzyme. The FM group showed lower ratio value for [lnXA]/[lnHK] than controls and CFS patients. The CFS group could not be distinguished from the control group. However, Model 2 (Supplementary Table 3) was better at explaining the changes in the [lnXA]/[lnHK] ratio, and in this model the differences between the FM patients, CFS patients and the control group disappeared, suggesting that BMI and nicotine use, rather than the FM group, is more likely to have an effect on this ratio.

The potential clinical relevance of this is not known. To our knowledge there are no other studies exploring the role of neither higher weight nor pain associations with lowered KAT II activity. Both higher BMI and pain scores are solid findings in our FM patients, and they had significantly higher use of nicotine. The initial finding of reduced KAT II activity in FM patients could be indicative of symptoms that follow this disorder. Chronic pain conditions are common and affect approximately 25% of the population (Landmark, Romundstad et al. 2012). The involvement of kynurenines in pain sensation and chronification is plausible due to the involvement of glutamate in the processing of pain (Jovanovic and Candido et al., 2020). Deviating findings of Try–Kyn metabolites such as elevated QA and XA have been reported in a large sample ($n = 17,834$) of chronic pain patients (Gunn et al., 2020), and serum samples of 119 chronic migraine patients showed increased levels of Try, AA and XA and decreased Kyn, KA, HK, HAA and QA (Curto et al., 2015). Pain intensity was associated with the Kyn/Try ratio and Try plasma levels in 17 patients with temporomandibular myalgia (Barjandi et al., 2019). Pain scores could explain a significant proportion of the [lnXA]/[lnHK] ratio in our study, and it is therefore likely that the increased severity of pain is related to lower [lnXA]/[lnHK] ratio. Interestingly, in the absence of any group differences, we also discovered that pain scores were positively associated with kynurenine Mono-Oxygenase activity [lnHK]/[lnKyn] (Supplementary Table 6), and negatively associated with kynureninase activity [lnHAA]/[lnHK] (Supplementary Table 7).

4.1.3.2. Neuroprotective ratio 1 – expressed by kynurenic acid (KA) and quinolinic acid (QA). The [lnKA]/[lnQA] ratio is regarded "neuroprotective" because of the anticipated neuroprotective properties of KA and the neurotoxic properties of QA (Schwarcz and Stone, 2017). Our findings suggest that the "neuroprotective ratio" in CFS is lower than in healthy controls. An imbalance between the neurotoxic and neuroprotective metabolites is described in several neurodegenerative disorders and depression (Maddison and Giorgini, 2015; Savitz, 2017), which

implies that similar mechanisms could be found in CFS patients, but further studies are warranted before any conclusions can be drawn.

4.1.3.3. Neuroprotective ratio 2 – expressed by kynurenic acid (KA) and 3-hydroxykynurenine (HK). Similar to the KA/QA ratio, the KA/HK ratio could also be regarded as "neuroprotective" because of the above-mentioned neuroprotective properties of KA and the anticipated neurotoxic properties of HK (Colin-Gonzalez et al., 2013). In this study group differences were found for [lnKA]/[lnHK], where the FM group showed lower ratio value than the control group when controlling for age, BMI, HADS-A and HADS-D (model 1). The FM group could not be distinguished from the CFS group. Model 2 was better overall at explaining the [lnKA]/[lnHK] ratio, and the differences between the FM group and controls disappeared (Supplementary Table 5). In this model, only pain scores could explain a significant proportion of the [lnKA]/[lnHK] ratio, with higher pain scores indicating lower [lnKA]/[lnHK] ratio. To our knowledge there are no other studies reporting these ratios in neither FM nor CFS.

4.1.4. Neuroprotection and neurotoxicity

The neuroprotective properties of KA lies with its ability to block *N*-methyl-D-aspartate (NMDA) glutamatergic receptors (Schwarcz and Stone, 2017). Excessive glutamate signalling through NMDA receptors leads to neuronal loss (Colin-Gonzalez et al. 2013; Schwarcz and Stone, 2017). It is suggested that QA has neurotoxic effects by binding to NMDA receptors, and by promoting oxidative stress (Santamaría et al., 2001). The neurotoxic properties of HK depend on its physiological concentrations and results should be interpreted with caution since it can act as both agonist and antagonist of NMDA receptors (Colin-Gonzalez et al., 2013). Glutamate signalling through NMDA receptors can increase pain sensation by producing hypersensitivity of spinal neurons (Bannister et al., 2017) and it is thus possible that enhanced HK or QA could lead to increased pain sensation independent of reduced KA. Interestingly, pain NRS scores were negatively associated to KA in our study (Supplementary Table 8). It is tempting to speculate that this indicates a negative neuroprotective state for patients with CFS of FM. It may offer an explanation to how QA influences the overall symptom picture of these patients, although the mechanisms behind these observations need further investigation.

