Dissertation

Immunological and microbial features of patients suffering from chronic pain due to Fibromyalgia-Syndrome

submitted by

Dr.med.univ. Thomas Michael WEBER

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Univ.-Prof. Dr. Peter HOLZER

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

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Disclosures

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"Fibromyalgia-associated hyperalgesia is related to psychopathological alterations but not to gut microbiome changes"

Weber T[1*], Tatzl E[1], Kashofer K[2], Holter M[3], Trajanoski S[4], Berghold A[3], Heinemann A[5], Holzer P[5], Herbert MK[1]

[1] Department of Anesthesiology and Intensive Care Medicine, Medical University of Graz, Austria
[2] Diagnostic & Research Institute of Pathology, Medical University of Graz, Austria
[3] Institute of Medical Informatics, Statistics and Documentation, Medical University of Graz, Austria
[4] Center for Medical Research, Medical University of Graz, Austria
[5] Division of Pharmacology, Otto Loewi Research Center, Medical University of Graz, Austria

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List of Abbreviations

ACR	American College of Rheumatology	MPS	Mechanical pain sensitivity
CBD	Cannabidiol	NMDA	N-methyl-D-aspartate
CCA	Canonical Correspondence Analysis	NRS	Numeric Rating Scale
CDT	Cold detection threshold	NSAID	Non-steroidal anti-inflammatory drug
COMT-gene	Catechol-O-Methyltransferase	OTC	Over-the-counter Medication
CPT	Cold pain threshold	OTU	Operational Taxonomic Unit
DASS	Depression-Anxiety-Stress Questionnaire	PAF	Platelet activating factor
DEseq2	Differential Expression of Sequence 2	PBS	Phosphate-buffered saline
DFNS	Deutscher Forschungsverband für neuropathischen Schmerz	PCR	Polymerase Chain Reaction
DNA	Desoxyribonucleic acid	PGE2	Prostaglandin E2
DMA	Dynamic mechanic allodynia	PHQ-15	Patient Health Questionnaire - 15
DRG	Dorsal root ganglia	PHS	Paradoxical heat sensation
ELISA	Enzyme-linked immunosorbent assay	PPI	Proton Pump Inhibitor
FACS	Fluorescence-activated cell sorting	PPT	Pressure pain threshold
FIQ	Fibromyalgia Impact Questionnaire	PTSD	Post-traumatic stress disease
FMLP	Formyl-Methionyl-Leucyl-Phenylalanine	QST	Quantitative Sensory Testing
fMRI	Functional magnetic resonance imaging	RIA	Radioimmunoassay
FMS	Fibromyalgia-Syndrome	RPMI	Cell Culture Media
FODMAP	fermentable oligo-, di- and monosaccharides and polyols	rRNA	Ribosomal Ribonucleic acid
FSC	Forward scatter	SCFA	Short chain fatty acids
FSC-A	Forward-Scatter in FACS	SERT	Serotonin transporter
GABA	Gamma-aminobutyric acid	SNRI	Serotonin-Noradrenaline-Reuptake-Inhibitor
HBO	Hyperbaric Oxygen Therapy	SSC	Side scatter
HC	Healthy Controls	SSC-A	Side-Scatter in FACS
HPA	Hypothalamic-pituitary-adrenal	SSS	Symptom Severity Index
HPT	Heat pain threshold	THC	Tetrahydrocannabinol
HRH-1	Histamine receptor 1	TMD	Temporomandibular disorders
IASP	International Association for the Study of Pain	TNFalpha	Tumor Necrotic Factor Alpha

IBS	Irritable Bowel syndrome	TRPV-1	Transient receptor potential cation channel subfamily V member 1
IL-1beta	Interleukin 1beta	TSL	Temperature sensory limen
LPS	Lipopolysaccaride	VDT	Vibration detection threshold
LTB4	Leukotriene B4	WDT	Warm detection threshold
MbFhW	Marburger Fragebogen zum habituellen Wohlbefinden	WHO	World Health Organisation
MDT	Mechanical detection threshold	WPI	Widespread Pain Index
MPT	Mechanical pain threshold	WUR	Wind-up ratio

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Zusammenfassung

Das Fibromyalgie-Syndrom (FMS) ist eine komplexe Erkrankung, welche aus einem Symptomenkomplex von „chronic widespread pain“, Depression, kognitiver Dysfunktion („fibro-fog“) und Verdauungsstörungen besteht. Ziel dieser Studie war es, die FMS-spezifischen Schmerzprofile, welche mittels Quantitativer Sensorischer Testung (QST) ermittelt wurden, gemeinsam mit psychologischen Auffälligkeiten in Korrelation mit dem gastrointestinalen Mikrobiom zu setzen.

Eingeschlossen wurden 25 Patient*innen mit FMS und 26 alters- und geschlechtsgerechte gesunde Proband*innen. Die medizinische und psychopathologische Anamnese, Ernährungsgewohnheiten sowie die Lebensqualität sind mittels Fragebögen erhoben worden. Die Stuhlproben wurden mittels 16S rRNA Genamplifikation und Sequenzierung analysiert. Eine QST ist anhand des Protokolls der Deutschen Gesellschaft für neuropathischen Schmerz durchgeführt worden.

Mittels QST konnte gezeigt werden, dass lemniskale und spinothalamische afferente Nervenfasern bei Patient*innen mit FMS beeinträchtigt sind und konsekutiv eine periphere und eine zentrale Sensibilisierung auftritt. Die psychologischen Fragebögen konnten erhöhte Werte für Depression, Angst und Stress darstellen. Beim gastrointestinalen Mikrobiom waren jedoch weder die Alpha- noch die Betadiversität signifikant verändert. Dementsprechend fanden sich bei Patient*innen mit FMS weder funktionelle Modifikationen neutrophiler und eosinophiler Granulozyten noch eine Änderung der Monozyten-Aktivierung durch Lipopolysaccharid.

Als Schlussfolgerung kann festgehalten werden, dass Patient*innen mit FMS sich signifikant von gesunden Proband*innen bezogen auf die Psychopathologie und QST unterscheiden, jedoch nicht hinsichtlich des Mikrobioms. Dies könnte einer der Limitationen der Studie geschuldet sein. Daher erscheint der Einfluss des Mikrobioms auf die Pathophysiologie des FMS begrenzt.

Abstract

Fibromyalgia syndrome (FMS) is a disease with multifocal symptoms that are primarily characterized by chronic widespread pain and further symptoms including major/minor depression, cognitive dysfunction (“fibro-fog”) and digestive disorders. In this study I examined whether FMS-related pain values assessed by quantitative sensory testing (QST) and psychological alterations are accompanied by changes of the fecal microbiome.

For this purpose, 25 patients with FMS and 26 age- and sex-matched healthy controls were recruited. Medical history, food habits, psychological tests and quality of life were evaluated with questionnaires. Stool samples were analyzed by 16S rRNA gene amplification and sequencing. QST was performed according to the protocol of the German Network for Neuropathic Pain.

QST showed that afferent pathways (lemniscal and spinothalamic nerve fibers) are altered in FMS relative to healthy controls and that peripheral and central pain sensitization processes are directly manifest. Psychometric results revealed high scores of depression, anxiety and stress. In contrast, neither the composition nor the alpha- and beta-diversity of the fecal microbiome was altered in patients with FMS. Likewise, the functional shape change response of neutrophilic/eosinophilic granulocytes and the activation of monocytes by lipopolysaccharide remained unchanged in FMS patients.

In summary, FMS patients differ from healthy controls in several parameters of QST and psychopathology, but not in terms of composition and diversity of the gut microbiome. Despite consideration of multinumerous confounding factors it is concluded that the contribution of the gut microbiome to the pathophysiology of FMS is limited.

1 INTRODUCTION

As the Institute of Medicine of the national academies reports, every year around 600 billion Dollars are spent for people that have to deal with chronic pain. Fibromyalgia-syndrome (FMS), as a chronic pain disease with a high incidence, affects about 2-4.7 % of the population worldwide. The average ratio of female to male is about 3:1 (1). Sarzi-Puttini et al. (2) studied the prevalence of musculoskeletal pain in an Italian population sample and found that fibromyalgia is number three, right after lumbar pain and osteoarthritis.

1.1 Diagnosis

For the correct diagnosis of FMS, a detailed anamnesis is essential. Screening tools (eg Fibromyalgia Rapid Screening Tool (3)) and specific questionnaires (eg revised Fibromyalgia Impact Questionnaire, FIQ (4)) may help to provide all information needed. Indeed, it can be crucial not to loose the diagnostic pathway if FMS is suspected. Historically, before 1990, FMS was called “fibrositis”. In 1990, the American College of Rheumatology (ACR) first described the term “fibromyalgia” (5). It was defined as “widespread pain noted as pain in all four quadrants (both the left and right side of the body, above and below the waist) plus axial skeletal pain”. It also included the use of so called *tender points*. In 2010, about 20 years later, the ACR revised those criteria (6). The tender points were now excluded, instead the ACR defined 19 different body areas, which the patient had to report as painful. In addition, it also included several attendant symptoms (eg fatigue, waking up unrefreshed, cognitive symptoms, somatic symptoms). In 2011, more attendant symptoms were added (eg headache, abdominal discomfort, depression) (7). Finally, in 2016, the ACR defined the criteria that are basically valid until now (see Table 1) (8).