Try is an essential amino acid that is converted into Kyn by indoleamine 2–3-dioxygenase (IDO) in macrophages and glial cells (Schwarcz et al., 2012), and thus the [lnKyn]/[lnTry] ratio is indicative of IDO-activity. IDO activity is increased by the pro-inflammatory cytokines IFN-γ and TNF-α. Although we did not find increased IFN-γ and TNF-α levels in the patients, we did find that CFS and FM patients had other increased inflammatory markers, such as CRP (Groven, 2011; Groven et al., 2019). Since hsCRP status can be a proxy of the CFS and FM status controlling for hsCRP in this study may conceal any associations between the patient groups and the kynurenine pathway. When we added CRP in the model, it did not alter the results.

Inflammatory agents such as IL-6 are also produced in fatty tissue and this explains the relationship between higher BMI and inflammation. Altered tryptophan breakdown index (Kyn/Try) has been reported in obese subjects with systemic inflammation (Cussotto et al., 2020). We also found that BMI was positively associated with [lnKyn]/[lnTry] ratio (Supplementary Table 9). The link between BMI and inflammation on enzyme activity and neurotoxic metabolites of the kynurenine pathway, and how this relates to CFS and FM is a potential useful approach for further studies.

As Try is also converted into serotonin, altered levels of Try and altered activity in the enzymes of the kynurenine pathway could affect the production of serotonin. Studies have indicated lower serotonin levels in FM patients (Alnigenis and Barland, 2001) and increased serum concentrations of serotonin in sub-groups of CFS patients (Badawy et al., 2005). Abnormal Try and Kyn metabolites and neuroprotective ratios / higher neurotoxic ratios are found in patients suffering from depression (Savitz, 2017). Depressive symptoms are often seen in CFS and FM, as also shown in this study (higher HADS-D score). Tricyclic antidepressants (TCAs) and selective serotonin and noradrenaline reuptake inhibitors (SSRI/SNRIs) (both increasing levels of serotonin in synapses) have been reported to have an effect on pain and depression (but not fatigue) in FM (Hauser et al., 2009; Macfarlane et al., 2017; Welsch et al., 2018). In this study we did not find any differences in the Try concentrations or [lnKyn]/[lnTry] ratio between CFS, FM and controls, nor did the use of anti-depressants alter any of ours results (data not shown). Serotonin was not measured.

4.2. Limitations and strengths

The present study is a cross sectional study and conclusions on causality cannot be drawn. The time of day for blood samples collection varied between 9 AM and 18 PM. There were no dietary restrictions prior to the collection of blood samples, and information on nutrition or supplements was not recorded. Furthermore, only females ages 18–60 were included, also limiting generalisability. For example, children and adolescents, elderly and people with other known somatic disorders may have symptoms of CFS and FM with completely different biological background. Another weakness is that the control group consisted of university and hospital staff, which may not be representative of the general population.

Strength of the study: Other factors that are linked to inflammation or a shift in immunological responses such as infection, age, pregnancy, and BMI have all been taken into account. Participants with active infections or pregnancy were excluded. Both patient populations were recruited from a specialist care clinic. This is a strength as diagnoses were thoroughly evaluated and confounding comorbidities were excluded. There were no inequalities in the socioeconomic status in our study population.

To our knowledge this is the first report comparing the kynurenine metabolites and their ratios in CFS and FM and the findings need to be further explored.

5. Conclusion

CFS patients may have lower neuroprotection due to higher levels of QA and lower neuroprotective ratio (KA/QA) than healthy controls. Fatigue and pain – central factors in CFS and FM – seem to be particularly related to AA, QA, and KAT II activity. Body weight reduction and smoking cessation may be beneficial in chronic fatigue and pain conditions. Kynurenine metabolites and ratios can be promising indicators and targets of diagnosis and treatment of both FM and CSF. However, caution should be taken because of the complexity of the symptoms in these patients, such as fatigue and pain, and their underlying mechanisms, independent of diagnostic groups.

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Conflict of interest

All the authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2021.105287.

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Appendix B

Supplementary Tables 1–4

Supplementary Table 1

Items of the Structured Interview

Questionnaire item	Question asked	Recorded as
1. Age	What is your age?	Years
2. Height (in cm)	What is your height? (Alternatively measured on site.)	m and kg. Converted to BMI: $\frac{\text{weight (kg)}}{[\text{height (m)}]^2}$
3. Weight (in kg)	What is your weight? (Alternatively measured on site.)	
4. Subjective symptoms of infection	Are you experiencing any signs of infection such as sore throat, runny nose fever etc.?	Yes/no
5. "Feverishness"	Do you experience a subjective feeling of «fever» or «influenza»? Are you experiencing this *feverishness* now (during interview)?	As a symptom related to CFS or FM. (Not related to the previous question.)
6. Comorbid disorders/diagnoses	Do you suffer from any other diseases or disorders?	Reported diseases and disorders.
7. Allergies	Do you have any allergies?	Yes/no Type of allergy
8. Medication	Which medication are you currently taking?	Reported medication. Time of consumption of medication.
9. Menstrual cycle	When was your last period?	Duration since last period in relation to cycle length and discussion with the participant was used to determine the current phase.
10. Use of contraceptives	Do you use any contraceptives?	Reported contraceptives. Type of contraceptives.
11. Menopause	Are you undergoing or have you undergone menopause?	Yes/no/maybe
12. Level of physical activity	How physically active are you?	Level of physical activity over the past two weeks.
13. Nicotine use	Do you smoke or use any nicotine substances regularly?	Yes/no
14. Duration of illness	When, in your experience, did the symptoms of your illness (CFS or FM) occur? (Only applicable for the patient groups.)	Years

Supplementary Table 2

Correlational Matrix and Effect Sizes of Confounding Factors on the Immunological Markers hsCRP, Cytokines and Chemokines

Effect size	Parameter			Immunological marker														
				hsCRP	IFN-γ	IL-1ra	IL-1β	IL-4	IL-6	IL-8	IP-10	MCP-1	TNF-α	TGF-β1	TGF-β2	TGF-β3	IL-10	IL-17A
r	Illness duration	+/- 3 years	Total (CFS+FM)					.20					.21	.26	.25	.26	.29	.24
			CFS										.33	.44	.43	.35	.37	
			FM															
	Nicotine	no - yes	Total N															
			Controls	-.28														
			CFS															
			FM															
	Activity level	Low - high	Total N	-.24			.26	.35	.23			-.22	.23	.25	.31	.23	.24	.28
			Controls															
			CFS									.36						
			FM															

Effect sizes: $r = \frac{z}{\sqrt{n}}$ and Spearman's rho correlation (ρ)

The effect sizes for the confounding factors from the structured interview and questionnaires when conducting correlational analyses (Spearman's ρ). Only the effect sizes that reached statistical significance levels of $p \leq .05$ are given.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

Correlational Matrix and Effect Sizes of Confounding Factors on the Immunological Markers hsCRP, Cytokines and Chemokines (cont.)

Effect size	Parameter		Immunological marker														
			hsCRP	IFN-γ	IL-1ra	IL-1β	IL-4	IL-6	IL-8	IP-10	MCP-1	TNF-α	TGF-β1	TGF-β2	TGF-β3	IL-10	IL-17A
ρ	Age	Total N								.25	.30						
		Controls									.41						
		CFS									.32						
		FM		-.33						.36	.42						
	BMI	Total N	.44					.19	.17								
		Controls	.38	.35						.43							
		CFS									.34						
		FM	.60				.27	.28							.28		
	HADS total score	Total N				-.17	-.24	-.21		.17		-.18	-.22	-.21	-.21		
		Controls															
		CFS															
		FM															
	HADS anxiety	Total N		-.16			-.20	-.18									
		Controls															
		CFS															
		FM															
	HADS depression	Total N	.18			-.20	-.25	-.21		.21	.21	-.20	-.26	-.30	-.26		
		Controls															
		CFS								.29							
		FM															

Effect sizes: $r = \frac{z}{\sqrt{n}}$ and Spearman's rho correlation (ρ)

The effect sizes for the confounding factors from the structured interview and questionnaires when conducting correlational analyses (Spearman's ρ). Only the effect sizes that reached statistical significance levels of $p \leq .05$ are given.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

Correlational Matrix and Effect Sizes of Confounding Factors on the Immunological Markers hsCRP, Cytokines and Chemokines (cont.)

Effect size	Parameter		Immunological marker														
			hsCRP	IFN-γ	IL-1ra	IL-1β	IL-4	IL-6	IL-8	IP-10	MCP-1	TNF-α	TGF-β1	TGF-β2	TGF-β3	IL-10	IL-17A
ρ	Fatigue	Total N			-.17	-.30	-.40	-.31			.18	-.26	-.23	-.32	-.23	-.20	-.26
		Controls															
		CFS	-.29														
		FM									-.29						-.27
	Pain	Total N	.16	-.18		-.20	-.31	-.19			.17	-.21	-.22	-.20	-.17	-.16	-.17
		Controls															
		CFS															
		FM															
	FS score	Total N	.18	-.21		-.25	-.40	-.24			.19	-.26	-.23	-.27	-.20	-.18	-.20
		Controls															
		CFS															
		FM											.34	.30	.32		

Effect sizes: $r = \frac{z}{\sqrt{n}}$ and Spearman's rho correlation (ρ)

The effect sizes for the confounding factors from the structured interview and questionnaires when conducting correlational analyses (Spearman's ρ). Only the effect sizes that reached statistical significance levels of $p \leq .05$ are given.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

Correlational Matrix and Effect Sizes of Confounding Factors on the Immunological Markers hsCRP, Cytokines and Chemokines (cont.)