Widespread Pain Index (WPI)	Symptom Severity Score (SSS)	Diagnosis
Chronic widespread pain in 4 out of 5 regions (left/right upper region, left/right lower region, axial region) WPI: 0-19 points of reported regions being painful in the past week	3 key symptoms (sum of severity) Fatigue, waking up unrefreshed, cognitive symptoms Additionally Headache, pain or cramps in the lower abdomen, depression in the last 6 months	WPI > 7 and SSS > 5; or WPI 4-6 and SSS > 9 Presence of generalized pain Similar level of symptoms ≥ 3 months

Table 1: Diagnostic criteria regarding FMS, adapted from the ACR (8).

To finish up the diagnostic approach, a clinical status is mandatory to exclude other reasons for chronic widespread pain. Differential diagnosis can sometimes be challenging, Table 2 shows the most important pathologies that need to be ruled out (9).

Categorically, FMS can be further divided into mild and severe FMS. Both scores from WPI and SSS are added. Patients with a sum of lower than 20 points are classified as having mild FMS, patients with a higher score than 20 points as having severe FMS (7,8).

Differential diagnosis	Example
Systemic inflammatory rheumatic disease	Rheumatic Arthritis
Nonrheumatic musculoskeletal conditions	Ehlers-Danlos Syndrome
Neurological diseases	Polyneuropathy
Spinal stenosis/Myelopathy	Spinal canal stenosis
Myopathy/Myositis	Dermatomyositis
Mental health disorders	Depression
Somatoform disorders	Somatization disorder

Table 2: Most important differential diagnostic categories and examples, which need to be excluded prior to the diagnosis of FMS, adapted from Häuser et al. (9).

1.2 Symptoms

As described previously, the symptom complex of FMS can be divided into two categories. Cardinal features are symptoms that are necessary for a correct diagnosis, while other symptoms, which can be very broad, supplement the diagnosis (see Table 3) (7–9).

Cardinal features are basically pain, which affects the whole body. Usually, the quality of pain is similar to neuropathic pain (burning). Two other symptoms that occur in most patients are fatigue due to unrestorative sleep and sleep disturbances, together with depression, brain fog, headache and abdominal cramps (8).

Category	Symptom
Psychiatric	Anxiety Depression Post-traumatic stress disorder Concentration/ Memory difficulties
Sleep	Insomnia Frequently waking up Non-restoring sleep
Autonomic Nervous System	Blurred vision Photophobia Xerostomia Morbus Raynaud Orthostatic hypotension
Pain	Generalized pain Headache / Migraine Dyspepsia Irritable Bowel Syndrome Dysmenorrhoea Vulvodynia Dysuria
Hypersensitivity	Hypersensitivity to light, odor, sound Chemical sensitivity
Fatigue	Physical mental

Table 3: The variety of symptoms that can occur in FMS (9).

As an example to show how patients mark the painful areas in the German Pain Questionnaire (10), in Figure 1 a patient with severe FMS is shown.

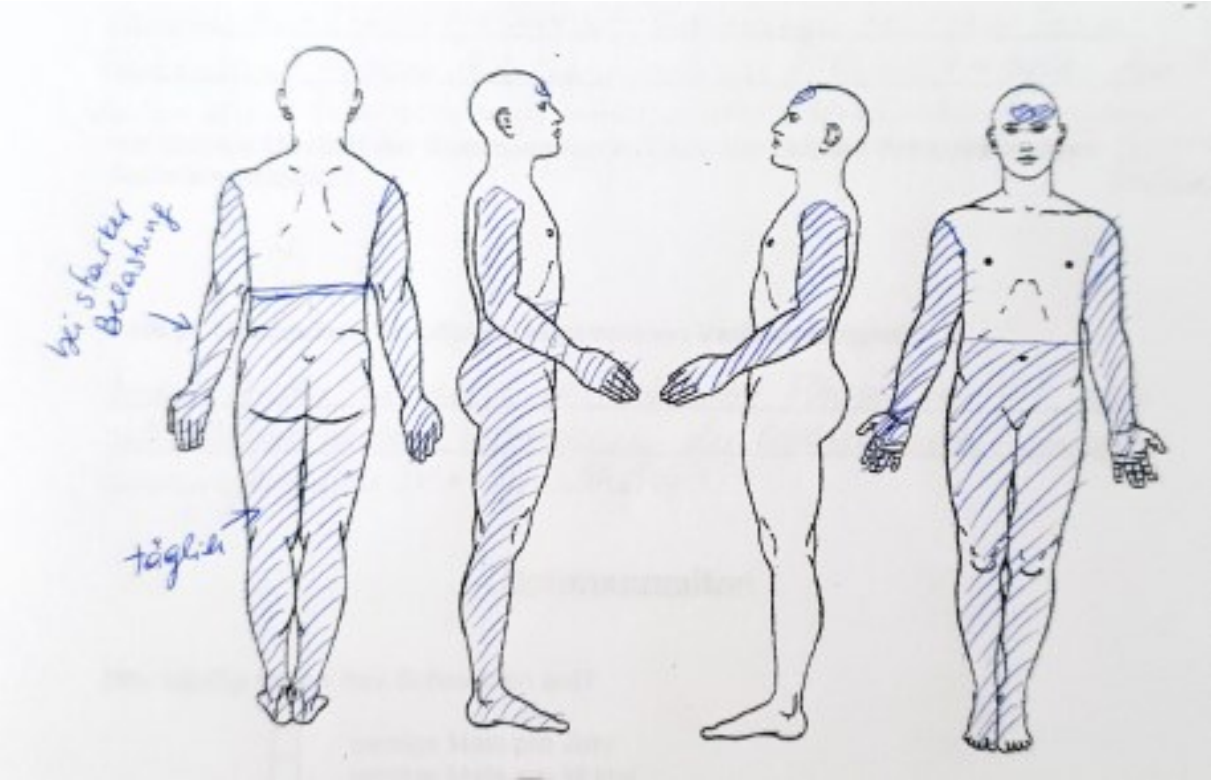


Figure 1: Example of a patient with FMS who colored all body areas that are painful with blue lines.

1.3 Pathophysiology of FMS

The pathophysiology of FMS appears to be very complex and is hardly understood. The International Association for the Study of Pain (IASP) has recently introduced the ICD-11 term of “nociplastic pain” as a new designation to describe pain in the absence of actual or threatened tissue damage (11). FMS is classified as a disease in which nociplastic pain plays a crucial role in its pathophysiology.

Some of the pathophysiologic mechanisms that may underlie FMS have been discussed by Weber et al. (12) and are quoted here in Italics under quotation marks.

“It has been proposed that both genetic and environmental predisposition might play a role, including bacterial infections (e.g. Borrelia) or a stressful life event (e.g. loss of partner) (2). *It is not clear whether peripheral inflammation or central nociplastic changes catalyze chronic widespread pain* (13). *Probably due to an interplay of peripheral and central mechanisms,*

neuromorphological changes are brought about that trigger further symptoms. Changes at spinal and supraspinal levels can also lead to reduced endogenous pain inhibition (14)*. Furthermore, significant alterations of the autonomic nervous system occur, which may explain many of the FMS-specific symptoms. For instance, Furlan et al.* (15) *showed that patients with FMS present with enhanced cardiovascular sympathetic activity, although there are currently controversies about basal levels of sympathetic activity. Other studies showed even lower sympathetic activity levels in FMS* (16,17)." (12).

Further pathophysiologic mechanisms are considered in more detail in the following paragraphs.

1.3.1 Central Sensitization

Pain involves a complex system of sensory, affective, motivational and cognitive dimensions (18). The primary element is represented by nociceptive afferent (Aδ and C) nerve fibres that react to mechanical, thermal and/or chemical stimuli, which are first transmitted to the posterior horn of the spinal cord. Via synaptic connections in the spinal cord, the pain signal is then forwarded by spinothalamic, spinoreticular and spinomesencephalic pathways to particular brain structures to cause pain perception and the other dimensions of pain experience (18). In addition, the spinoreticular and mesencephalic pathways also activate descending pathways that modulate/inhibit the afferent input to the spinal cord. Besides reaching the neocortex which enables the brain to make an anatomical mapping of pain origin and to perceive pain, pain signals also reach the limbic system which is relevant to emotional pain processing (18).

Physiologically, pain serves as a protective shield for potentially harmful exogenous stimuli. For example, if you put your hand close to a burning fire, the pain that results from the heat stimuli warns the body of a potential harmful situation (18). Pathologically, however, pain, especially chronic pain, may become a disease in its own right as reflected by the IASP term of "nociplastic pain" that is also applied to FMS (11). Chronic pain may involve both peripheral and central sensitization processes. Peripheral sensitization relates to increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields (19). Endogenous (eg leukotrienes, prostaglandins) and exogenous (eg capsaicin) noxae are able to modify the threshold of the nociceptors (19).