Effect size	Parameter		Immunological marker														
			hsCRP	IFN-γ	IL-1ra	IL-1β	IL-4	IL-6	IL-8	IP-10	MCP-1	TNF-α	TGF-β1	TGF-β2	TGF-β3	IL-10	IL-17A
ρ	Fatigue	Total N			-.17	-.30	-.40	-.31			.18	-.26	-.23	-.32	-.23	-.20	-.26
		Controls															
		CFS	-.29														
		FM									-.29						-.27
	Pain	Total N	.16	-.18		-.20	-.31	-.19			.17	-.21	-.22	-.20	-.17	-.16	-.17
		Controls															
		CFS															
		FM															
	FS score	Total N	.18	-.21		-.25	-.40	-.24			.19	-.26	-.23	-.27	-.20	-.18	-.20
		Controls															
		CFS															
		FM											.34	.30	.32		

Effect sizes: $r = \frac{z}{\sqrt{n}}$ and Spearman's rho correlation (ρ)

The effect sizes for the confounding factors from the structured interview and questionnaires when conducting correlational analyses (Spearman's ρ). Only the effect sizes that reached statistical significance levels of $p \leq .05$ are given.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

Supplementary Table 3

Comparisons of the Kynurenine Pathway Metabolites and Ratios with Group Effects

Metabolite	Model	Adjusted R^2 *	Control				CFS						FM						
			M	*SE*	95% CI		*M*	*SE*	95% CI		β[a]	*p*[a]	*M*	*SE*	95% CI		β[a]	*p*[a]	*p*[b]
lnTry[c,d]	0	.047	4.170	0.028	4.144	4.225	4.081	0.029	4.023	4.140	-.088	**.032**	4.051	0.027	9.998	4.104	-.118	**.003**	.454
	1	.044	4.155	0.033	4.091	4.220	4.089	0.033	4.025	4.154	-.066	.168	4.063	0.031	4.002	4.123	-.093	**.056**	.564
	2	**.096**	4.113	0.050	4.014	4.212	4.126	0.039	4.048	4.203	.013	.865	4.063	0.040	3.984	4.143	-.050	.526	.244
lnKyn[c,e]	0	.024	0.336	0.300	0.277	0.394	0.250	0.031	0.189	0.312	-.085	**.048**	0.344	0.028	0.288	0.400	.008	.841	**.027**
	1	.055	0.354	0.033	0.288	0.420	0.289	0.033	0.223	0.355	-.065	.186	0.304	0.031	0.242	0.365	-.050	.307	.755
	2	**.100**	0.231	0.049	0.134	0.327	0.347	0.038	0.272	0.422	.116	.107	0.360	0.039	0.282	0.437	.129	.091	.804
lnKA[c]	0	.029	3.811	0.044	3.723	3.898	3.644	0.047	3.551	3.736	-.167	**.011**	3.724	0.043	3.640	3.808	-.086	.163	.203
	1	.072	3.816	0.051	3.716	3.916	3.697	0.051	3.596	3.797	-.119	.112	3.688	0.048	3.594	3.782	.088	-.128	.898
	2	**.165**	3.589	0.074	3.443	3.736	3.783	0.058	3.668	3.897	.194	.079	3.814	0.060	3.696	3.932	.225	**.054**	.694
lnAA[c]	0	.028	2.676	0.043	2.593	2.761	2.581	0.045	2.429	2.606	-.159	**.012**	2.606	0.041	2.256	2.687	-.070	.238	.146
	1	.026	2.686	0.050	2.588	2.785	2.541	0.050	2.443	2.639	-.145	**.049**	2.593	0.046	2.502	2.685	-.093	.207	.455
	2	**.159**	2.485	0.073	2.341	2.629	2.640	0.056	2.529	2.751	.155	.152	2.694	0.057	2.581	2.807	.209	.066	.482
lnHK[e]	0	.022	3.751	0.045	3.662	3.841	3.719	0.047	3.626	3.812	-.033	.065	3.860	0.043	3.776	9.945	.109	.062	**.028**
	1	**.085**	3.761	0.051	3.661	3.861	3.786	0.050	3.687	3.886	.025	.735	3.802	0.047	3.709	3.895	.041	.583	.824
	2	.055	3.737	0.080	3.579	3.895	3.814	0.062	3.692	3.937	.077	.513	3.792	0.063	3.668	3.916	.055	.657	.788
lnXA[d]	0	.010	2.872	0.069	2.737	9.008	2.787	0.072	2.645	2.929	-.085	.393	2.691	0.066	2.562	2.821	-.181	.058	.327
	1	.014	2.853	0.080	2.696	3.011	2.846	0.080	2.688	3.004	-.007	.950	2.666	0.075	2.518	2.814	-.187	.114	.111
	2	.015	2.766	0.124	2.522	3.011	2.902	0.097	2.710	3.093	.135	.461	2.687	0.099	2.490	2.883	-.080	.680	.105
lnHAA	0	.003	3.682	0.051	3.581	3.783	3.592	0.053	3.487	3.697	-.090	.222	3.652	0.048	3.557	3.748	-.030	.672	.401
	1	.010	3.671	0.059	3.555	3.788	3.628	0.059	3.512	3.744	-.043	.621	3.641	0.055	3.532	3.749	-.031	.724	.882
	2	.007	3.554	0.091	3.374	3.735	3.682	0.071	3.542	3.822	.128	.347	3.688	0.072	3.546	3.830	.133	.347	.951
lnQA	0	.005	5.788	0.034	5.720	5.856	5.843	0.036	5.771	5.914	.055	.275	5.819	0.033	5.754	5.884	.031	.516	.629
	1	.078	5.796	0.038	5.721	5.872	5.888	0.038	5.812	5.964	.092	.106	5.755	0.036	5.703	5.846	-.022	.700	**.036**
	2	**.096**	5.682	0.056	5.571	5.793	5.934	0.044	5.847	6.021	.252	**.003**	5.829	0.045	5.739	5.918	.146	.098	.080
lnPic	0	.001	3.780	0.056	3.669	3.891	3.668	0.059	3.551	3.785	-.112	.171	3.696	0.054	3.590	3.803	-.083	.285	.721
	1	.034	3.729	0.064	3.603	3.855	3.723	0.064	3.596	3.849	-.007	.944	3.732	0.060	3.613	3.850	.003	.977	.917
	2	.045	3.580	0.098	3.385	3.774	3.764	0.077	3.612	3.916	.185	.205	3.842	0.079	3.686	3.998	.262	.090	.460

Comparisons of the Kynurenine Pathway Metabolites and Ratios with Group Effects (cont.)

Metabolite	Model	Adjusted R^2	Control				CFS						FM						
		*	*M*	*SE*	95% CI		*M*	*SE*	95% CI		β[a]	*p*[a]	*M*	*SE*	95% CI		β[a]	*p*[a]	*p*[b]
[lnKyn]/[lnTry][d,f]	0	.058	-3.834	0.029	-3.894	-3.774	-3.831	0.032	-3.895	-3.768	.003	.950	-3.708	0.029	-3.765	-3.650	.126	**.003**	**.005**
	1	**.145**	-3.801	0.034	-3.868	-3.735	-3.800	0.034	-3.867	-3.733	.001	.979	-3.759	0.032	-3.822	-3.696	.042	.398	.389
	2	.138	-3.883	0.052	-3.985	-3.780	-3.779	0.041	-3.859	-3.699	.104	.178	-3.704	0.042	-3.786	-3.621	.179	**.048**	.176
[lnKA]/[lnKyn]	0	.006	-0.359	0.047	-0.451	-0.267	-0.438	0.049	-0.535	-0.341	-.079	.247	-0.327	0.045	-0.415	-0.239	.032	.622	.097
	1	.088	-0.339	0.052	-0.443	-0.236	-0.392	0.052	-0.496	-0.289	-.053	.492	-0.375	0.049	-0.472	-0.278	-.036	.645	.814
	2	**.132**	-0.524	0.079	-0.680	-0.368	-0.343	0.062	-0.465	-0.221	.181	.123	-0.249	0.063	-0.375	-0.124	.275	**.028**	.268
[lnXA]/[lnHK][d,f]	0	.089	-0.869	0.056	-0.979	-0.759	-0.932	0.058	-1.05	-0.818	-.063	.432	-1.169	0.053	-1.273	-1.065	-.300	**.015**	**< .001**
	1	.113	-0.898	0.064	-1.025	-0.772	-0.941	0.064	-1.067	-0.815	-.043	.647	-1.135	0.060	-1.252	-1.017	-.236	**.013**	**.032**
	2	**.151**	-0.949	0.098	-1.143	-0.756	-0.923	0.076	-1.073	-0.774	.026	.857	-1.108	0.077	-1.259	-0.956	-.158	.296	.073
[lnHK]/[lnKyn][g]	0	.013	3.415	0.036	3.343	3.487	3.469	0.038	3.394	3.544	.054	.309	3.516	0.035	3.448	3.585	.101	**.045**	.355
	1	.050	3.406	0.042	3.324	3.489	3.497	0.042	3.415	3.579	.091	.140	3.498	0.039	3.421	3.575	.092	.137	.987
	2	.088	3.510	0.064	3.384	3.636	3.466	0.049	3.368	3.563	-.044	.639	3.432	0.050	3.333	3.530	-.078	.428	.611
[lnAA]/[lnKyn]	0	.003	2.340	0.040	2.262	2.418	2.268	0.041	2.186	2.349	-.072	.210	2.262	0.038	2.188	2.337	-.078	.158	.924
	1	.000	2.331	0.046	2.240	2.422	2.252	0.046	2.162	2.343	-.079	.243	2.289	0.043	2.205	2.374	-.042	.537	.563
	2	.065	2.258	0.069	2.122	2.395	2.291	0.053	2.186	2.397	.033	.746	2.334	0.054	2.226	2.441	.482	.075	.560
[lnHAA]/[lnHK]	0	.015	0.982	0.011	0.959	1.004	0.968	0.012	0.945	0.991	-.014	.400	0.949	0.011	0.928	0.971	-.033	**.039**	.244
	1	.040	0.975	0.013	0.950	1.001	0.962	0.013	0.936	0.987	-.014	.478	0.962	0.012	0.938	0.986	-.014	.474	.994
	2	**.115**	0.953	0.020	0.914	0.992	0.967	0.015	0.937	0.997	.014	.633	0.976	0.015	0.945	1.006	.023	.454	.665
[lnKA]/[lnQA][c]	0	.043	-1.977	0.051	-2.077	-1.877	-2.199	0.053	-2.304	-2.094	-.222	**.003**	-2.095	0.048	-2.190	-1.999	-.117	.096	.149
	1	.045	-1.980	0.059	-2.097	-1.864	-2.191	0.059	-2.308	-2.075	-.211	**.016**	-2.087	0.055	-2.196	-1.978	-.106	.223	.207
	2	.033	-2.093	0.091	-2.273	-1.914	-2.151	0.071	-2.291	-2.011	-.058	.665	-2.015	0.073	-2.159	-1.871	.078	.580	.159
[lnKA]/[lnHK][c,d]	0	.037	0.017	0.014	-0.010	-1.877	-0.023	0.014	-0.051	0.005	-.040	**.042**	-0.034	0.013	-0.059	-0.009	-.051	**.007**	.567
	1	.041	0.017	0.016	-0.014	0.048	-0.027	0.016	-0.058	0.004	-.043	.061	-0.029	0.015	-0.058	-0.001	-.046	**.048**	.904
	2	**.122**	-0.034	0.023	-0.080	0.012	-0.014	0.018	-0.050	0.022	.020	.565	0.005	0.018	-0.031	0.041	.039	.281	.433