Central sensitization denotes increased responsiveness of pain-processing neurons in the central nervous system to their normal or subthreshold afferent input (20). Central sensitization is a key element of altered pain processing in FMS (13). Inflammation, neural injury and physical activity might trigger this pathway. Increased membrane excitability and synaptic efficacy and enhanced threshold of inhibitory pain pathways are changes that seem to be

responsible for central sensitization. Pain is therefore decoupled from the presence, intensity or duration of any noxious stimuli. Furthermore, harmless sensations can lead to pain because of altered pain sensitivity caused by central sensitization (20). Central pain sensitization appears to be driven by neuroinflammation brought about by activation of microglia which release proinflammatory cytokines and chemokines (20). This contrasts with the physiological role of microglia in the central nervous system in the support of neural precursor cells, neuroplasticity and immune defense (21).

1.3.2 Microbiome

The human microbiome is a system that inhabits multiple organs and can, broadly speaking, affect metabolism and other body functions. Owing to its extent, the microbiome in the human gut has recently attracted particular interest and research efforts. Several non-modifiable factors as well as modifiable factors shape the individual profile of each human gut microbiome and are summarized in Table 4 (22).

Non-modifiable factors	Modifiable factors
Gut architecture	Diet
Genetics	Lifestyle
Birthing history	Traumatic memories
Early feedings patterns	Interventions
Age	Prescription drugs
Geographic location	Exposure
Members of minorities	

Table 4: Non-modifiable and modifiable factors of the gut microbiome (22).

Physiologic and metabolic functions are changed by dysbiosis, which refers to an imbalanced composition and diversity of the microbial community and which can influence processes within the gut, such as gastrointestinal permeability, and outside the gastrointestinal tract, such as pain sensitivity and other brain functions (22–25). Predisposing diseases, such as diabetes mellitus, also can increase the permeability of the intestinal wall to antigens, which can disturb immune processes and result in systemic inflammation (22–25). The so called *inflammatory soup*, which is characterized by elevated metabolites of various origin including the gut microbiome, neuroactive mediators and altered cytokine production, has an impact on how noxious stimuli are perceived and processed (24,25). For example, noxious stimuli may result in an elevated response to pain (which is called hyperalgesia and may be associated with

lowered pain thresholds), while non-noxious stimuli are perceived as pain (which is called allodynia) (13).

Much research has been done on the irritable bowel syndrome (IBS), which is thought to involve a disturbed communication between the gut (including the microbiome) and the brain and is associated with visceral pain (26). This relationship between the gut microbial community and brain also predisposed me for taking a close look at the gut microbiome in FMS patients. Ford et al. (26) showed that the alpha and beta diversity of the gut micobiome of patients suffering from IBS compared to healthy controls (HC) are similar, but bacterial taxa are differentially abundant (eg Bifidobacterium, Faecalibacterium, *Lactobacillacae*, Bacteroides, *Enterobacteriacae*). Based on the microbiome composition it appears possible to predict the phenotype in IBS (diarrhea vs obstipation). The reported association between gut microbiome disturbances and altered pain processing made a worthwhile case for investigating the gut microbiome as a factor relevant to the pathogenesis of FMS.

The biggest study of the gut microbiome in FMS was performed by Clos-Garcia et al. in 2019 (27). They compared 105 patients with FMS vs. 54 HC and analyzed the gut microbiome and serum metabolites. As a result, alpha microbiome diversity and core microbiome were found to be significantly altered. Especially the abundance of Bifidobacterium and Eubacterium genera is significantly reduced, which is of main interest because these bacteria are involved in the production of short chain fatty acids (SCFAs). A deficiency of SCFA is thought to lower the production of tight junction proteins and thus to increase gut permeability, contribute to a leaky gut syndrome and promote inflammatory processes.

Another study from Israel, performed by Minerbi et al. in 2019 (28) with 77 FMS and 79 HC, took a close look at the gut microbiome and serum levels of metabolites. In a first analysis, they found no significant alterations in gut microbiome. A further analysis, performed at a higher resolution, revealed significant differences in the beta-diversity of the microbiome in FMS. Specifically, they could identify 19 differentially abundant bacteria in FMS patients vs HC. In an exemplary workflow, they correlated the clinical features of FMS (pain, fatigue, cognitive symptoms) with the gut microbiome and found quantitative associations between the abundance of several taxa and the severity of symptoms related to FMS. Furthermore, a machine learning system was able to highly predict FMS vs HC according to individual microbiome features.

Another study, performed by Freidin et al. in 2021 (29) with 113 patients suffering from chronic widespread pain and 1623 HC showed that the alpha diversity of the gut microbiome is reduced. In addition, there was a depletion of Coprococcus comes although further genetic

analysis did not prove a causal role of this bacterium in the development of chronic widespread pain.

1.3.3 Metabolomics

Another piece of the puzzle as to how FMS is brought about might be found in the large number of metabolites that are produced and released by the gut microbiome or under the control of microbial factors (30–33). Metabolites can also be used as a diagnostic tool. Current research on that topic is made in other specialities, such as cancer, cardiovascular and diabetes mellitus research. Metabolomics represent therefore an important technique to study the complex array of circulating metabolites that are altered in chronic pain syndromes (30,31). SCFAs are metabolites of the gut microbiome that have attracted much attention because they have a wide range of actions in the gut, immune system and brain (32). Although some experimental studies indicate a pronociceptive effect under certain conditions (32), SCFAs are in general thought to act rather as antiinflammatory and antinociceptive mediators (33). They have been shown to strengthen the mucosal barrier in the gut and thus to reduce the influx of factors into the gastrointestinal wall, which has a beneficial effect on the gut, the immune system, pain and depression (32,33). Vice versa, a depletion of the bacteria that produce SCFAs is envisaged to result in a deficiency of SCFAs, an increase in gut mucosal permeability (leaky gut) and adverse effects on immune system, nociception and emotional-affective processing.

There are many other metabolites and metabolism-active factors, in part related to the gut microbiome, which may be of relevance to pain syndromes. Using metabolomics, Caboni et al. (34) examined 22 FMS patients and 21 HC for differences in their metabolite profile. They reported that FMS patients can be discriminated by an excess of lysophosphocholines, which can be generated by oxidative stress with lipid peroxidation, and a lysophosphocholine-induced activation of the platelet activating factor (PAF) system. Given that PAF is known to elevate the levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNFalpha) and interleukin 1beta (IL-1beta) and to lead to pain hypersensitivity and allodynia, Caboni et al. (34) argue that lysophosphocholines could be biomarkers of FMS patients with a particular phenotype.

Fais et al. (35), analyzing 22 FMS patients vs 22 HC, focused on the role of purines in pain transmission. The purine derivative adenosine is known to have an antinociceptive effect when acting from the extracellular space. It acts on leukocytes to suppress systemic inflammation and can further reduce pain transmission via an action on spinal microglia. The study group found that a higher conversion of adenosine to inosine via an elevated activity of adenosine

deaminase diminishes the extracellular concentration of adenosine which, arguably, might be responsible for lowered pain threshold and central sensitization in FMS patients.

Malatji et al. (36,37) compared 18 patients with FMS vs 3 control groups (n=41). They found that lactic acid, hippuric acid and succinic acid, which are metabolites originating from the gut microbiome, are elevated in FMS vs HC. Furthermore, the combination of succinic acid, taurine and creatine directly correlates with pain and fatigue symptoms (measured with FIQ). The authors speculate that these metabolites, which are associated with the total energy household, might influence the gut-brain axis and in this way lead to altered pain transmission and processing.

A number of other studies have tried to identify biomarkers for chronic pain syndromes. In their study of the gut microbiome (105 FMS patients, 54 HC), Clos-Garcia et al. (27) found the serum levels of glutamate elevated in FMS patients (n=105) vs HC (n=54). As glutamate is known for its role in central sensitization, the authors argue that excess glutamate could have a role in the pain of FMS patients. Tryptophan metabolism is a process that can be influenced by the gut microbiome (22), and Hackshaw et al. (38) found that patients with FMS (n=15) differed from patients with rheumatoid arthritis and osteoarthritis (n=17) with regard to tryptophan catabolism, which advocates further research in the biomarker-based discrimination of chronic pain syndromes. In a study of female patients with neck pain (n=30) or chronic widespread pain (n=16) vs HC (n=39) by Hadrevi et al. (39), metabolomics revealed an increased abundance of arginine and aminomalonic acid in chronic widespread pain but a decreased abundance in neck pain.

1.3.4 Neuroimmunological mechanisms

Given that both immunological and neuronal mediators and processes are altered in FMS, great efforts have been undertaken to elucidate potential neuroimmunological mechanisms underlying fibromyalgia (14,20,40). However, as with metabolic factors a conclusive concept to explain FMS by a purely neuroimmunological pathophysiology has not been achieved, although immunomodulating drugs are part of the therapeutic approaches (41).

Neuropathic pain is involved in the hypersensitivity associated with FMS (14), yet how neuropathic pain is generated in this disorder is little understood. In neuropathic pain resulting from nerve lesions the expression and sensitivity of transient receptor potential channels (TRP) and sodium channels in dorsal root ganglion (DRG) neurons is dysregulated (42). Altered channel function leads to ectopic activity of nociceptive DRG neurons, which via chemokines stimulates spinal microglia to express cytokines and chemokines that contribute to a pro-inflammatory state (42). In addition, leukocytes, particularly macrophages, migrate to the site

of nerve injury and produce pro-inflammatory chemokines and cytokines, which attests to a prominent neuroinflammatory component in neuropathic pain (43).

Small fiber neuropathy is a condition that affects the small, unmyelinated nerve fibers (C-fibers) in the peripheral nervous system and can be associated with many medical conditions (14, 44 - 46). Symptoms vary and include, but are not limited to, pain, burning, tingling and numbness. Usually, hands and feet are affected first. Alterations in pain sensitivity can include both hypersensitivity (hyperalgesia) and allodynia while the sensitivity to temperature, cold and pinprick may be reduced (hypesthesia) (46). Furthermore, abnormal autonomic symptoms (such as abnormal sweating, changes in skin color, gastrointestinal symptoms) can occur (44,46). Small fiber neuropathy may also play a role in some FMS patients although the pathophysiology is not completely clear in this condition (14, 45). Several factors are discussed to contribute to the development of small fiber neuropathy, such as metabolic, infectious, toxic, inflammatory and autoimmunological disturbances (46). In an experimental study with mice, IgG were transferred from patients with FMS to mice and they consecutively expressed symptoms of FMS (47). The authors suggest that an autoimmunological process might be behind the pathophysiology of FMS and that plasmapheresis could be a strategy to treat FMS patients in future investigations (47).

Both small fiber neuropathy and FMS can be associated with particular metabolic changes and disturbances (36, 48). Such changes might also arise from an altered gut microbiome and could include an overproduction of reactive oxygen species, which causes oxidative stress and inflammation, damaging nerve fibers. Also, accumulation of toxic agents and medications have been considered to contribute to cause small fiber neuropathy (either via direct toxicity, vascular damage, or inflammation) (49).

Üceyler et al. (14) summarized and discussed a number of studies on the relationship between small fiber neuropathy and FMS. Taken together, the findings suggest a potential link between clinical characteristics of FMS and small fiber neuropathy in some FMS patients. Using immunocytochemistry with antibodies against protein gene product 9.5 (a neuronal marker) in skin biopsies it has been shown that the density of small nerve fibers in FMS patients is significantly lower than in HC (14). This suggests that some symptoms in FMS (such as chronic widespread pain evaluated, eg, by QST) might be caused by small fiber neuropathy, although this relationship is not specific for FMS (14).

Pain hypersensitivity in FMS and other chronic pain syndromes may arise from an up-regulation of specific receptors and ion channels on nociceptive afferent neurons. Among others, TRPV1, which is an ion channel responsive to capsaicin, heat, acidosis and

endovanilloids, has long been addressed as a molecular entity that contributes to hyperalgesia. Studies using experimental models of fibromyalgia in mice have provided evidence that hyperalgesia is associated with up-regulation of TRPV1 and that blockade of TRPV1 signaling reduces pain hypersensitivity (50-56). The role of TRPV1 in FMS patients has not yet been examined due to a lack of appropriate tools, but there is information from other chronic pain conditions that TRPV1 may play a role in humans as well. It need be mentioned in this context that TRPV1-expressing sensory neurons can be defunctionalized for a prolonged time by exposure to an excess amount of capsaicin, the archetypical stimulant of TRPV1. This action is used to treat neuropathic pain syndromes, such as postherpetic neuralgia, by short-term attachment of a patch containing capsaicin (8%) to the affected skin area under specific precautions to control capsaicin's acute burning effect (57).

Wouters et al. (58) hypothesized that an up-regulation of TRPV1 could be responsible for visceral hypersensitivity in IBS, which occurs in about 30-70% of patients suffering from FMS (59). They found that submucosal neurons in rectal biopsies from IBS patients diagnosed according to ROME III criteria are hypersensitive to TRPV1 and that material isolated from the gut of IBS patients is able to sensitize TRPV1 in healthy tissue (58). As this sensitization of TRPV1 can be mimicked by histamine acting via the histamine receptor H1 (HRH-1), Wouters et al. (58) treated IBS patients and HC with an HRH-1 antagonist or placebo for 12 weeks. They found that the HRH-1 antagonist resulted in a significant reduction of abdominal pain and visceral hyperalgesia, which indicates that sensitization of TRPV-1 mediated by HRH-1 is involved in IBS (58).

Lastly, immunological and inflammatory processes cannot be neglected in the pathogenesis of FMS, given that infection, immune activation and inflammation play a role in many pain syndromes. Neutrophils are the first cells to migrate towards a site of infection, injury or inflammation whereby their function and shape are dynamically changed. In an attempt to examine a role of neutrophils in FMS, I briefly explain here in which way I have addressed neutrophil activity in this thesis. A shape change is a crucial feature when neutrophils change their function. In the basal state, they have a spherical shape (60). Activation due to immunological stimuli causes morphological changes as the neutrophils attain a flattened shape. In addition, they develop pseudopods (arm-like projections) that help the cells to move towards a higher concentration of chemokines released from the site of a proinflammatory insult (60). At the same time, they begin to adhere to the wall of blood vessels, a process that is regulated by a complex and highly diverse array of signaling molecules (61). Neutrophils then migrate out of the bloodstream into tissue and destroy pathogens (60).

Neutrophils have recently been shown to play a causal role in an experimental model of fibromyalgia/chronic widespread pain (62). The model was based on consecutive intramuscular injections of the proinflammatory compound carrageenan to mice, which induced persistent mechanical hypersensitivity. This state of hyperalgesia could be transferred by blood, but not by serum, to naive recipient mice, indicating that blood cells carry the state of hyperalgesia (62). Since neutrophil depletion prevented the transfer of hyperalgesia, it was concluded that neutrophils are the cells that confer mechanical hyperalgesia to the recipient mice. A similar mechanism appears to operate in humans, given that the transfer of neutrophils from FMS patients, but not HC, to naive mice also evoked hyperalgesia in the recipient animals. Further experiments revealed that, in the mouse model, neutrophils are able to infiltrate DRGs, which led the authors to conclude that neutrophils are fundamental for the development of chronic widespread pain (62).

Abnormal changes in the shape and function of neutrophils contribute to a chronic inflammatory state and can, like neutropenia, predispose to a variety of infectious diseases (63). Leukocyte adhesion deficiencies, where the adherence to blood vessels and the ability to migrate to an infectious site is impaired, are genetically determined and are the cause for a higher incidence of infections (64). Infectious diseases are also a major clinical manifestation of Hyper-IgM-Syndromes that result from multiple gene mutations, some of which may also affect the functionality of neutrophils (65). The findings of Caxaria et al. (62) support the hypothesis that the function of neutrophils is also changed in FMS in a way that enables them to infiltrate DRGs.

1.4 Therapeutic strategies

Therapeutic strategies in FMS are published by the American College of Rheumatology and include, after a detailed explanation of the disease, exercise, psychological treatment, pharmacotherapy and multimodal rehabilitation programs (66,67).

The most important part of therapy is patient education which is crucial for effective pain management. Patients suffering from FMS must understand the condition, including the symptoms of chronic widespread pain, fatigue, sleep patterns and mental distress. Managing strategies include a resolution about the fact that FMS is not curable while early multimodal strategies can stabilize the disease. Patients must also be aware of the high incidence of co-diseases, and regular check-ups are necessary to make sure that co-diseases are treated adequately (66).

Medical treatment includes several antidepressants (duloxetine, milnacipran) that can help alleviating pain and fatigue. The older tricyclic antidepressant, amitriptyline, can at low doses promote sleep. Anti-seizure drugs that act on the α2δ subunit of voltage-dependent calcium channels (pregabalin, gabapentin) are able to reduce pain and ameliorate the symptoms of small fiber neuropathy. Over-the-counter (OTC)-medications such as ibuprofen and other non-steroidal anti-inflammatory drugs (NSAIDs) should only be used for short-time relief of non-specific back pain and be carefully monitored due to their potential adverse effects (66).

Weak opioids like tramadol are recommended for a defined time frame to relieve pain. Besides an action on opioid receptors, tramadol is also a serotonin-noradrenaline-reuptake-inhibitor (SNRI) and in this combined mechanism of action can ameliorate neuropathic pain. Due to common side effects (including nausea), it is limited to severe cases (66).

Physical therapy seems to be the most important, self-healing tool without any side effects for patients with FMS if taken on a regular basis. The WHO suggests to engage three times a week in 30 minutes of anaerobic training. In FMS, patients should try to reach that goal without exceeding their personal limits. Pacing strategies need to be learned in that manner. Furthermore, cognitive behavioral therapy can help to manage the impact of FMS and to improve coping strategies (66).

Lifestyle changes are recommended for every patient such as to do regular exercise, live on a balanced diet, have regular sleep and acquire stress managing strategies (66). Short-term medication may sometimes be necessary to obtain a regular sleep-awake cycle (66).

Alternative therapeutic strategies include acupuncture, tai chi, yoga and biofeedback which is a strategy that helps one to learn to control body functions and to gain more control over breathing, muscular tenderness and heart rate. It reduces stress, improves sleep and optimizes pain management via a higher awareness of physiological responses to pain (67).

In addition to established treatment strategies, a number of experimental therapeutic approaches are developed and tested in order to improve the quality of life of FM patients. Examples of these efforts include:

- Hyperbaric oxygen therapy (HBO) involves breathing pure oxygen in a pressurized room. This type of therapy can increase the amount of oxygen delivered to tissues, promotes healing and reduces inflammation (68).
- Based on the premise that gut microbial dysbiosis might contribute to FMS, there are ongoing trials to investigate whether transfer of fecal bacteria from healthy donors may

be beneficial for FMS patients. This strategy follows an approach already proved in IBS patients to have a therapeutic effect (69,70).