Covariates Model 0: None. Covariates Model 1: Age; BMI (Body mass index); and anxiety and depression scores (from the hospital anxiety and depression scale [HADS]). Model 2 confounding factors: Same as Model 1 with additional: NRS pain (Numeric rating scale [0–10] of pain severity experienced at present); Fibromyalgia severity score (from the fibromyalgia survey diagnostic criteria [FSDC]); Fatigue scores (from the Chalder fatigue questionnaire ([CFQ]); Nicotine (Use of nicotine by smoking or snuff [binominal score]); and IgE from serum. SE = standard error. CI = confidence interval. ***Bold font** = strongest model for adjusted R^2 when relevant. **Red, bold font** = $p < .05$. [a]The control group is set as the reference groups, and the values are estimjated from the controlgroup compared to CFS and FM, respectively. [b] Significance levels for pair-wise comparison between CFS and FM.

Supplementary Table 4

Confounding Factor Comparisons of the Kynurenine Pathway Metabolites and Ratios

Confounder	Model	lnTry			lnKyn			lnKA			lnAA			lnHK			lnXA			lnHAA		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Groupp overall			.059	**.009**		.036	**.057**		.041	**.037**		.041	**.040**		.035	.064		.023	.165		.010	.462
CFS vs. control	0	**-.088**	.029	**.032**	**-.085**	.025	**.048**	**-.167**	.041	**.011**	**-.159**	.040	**.012**	-.033	.002	.618	-.068	.003	.516	-.090	.010	.222
FM vs. control		**-.118**	.056	**.003**	.008	.000	.841	-.086	.012	.163	-.070	.009	.238	.109	.019	.083	**-.196**	.026	**.048**	-.030	.001	.672
Groupp overall			.026	.148		.013	.390		.024	.172		.027	.140		.003	.804		.023	.182		.002	.880
CFS vs. control	1	-.066	.013	.168	-.065	.012	.186	-.119	.017	.112	**-.145**	.026	**.049**	.025	.001	.735	-.007	.000	.950	-.043	.002	.621
FM vs. control		**-.093**	.025	**.056**	-.050	.007	.307	-.128	.020	.088	-.093	.011	.207	.041	.002	.583	-.187	.017	.114	-.031	.001	.724
Groupp overall			.010	.500		.022	.210		.028	.140		.025	.183		.003	.804		.020	.256		.007	.604
CFS vs. control	2	.013	.000	.865	.116	.019	.107	.194	.022	.079	.155	.015	.152	.077	.003	.513	.135	.004	.461	.128	.006	.347
FM vs. control		-.050	.003	.526	.129	.021	.091	.225	.027	.054	.209	.024	.066	.055	.001	.657	-.080	.001	.680	.133	.006	.347
Age	1	-.001	.003	.517	**.004**	.042	**.013**	**.008**	.058	**.003**	**.005**	.025	**.053**	.003	.012	.193	.000	.000	.939	-.002	.003	.519
	2	-.001	.002	.590	**.005**	.060	**.004**	**.008**	.064	**.002**	.004	.015	.149	.003	.010	.247	-.001	.000	.833	-.001	.002	.628
BMI	1	-.004	.009	.261	.006	.016	.122	.009	.015	.135	-.004	.004	.472	**.019**	.073	**.001**	.005	.002	.602	.009	.012	.178
	2	-.006	.017	.121	.003	.005	.409	.008	.014	.168	-.004	.004	.456	**.017**	.052	**.007**	.004	.001	.657	.008	.008	.285
HADS anxiety	1	.003	.002	.590	.005	.004	.419	.001	.000	.909	-.001	.000	.898	.015	.019	.098	.025	.021	.077	.010	.007	.308
	2	.004	.003	.513	.007	.010	.234	.008	.006	.370	.004	.002	.597	.011	.010	.243	**.028**	.027	**.053**	.013	.011	.213
HADS depression	1	-.007	.008	.273	.001	.000	.908	.001	.000	.894	.004	.001	.642	-.012	.011	.213	-.027	.023	.067	-.014	.011	.207
	2	-.004	.004	.481	.005	.004	.454	.009	.006	.364	.011	.010	.235	-.010	.008	.307	-.022	.015	.156	-.009	.004	.440
Pain	2	-.006	.002	.612	-.005	.002	.605	**-.034**	.030	**.039**	.005	.001	.762	.022	.012	.191	-.035	.012	.201	-.026	.013	.182
Fatigue	2	-.008	.024	.071	**-.008**	.026	**.059**	-.012	.025	.063	**-.014**	.036	**.025**	-.005	.005	.421	-.014	.013	.177	-.010	.012	.208