- Low-dose ketamine infusions over a defined time frame may be considered in severe cases as third-line treatment under continuous monitoring. Ketamine is commonly used in anesthesia and blocks the NMDA-type ionotropic glutamate receptor which contributes to neuropathic pain. A small study has shown that ketamine is able to reduce pain in FMS (71).
- As reviewed by Khurshid et al. (72), delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) have been shown to alleviate FMS symptoms and are considered as third-line drugs on an individual decision. The review by Khurshid et al. (72) reports that pain severity and stiffness can be diminished if patients with FMS are treated with medical cannabis and that the quality of sleep may be improved. CBD can be added as it has anti-inflammatory properties (72).

Individualized medical therapy is often necessary as a last resort. Particularly, chronic fatigue together with brain fog and bad sleep are hard to treat. Younger et al. (73) have published a small study that involved 30 patients with FMS on low dose naltrexone and showed an improved quality of life in at least 50% of patients. Besides being an opioid receptor antagonist, naltrexone is also a potent antagonist at Toll-like receptor 4 (TLR-4) on microglia. Symptoms that can be explained as a result of microglial activation can therefore be expected to be improved by low dose naltrexone (73). This observation is in line with the concept that targeting microglia in the central nervous system could be a potential future strategy to manage chronic pain syndromes (20).

1.5 Objective of the dissertation

The main objective was to explore whether monocytes or neutrophilic/eosinophilic granulocytes obtained from FMS patients react differently to immune challenge relative to healthy controls. This question can be examined by the release of proinflammatory mediators from these cells in response to lipopolysaccharide (LPS) or N-formyl-methionyl-leucyl-phenylalanine (FMLP), two factors that can originate from the human gut microbiome, especially under conditions of a leaky gut. The proinflammatory mediators might lead to sensitization of nociceptive afferent neurons, on the one hand, and central sensitization, on the other hand, both processes contributing to hyperalgesia in FMS.

The primary hypothesis under study was therefore:

- The microbiome in patients with FMS shows alterations in its composition and diversity as well as specific patterns regarding gram-negative bacteria compared to HC.

Related to the primary hypothesis I addressed a number of secondary hypotheses:

- Biochemical parameters in stool that suggest a leaky gut (alpha 1 antitrypsin, histamine, zonulin, calprotectin) are altered in patients with FMS vs. HC.
- The reactivity of neutrophilic/eosinophilic granulocytes (shape change) after stimulation with chemoattractants is altered in patients with FMS vs. HC.
- The reactivity of monocytes after stimulation with LPS or FMLP regarding secretion of prostaglandin E2 (PGE2) and tumor necrosis factor (TNF)-alpha is elevated in patients with FMS vs. HC.
- Quantitative sensory testing shows specific patterns regarding hyperalgesia and nociceptive sensitization in patients with FMS.
- The psychopathological profile of FMS patients with regard to psychological stress, anxiety and depression is altered relative to that of HC.

2 MATERIAL AND METHODS

The description of Materials and Methods is in part re-using the methods description presented in Weber et al. (12). Wherever text from Weber et al. (12) is reused in unchanged form, it is written in italics and designated by quotation marks.

"This study was a single-center, case-control study, which was performed at the Medical University of Graz, Austria, from December 2018 until December 2019. A permission of the local ethics committee (Medical University of Graz, Austria) was obtained prior to the beginning of the study (registration number: EK 31-012 ex 18/19). This study was performed according to the Declaration of Helsinki in 1964 and the current STROBE guidelines for reporting observational studies. Informed consent was obtained from all study participants" (12).

"For the study 25 patients with FMS and 26 healthy control (HC) subjects (age- and sex-matched) were recruited. FMS was diagnosed according to the criteria of the American College of Rheumatology published in 2016 (criteria catalogue and physical examination) (8) *and FMS symptom-severity was quantitated according to the Patient-Health-Questionnaire-15 (PHQ-15). The inclusion and exclusion procedure is illustrated in graph* (Figure) *2."* (12).

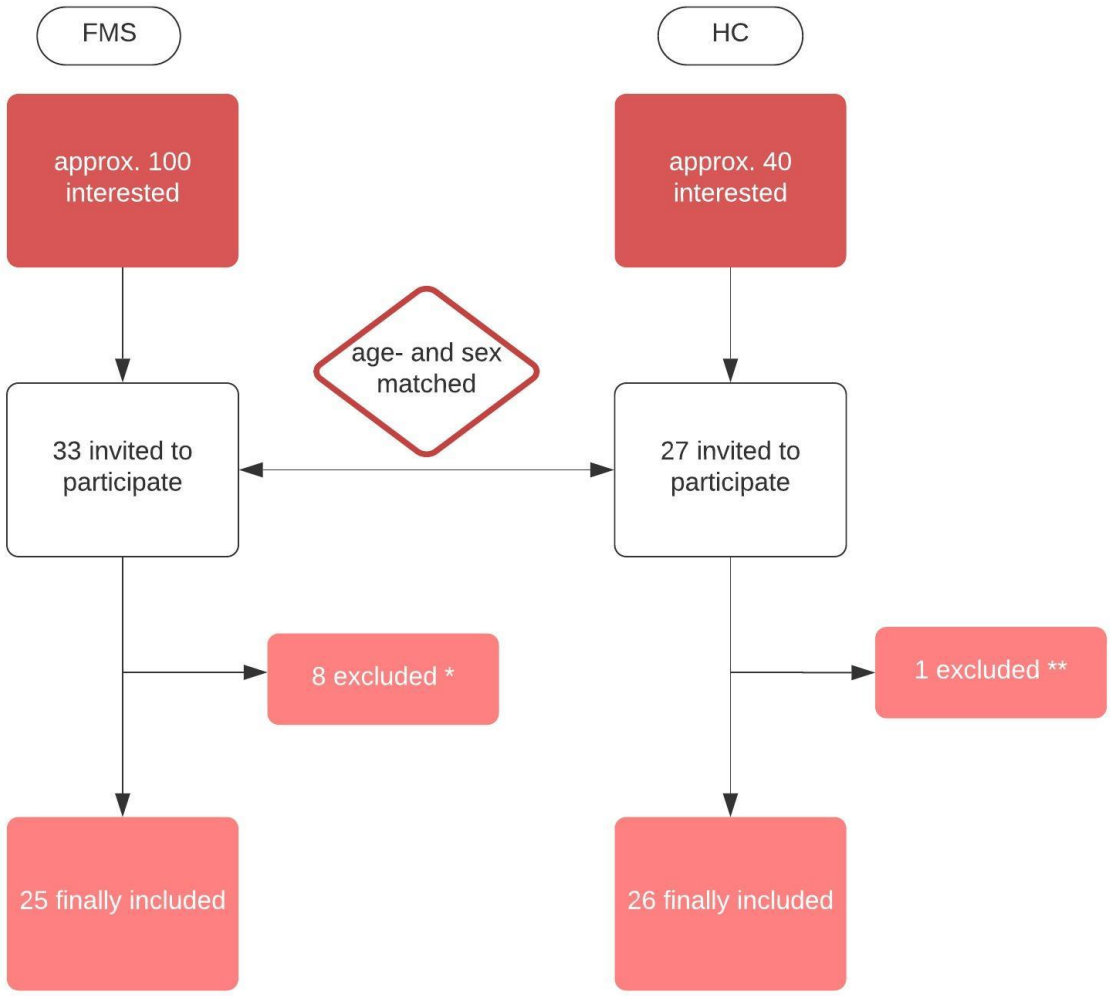


Figure 2: Flow chart of how many study participants were included/excluded in the study. * 8 subjects were excluded in the FMS group for the following reasons: 1 Ehlers-Danlos syndrome, 1 unclear autoimmune disease, 1 stroke in anamnesis, 1 acute severe pneumonia, 2 non-compliant, 2 lost contact. ** 1 was excluded in the healthy control (HC) group because of autoimmune disease. Graph reproduced from Weber et al. (12).

"FMS patients were recruited via an invitation letter at the local pain clinic. Age- and sex-matched voluntary employees at the University Hospital were asked to join the study as healthy controls. After they signed an informed consent, they had to complete one single study visit. After checking the inclusion- and exclusion criteria, all patients underwent a physical neuro-orthopedic examination to exclude reasons for musculoskeletal pain other than FMS. First, we took blood samples for analysis of immunological parameters. Patients and healthy probands then underwent quantitative sensory testing (QST). Last, the study participants were given detailed information about how to complete several questionnaires and to handle the stool samples. Patients and healthy controls were instructed to fill the stool collection tubes (4 stool

collection tubes) at home and then send them to the laboratory with a prepared, cooled package. An electronic thermometer was included for tracking a constant temperature (maximum temperature allowed +15 °C for 24 hours) at each parcel. All samples were then immediately frozen at -80° C." (12).

Inclusion criteria:

- Age 18 – 65 years
- Diagnosis of Fibromyalgia according to the criteria of the American College of Rheumatology in 2016

Exclusion criteria

- Pregnancy and current breast feeding
- Severe comorbidity in anamnesis (heart attack, stroke, cancer)
- Other rheumatologic or autoimmune diseases than FMS
- Intake of oral antibiotics within the last 2 weeks

2.1 Demographic data

"Demographic data and detailed medication anamnesis were obtained via a structured talk and documented on the German Pain Questionnaire (10)*. Furthermore, all study participants were asked to answer the validated German version of the Depression-Anxiety-Stress Scale (DASS-G)* (74)*, the ICD-10 Symptom-Rating Brief Description (ISR-10)* (75) *and the Marburg Questionnaire for Quality of Life (MbFhW)* (76)*"* (12). Patients with FMS only had to complete the Fibromyalgia Impact Questionnaire (FIQ).

"Patients and HC subjects also had to complete a questionnaire about their dietary habits. This questionnaire was derived from the Food-Frequency-Questionnaire of the Robert-Koch-Institute and asks about the most important dietary habits (12 items) for a balanced diet. (77)*"* (12).
Patients and probands were allowed to continue their dietary habits.

2.2 Microbiome analysis

"Stool samples were collected in Stool Collection Tubes with DNA Stabilizer (Stratec Molecular, Berlin, Germany) and then frozen at -80 °C. Bacterial DNA was extracted with the Maxwell RSC Blood DNA Kit (Promega, Mannheim, Germany) according to the manufacturer's instructions with slight modifications for stool samples. The stool samples were homogenized with lysis buffer on a MagNA Lyser Instrument using MagNA Lyser Green Beads (Roche

Diagnostics GmbH, Mannheim, Germany). The samples were treated then with 2.5 mg/ml lysozyme (Roth GmbH, Karlsruhe, Germany) for 30 min at 37 °C followed by digestion with 1 mg/ml proteinase K for 60 min at 56 °C. The enzyme was inactivated at 95 °C for 10 min. For the DNA isolation in the Maxwell RSC, 600 µl of lysate was taken. The concentration of DNA was determined by Picogreen fluorescence. Then, the variable V4 region of the bacterial 16S rRNA gene was amplified using the Mastermix 16s Complete PCR Kit (Molzym, Bremen, Germany) according to the manufacturer's instructions from 20 ng DNA using oligonucleotide primers 16s_515_fwd: TGCCAGCAGCCGCGGTAA and 16s_806_rev: GGACTACCAGGGTATCTAAT. Afterwards PCR products were subjected to agarose gel electrophoresis and the band of the expected length (350 nt) was excised from the gel and purified using the QiaQick (Qiagen, Hilden, Germany) gel extraction system. The amplicon DNA concentration was measured by Picogreen fluorescence.

Amplicons from 30 samples were pooled equimolarly and subjected to emulsion PCR in the Ion Chef™ Instrument according to the manufacturer's protocols using the Ion 400BP workflow and the Ion 530™ Chip Kit. Sequencing reactions were performed on the Ion GeneStudio S5 System running for 1000 flows (all reagents from Thermo Fisher Scientific, MA, USA). The sequence files were analyzed with GALAXY using the QIIME 2019.7 workflow (78-82)*."* (12)*.*

2.3 Biochemical parameters in stool

For detailed analysis of biochemical parameters in stool, two more specific stool tubes obtained from Biovis, Germany, were collected. All tubes were first handled as described in the microbiome section and stored at – 80°Celsius. They were then transported deep-frozen for analysis to Heidelberg, Germany. According to Biovis, all samples were analysed with ELISA according to standard protocols with the commercially available ELISA kit (Immundiagnostik AG, Bensheim, Germany). Biovis listed standard values that are given for healthy human beings (listed in Table 5) (83).

Alpha 1 antitrypsin	below 27.5 mg/dl
Zonulin	below 55 ng/ml
Calprotectin	below 50 mg/l
Histamine	below 959 ng/ml
Tryptophan	above 80 nmol/g

Table 5: Physiological standard values for the biochemical parameters analyzed in stool, as published by Biovis (83).

2.4 Immunological parameters

The analysis of immunological parameters was done by a biomedical specialist (Eva Tatzl).

2.4.1 Neutrophilic shape change

Blood samples (4 ml) were put in a dilution row and stimulated with increasing concentrations of leukotriene B4 (LTB4) (0.06, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15 nM) and FMLP (0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10 nM) for 4 minutes at 37° Celsius. Next, erythrocytes were lysed (buffer: ammonium chloride), and the leukocytes left were washed out (with centrifugation; 300xg) and resuspended in fixative buffer (0.25 parts of water and 0.1 parts of Facs-Flow, Cellfix; Becton Dickinson, Vienna, Austria). Then, the neutrophilic shape change was measured by fluorescence-activated cell sorting (FACS) with a FacsCalibur flow cytometer (Becton Dickinson, Mountainview, CA, USA) as an increase in forward scatter. Identification of neutrophils among other leucocytes was done in a scatter plot (with neutrophils and eosinophils together) by high side scatter in gating of the granulocyte region (Figure 3). Finally, neutrophils only were determined as cells with low autofluorescence (488 nm laser, FL-1 channel 530/30 nm) (84).

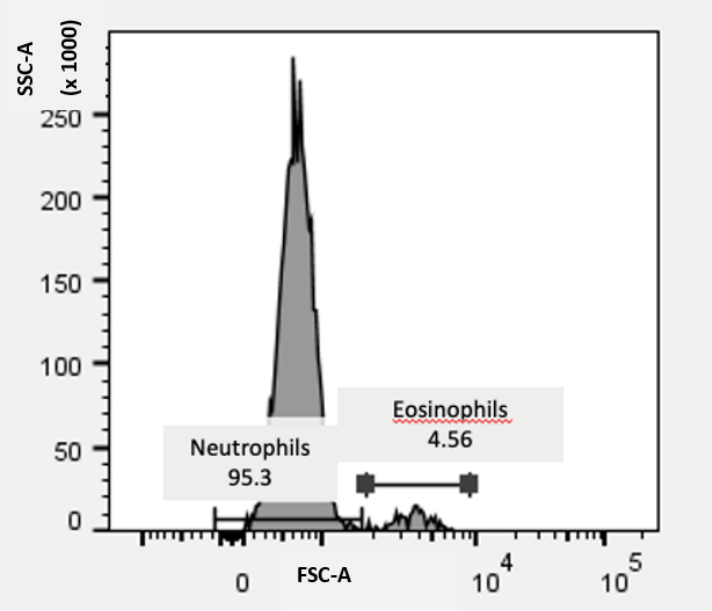


Figure 3: Neutrophils vs Eosinophils in FACS. FSC=forward scatter, SSC=side scatter.

2.4.2 PGE2 and TNF-alpha secretion of monocytes after stimulation with LPS

Whole blood (40 ml, citrated) was used for the analysis. Platelet-rich plasma was separated with centrifugation (300xg for 20 min) and erythrocytes were removed by dextran sedimentation. Peripheral mononuclear cells (monocytes, lymphocytes) were then isolated (Histopaque gradient centrifugation) and washed (phosphate-buffered saline; 5.5 mM glucose, 2.7 mM KCl). Then, the peripheral mononuclear cells were resuspended in RPMI 1640 medium, which contains penicillin (100 U/ml), streptomycin (100 U/ml), glutamine (20 mM), non-essential amino acids, HEPES (0.05 M) and sodium pyruvate (10 mM) (Gibco, Thermo Fisher Scientific, Vienna, Austria). Mononuclear cells ($0.5x10^6$/0.5 ml) were transferred to 24-well plates, and 25 µl of human AB serum was added. After adequate incubation, non-adherent cells were aspirated and removed, and then the 24-well plates were washed with phosphate-buffered saline (PBS). All adherent monocytes were cultured in 1 ml supplemented RPMI 1640 medium and then exposed to endotoxin (LPS, Escherichia coli serotype 055:B5) at a concentration of 10 ng/ml and then incubated for 24 hours. Lastly, the supernatants were collected for RIA of PGE2 (Tracer: (5,6,8,11,12,14,15(N)-3H) PGE2 from NEN, Vienna Austria) and ELISA for TNF-alpha (Kit: PeproTech, Rocky Hill, NJ, USA) (85).

2.5 Quantitative sensory testing (QST)

"QST was carried out according to the protocol of the German Network for the Treatment of Neuropathic Pain (DFNS) (86) *on the non-dominant hand. To measure heat and cold sensory levels and heat and cold pain threshold, the pain & sensory evaluation system PATHWAY (Medoc, Ramat Yishai, Israel) was used. Vibration detection threshold was measured with VSA-3000 (Medoc, Ramat Yishai, Israel), pressure detection threshold with Force dial FDK/FDN series (Wagner, Greenwich, CT, USA), mechanical-tactile threshold with von Frey filaments (MARSTOCKnervtest, Marburg, Germany), and dynamic-mechanical sensory threshold with Pinprick (MRC Systems, Heidelberg, Germany). Patients and healthy controls were always tested by the same examiner at the same room temperature, given that the ambient temperature has an influence on pain sensitivity in FMS* (86,87)." (12).

The PATHWAY system can be seen in Figure 4.

Figure 4: PATHWAY Pain & Sensory Evaluation System from Medoc Ltd. (2005). With permission from (88).