FS scores	2	.003	.003	.533	-.006	.013	.172	-.009	.010	.230	-.011	.018	.115	-.003	.001	.703	.011	.006	.357	.002	.000	.849
Nicotine	2	-.018	.001	.662	-.005	.000	.903	-.056	.006	.355	**-.185**	.070	**.002**	.043	.003	.496	-.126	.011	.213	-.109	.016	.137
IgE	2	-.005	.001	.711	-.006	.002	.601	-.020	.009	.264	-.024	.014	.171	-.014	.004	.465	-.016	.002	.594	.005	.000	.804

SE = standard error. CI = confidence interval.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

Confounding Factor Comparisons of the Kynurenine Pathway Metabolites and Ratios (cont.)

Confounder	Model	lnQA			lnPic			[lnKyn]/[lnTry]			[lnKA]/[lnKyn]			[lnXA]/[lnHK]			[lnHK]/[lnKyn]			[lnAA]/[lnKyn]		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Groupp overall	0		.008	.546		.013	.355		.069	**.004**		.018	.240		.100	**< .001**		.026	.134		.015	.302
CFS vs. control		.055	.008	.275	-.112	.012	.171	.003	.000	.950	-.079	.009	.247	-.063	.004	.432	.054	.007	.309	-.072	.010	.210
FM vs. control		.031	.003	.516	-.083	.007	.285	**.126**	.054	**.003**	.032	.002	.622	**-.300**	.090	**< .001**	**.101**	.026	**.045**	-.078	.013	.158
Groupp overall	1		.033	.087		.000	.994		.007	.610		.003	.785		.049	.026		.019	.242		.009	.505
CFS vs. control		.092	.018	.106	-.007	.000	.944	.001	.000	.979	-.053	.003	.492	-.043	.001	.647	.091	.015	.140	-.079	.009	.243
FM vs. control		-.022	.001	.700	.003	.000	.977	.042	.005	.398	-.036	.001	.645	**-.236**	.041	**.013**	.092	.015	.137	-.042	.003	.537
Groupp overall	2		.068	**.007**		.021	.236		.036	.082		.035	.086		.024	.187		.005	.714		.004	.739
CFS vs. control		**.252**	.063	**.003**	.185	.012	.205	.104	.013	.178	.181	.017	.123	.026	.000	.857	-.044	.002	.639	.033	.001	.746
FM vs. control		.146	.020	.098	.262	.021	.090	**.179**	.034	**.029**	**.275**	.035	**.028**	-.158	.008	.296	-.078	.005	.428	.075	.004	.482
Age	1	.002	.007	.294	.006	.021	.075	**.005**	.062	**.002**	**.009**	.069	**.001**	-.004	.008	.274	-.001	.001	.662	.001	.001	.784
	2	.003	.013	.176	.005	.018	.116	**.006**	.073	**.001**	**.009**	.071	**.002**	-.004	.010	.241	-.002	.005	.406	-.001	.003	.557
BMI	1	**.017**	.094	**< .001**	**-.014**	.024	**.059**	**.010**	.044	**.010**	**.013**	.031	**.033**	**-.015**	.029	**.039**	**.013**	.053	**.005**	**-.010**	.024	**.059**
	2	**.014**	.069	**.002**	-.014	.024	.066	**.009**	.036	**.024**	**.014**	.037	**.023**	-.013	.022	.084	**.014**	.054	**.006**	-.007	.014	.165
HADS anxiety	1	.008	.010	.235	.006	.002	.603	.002	.001	.781	-.002	.000	.823	.009	.004	.419	.010	.013	.168	-.006	.003	.475
	2	.011	.020	.095	.010	.006	.379	.003	.002	.629	.004	.001	.671	.017	.016	.140	.004	.002	.601	-.003	.001	.752
HADS depression	1	-.007	.007	.319	-.018	.016	.128	.007	.009	.242	.008	.005	.416	-.015	.010	.215	-.013	.018	.108	.003	.001	.682
	2	-.004	.002	.594	-.015	.010	.237	.009	.014	.168	.013	.012	.195	-.012	.007	.320	-.015	.025	.063	.006	.004	.474
Pain	2	-.010	.005	.406	-.013	.003	.528	.000	.000	.996	-.028	.019	.105	**-.059**	.055	**.006**	**.028**	.029	**.044**	.010	.003	.502
Fatigue	2	-.006	.013	.181	-.002	.001	.761	.000	.000	.983	-.004	.003	.551	-.007	.005	.387	.003	.002	.621	-.006	.007	.325
FS scores	2	-.004	.005	.422	-.013	.013	.186	-.009	.025	.061	-.012	.017	.129	.014	.017	.121	.004	.003	.546	-.004	.003	.504
Nicotine	2	**-.093**	.029	**.043**	-.029	.001	.718	.013	.001	.758	-.038	.003	.555	**-.170**	.034	**.030**	.048	.007	.344	**-.181**	.073	**.001**
IgE	2	-.006	.001	.662	-.031	.012	.193	-.002	.000	.895	-.016	.005	.415	-.002	.000	.927	-.008	.002	.609	-.018	.008	.286