2.6 Statistical analysis

"The demographic data were described by mean, standard deviation, median, minimum and maximum as appropriate for continuous variables whereas categorical variables were described by absolute and relative frequencies. The demographic data and nutrition scores of FMS patients and healthy controls were compared by t-test and Fisher's exact test.

*Statistical analysis was performed with SPSS version 27, IBM®, $p<0.05$ was defined as statistically significant. G*Power was used for calculating the number of study participants in*

FMS and healthy controls. A sample size of 26 in each group will have 80% power to detect an effect size of 0.8 using a two group t-test with a 5% two-sided significance level (89).

For analysis of the human gut microbiome, the Galaxy web platform (http://galaxy.medunigraz.at) was used. Operational taxonomic units (OTUs) were displayed as OTU tables created with QIIME2 implementation in Galaxy (Version 2019.7) DADA2-based workflow and visualized as principal coordinates analysis (PCoA) plots, and according bar charts were also generated with QIIME2. For the taxonomic classification SILVA rRNA database ver. 132 was used. Significant differences between FMS and HC ($p<0.01$) were analyzed using the Adonis test, and significant differences in individual bacterial strains were calculated by the Kruskal-Wallis test. Canonical correspondence analysis (CCA) and differential taxa abundance analysis with DeSeq2 were performed in R according to standard protocols (90).

Raw data of QST underwent further data preparation. After checking the standard distribution with the Shapiro-Wilk test (values above $p>0.05$ were considered as a normal curve of distribution), a small constant (+0.1; Bartlett-Procedure) was added according to Rolke et al. (86) *where applicable. Standard logarithmic transformation (ln) was done for all values except paradoxical heat sensations, cold and heat pain threshold and vibration detection threshold. Parameters were then compared with ANOVA or t-test. Further data preparation for interpretation of gain or loss of function made it necessary to z-transform all values. For FMS, z-scores (z-score = $(x_{single\ participant} - mean_{norms})/SD_{norms}$) were calculated and then displayed with Excel 2016, Microsoft®. Values above 0 indicate a gain of function, values below 0 a loss of function"* (12).

Biochemical markers in stool and immunological parameters in blood were statistically evaluated with the Mann-Whitney U test if the data showed a non-parametric distribution, and with one-way ANOVA if the data distribution was normal as was the case with zonulin.

3 RESULTS

In this section, some original results are shown that have previously been published in Weber et al. (12). Wherever text from Weber et al. (12) is reused in unchanged form, it is written in italics and designated by quotation marks.

3.1 Demographic data and general clinical assessment

3.1.1 Demographic data

Main demographic characteristics are shown in Table 6. Both FMS patients and healthy control subjects were comparable regarding sex, age and BMI, but significantly more patients with FMS than HC (6 vs 0, respectively) reported smoking habits.

	FMS N=25	**Healthy controls** N=26	**p**
Demographics			
Female, n (%)	22 (88)	21 (81)	.69
Age in years, mean (SD)	49.8 ±8.6	50.0 ±8.0	.91
BMI kg/m², mean (SD)	25.6 ±5.6	23.8 ±4.0	.11
Smoker, n (%)	6 (24)	-	.01 (*)

Table 6: Demographic data characterizing patients with FMS and healthy controls. The data have previously been presented in Weber et al. (12).

3.1.2 Clinical assessment and characteristics of FMS

All patients with FMS were characterized according to the criteria of the College of Rheumatology published in 2016 (8). For this purpose a standardized questionnaire (see appendix) was used that included a Symptom Severity Index and a Regional Pain Index. According to this, I could divide the patients into mild vs. severe FMS (13 vs. 12, respectively). Data regarding the Symptom Severity Index, the Regional Pain Index and the Patient Health Questionnaire 15 (PHQ-15) are displayed in Table 7. On average, patients with FMS reported a duration of 12.4 years since the onset of symptoms. Many of them were able to find a reason or reasons for FMS, which are displayed in Table 8. I also asked about the working status, and

only 14 patients with FMS were currently employed, all others were either retired or obtained help from a local authority.

NRS	6.1 (1.7)
Symptom Severity Index	8.5 (2.0)
Regional Pain Index	11.9 (2.8)
Score	20.4 (4.1)
PHQ-15	16.4 (3.7)

Table 7: Characterization of patients with FMS. Numeric Rating Scale (NRS; range 0-10; 10 is maximum pain) is the current pain score at the study visit. Score is the calculated sum of Symptom Severity Index (0-12 points) and Regional Pain Index (0-19 points). PHQ-15 is the Patient Health Questionnaire 15.

Trigger for FMS	n
Death of family member	1
Emotional distress	10
Rheumatologic disease	2
Lyme disease	4
Mumps meningitis	1
Shoulder surgery	1
Birth	1
Epstein-Barr-Virus	1
Accident	1

Table 8: Patient-reported primary reasons as a trigger of FMS; 3 subjects were unable to report a trigger.

3.1.3 Nutrition

In Table 9, the dietary habits and the nutrition score (as described in section 2.1) of the study subjects are displayed. I was unable to find any differences regarding an omnivore/vegetarian/vegan diet between the research groups.

	FMS N=25	Healthy controls N=26	p
Diet	**Nutrition score**		
Omnivore, n (%)	17 (68)	21 (84)	.16
Vegetarian, n (%)	7 (28)	5 (16)	.25
Vegan, n (%)	1 (4)	-	.50
Nutrition Score, mean (SD)	5.52 ±1.39	4.32 ±1.49	.78

Table 9: Dietary habits of patients with FMS vs. healthy controls. The data have previously been presented in Weber et al. (12).

3.1.4 Medication status

Results regarding medication anamnesis are displayed in Table 10. I found a significantly higher number of patients with FMS under antidepressant drugs, proton pump inhibitors (PPI) and THC/CBD.

	FMS N=25	Healthy controls N=26	p
Medications			
NSAIDs, n (%)	17 (68)	8 (31)	.17
Antidepressants, n (%)	9 (36)	2 (8)	.01(*)
Antihypertensive Drugs, n (%)	5 (20)	2 (8)	.12
PPI, n (%)	6 (24)	2 (8)	.01(*)
Antibiotics, n (%)	3 (12)	-	.20
THC/CBD, n (%)	10 (40)	-	.01(*)

Table 10: Current medication intake of the study subjects (within the last 3 months and currently active). NSAIDs = non-steroidal anti-inflammatory drugs; PPI = proton pump inhibitors; THC = tetrahydrocannabinol; CBD = cannabidiol. The data have previously been presented in Weber et al. (12).

3.1.5 Psychometric questionnaires

In Table 11, all results obtained with the psychometric questionnaires are displayed. The scores for depression, anxiety, stress as well as symptom rating were significantly higher in the FMS group vs. HC whereas the quality of life rating was significantly lower.

	FMS N=25	Healthy controls N=26	p
Psychometric questionnaires			
Depression, Median (IQR)	6.90 (8.00)	1.25 (1.00)	.01(*)
Anxiety, Median (IQR)	7.39 (5.00)	1.25 (1.75)	.01(*)
Stress, Median (IQR)	11.20 (8.00)	2.45 (3.00)	.01(*)
Symptom Rating, Median (IQR)	1.06 (0.84)	0.30 (0.31)	.01(*)
Quality of Life, Median (IQR)	16.80 (13.00)	37.64 (6.00)	.01(*)
Fibromyalgia Impact Questionnaire (IQR)	49.03 (14.5)	-	

Table 11: Calculated scores from several questionnaires (DASS, MbFhW, FiQ). The data have previously been presented in Weber et al. (12).

3.2 Microbiome analysis

"Stool samples were sequenced with a total of 3,491,933 reads and an average of 68,838 ± 73,362 reads per sample. Detailed analysis showed that the Shannon alpha-diversity, Evenness vector, Faith's phylogenetic diversity and observed OTUs (Table 12) did not differ between the two groups.

The distribution of bacterial taxa in FMS patients and healthy controls is displayed in Figure 5.

Although subtle differences in the distribution of bacterial taxa between the two groups were observed, these differences were not statistically significant (Table 13).

Canonical correspondence analysis (CCA) of the bacterial composition in FMS patients and healthy controls was also unable to disclose a significant difference (Figure 5). Furthermore, differential taxa abundance analysis with DESeq2 (Figure 6) likewise failed to reveal clinically relevant significant differences between the bacterial strains in either study group." (12)

	FMS	Healthy controls	p
Alpha-diversity			
OTUs	194.85 (42.98)	197.99 (49.69)	.81
Faith-PD	16.07 (2.71)	16.13 (2.98)	.95
Evenness vector	0.73 (0.05)	0.73 (0.05)	.96
Shannon Diversity	5.59 (0.56)	5.59 (0.56)	.93

Table 12: Alpha-diversity statistics for the microbiome in FMS patients vs. healthy controls. The values shown are means, with SD given in parenthesis. P-values were calculated with Kruskal-Wallis test. The data have previously been presented in Weber et al. (12).

	FMS	Healthy controls	F-Statistics	p
Beta-diversity				
Bray-Curtis	0.99±0.01	0.99±0.02	0.0238	.238
Jaccard distance	1.0±0.01	0.99±0.02	0.0215	.357
Unweighted unifracture	0.15±0.03	0.15±0.03	0.0231	.259
Weighted unifracture	0.53±0.11	0.52±0.12	0.0208	.425

Table 13: Statistical analysis of microbial beta-diversity in FMS patients vs healthy controls. The values shown are means with SD. ANOVA was used to calculate p-values. The data have previously been presented in Weber et al. (12).