SE = standard error. CI = confidence interval.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

Confounding Factor Comparisons of the Kynurenine Pathway Metabolites and Ratios (cont.)

Confounder	Model	[lnHAA]/[lnHK]			[lnKA]/[lnQA]			[lnKA]/[lnHK]		
		β	ΔR^2	*p*	β	ΔR^2	*p*	β	ΔR^2	*p*
Groupp overall	0		.027	.116		.055	**.012**		.049	**.020**
CFS vs. control		-.014	.005	.400	**-.222**	.055	**.003**	-.040	.026	.042
FM vs. control		**-.033**	.027	**.039**	-.117	.018	.096	**-.051**	.046	**.007**
Groupp overall	1		.004	.720		.039	.054		.032	.091
CFS vs. control		-.014	.003	.478	**-.211**	.039	**.016**	-.043	.024	.061
FM vs. control		-.014	.004	.474	-.106	.010	.223	**-.046**	.027	**.048**
Groupp overall	2		.004	.747		.014	.369		.010	.519
CFS vs. control		.014	.002	.633	-.058	.001	.665	.020	.002	.565
FM vs. control		.023	.004	.454	.078	.002	.580	.039	.008	.281
Age	1	**-.001**	.028	**.042**	.006	.024	.060	.001	.016	.126
	2	-.001	.023	.076	.005	.020	.096	.001	.020	.093
BMI	1	-.002	.016	.120	-.008	.010	.214	-.003	.015	.143
	2	-.002	.013	.188	-.006	.005	.399	-.002	.009	.259
HADS anxiety	1	-.001	.001	.684	-.007	.003	.498	-.004	.012	.190
	2	.001	.001	.748	-.003	.001	.758	-.001	.001	.793
HADS depression	1	-.001	.001	.641	.008	.004	.444	.004	.011	.208
	2	.000	.000	.906	.012	.008	.284	.005	.021	.088
Pain	2	**-.012**	.060	**.004**	-.023	.010	.237	**-.015**	.066	**.002**
Fatigue	2	-.001	.002	.598	-.005	.004	.487	-.001	.003	.525
FS scores	2	.001	.003	.541	-.004	.002	.627	-.001	.002	.574
Nicotine	2	**-.040**	.045	**.012**	.038	.002	.611	-.028	.017	.130
IgE	2	.005	.009	.256	-.014	.003	.519	-.001	.000	.895

SE = standard error. CI = confidence interval.
BMI = Body Mass Index. HADS = Hospital Anxiety and Depression Scale. Pain = pain severity score experienced at present (Scale: 0–10). Fatigue = Scores from the Chalder fatigue questionnaire (Scale: 0–33). FS score = Fibromyalgia Severity from the Fibromyalgia Survey Diagnostic Criteria (FSDC).

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Norwegian University of
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