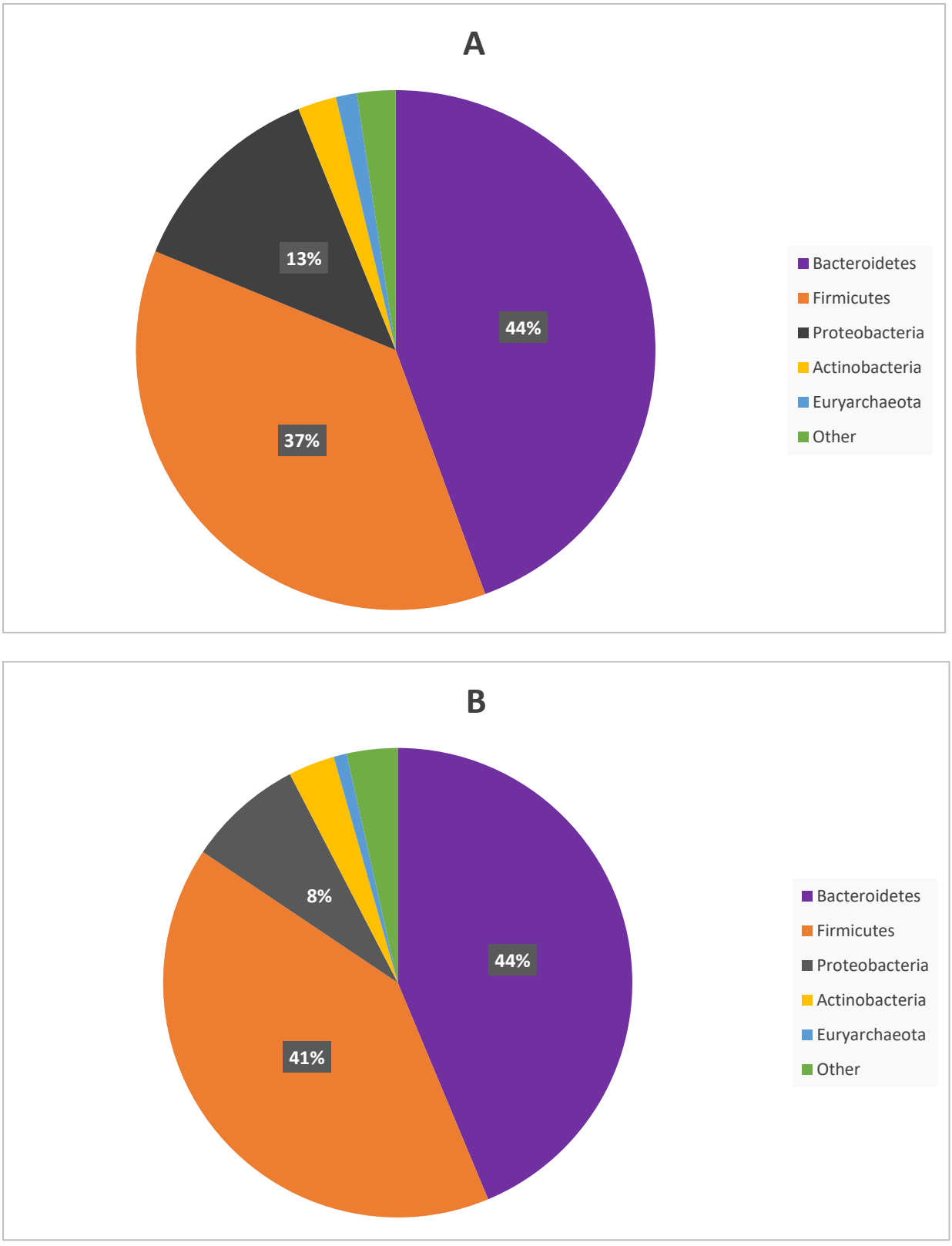


Figure 5: Taxonomic composition of bacterial phyla in FMS patients (A) and healthy controls (B). In FMS, most dominant were Bacteroidetes (44%), followed by Firmicutes (37%) and Proteobacteria (13%). In healthy controls, the predominant phyla were Bacteroidetes (44%), Firmicutes (41%) and Proteobacteria (8%). Graph reproduced from Weber et al. (12).

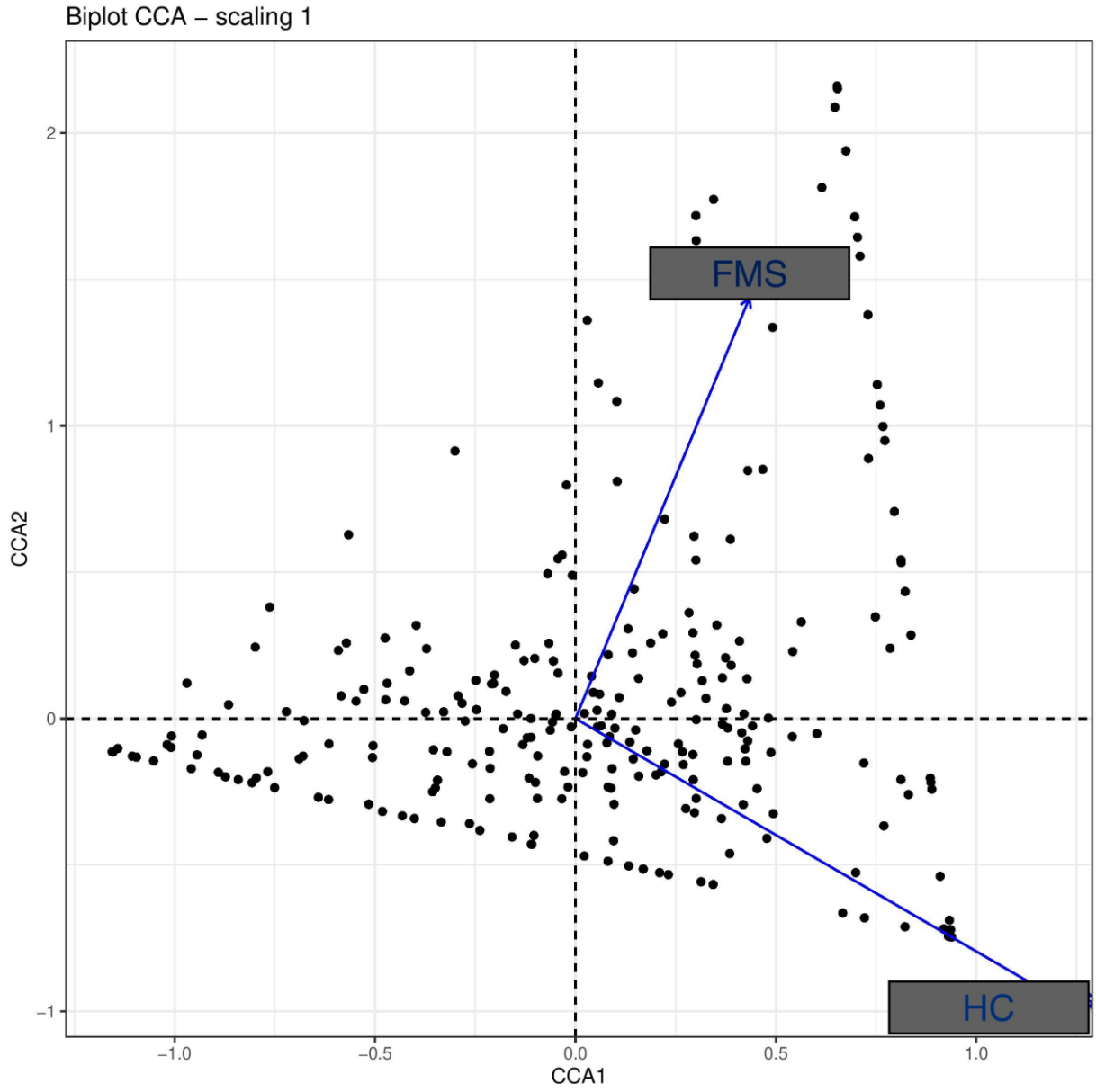


Figure 6: Canonical correspondence analysis of the microbiota in FMS patients and healthy controls (HC), which failed to reveal a distinct “cluster” to each group. Graph reproduced from Weber et al. (12).

3.3 Biochemical parameters

All biochemical parameters (alpha 1 antitrypsin, calprotectin, histamine, zonulin and tryptophan) that were analyzed from stool samples failed to display significant differences between the two study groups (Figures 7-11). Zonulin was the only parameter that showed a normal data distribution which was subjected to ANOVA analysis whereas the data for the other parameters were analyzed with the Mann Whitney-U test. Zonulin was also the only

analyte that tended to be decreased in FMS patients relative to HC, although in a non-significant manner. After primary analysis, extreme values (values outside ± 2 SD) were excluded and the data again subjected to statistical analysis, which also was unable to show a significant difference. Physiological standard values, as stated by Biovis ®, are given in the legends of Figures 7-11 for comparison.

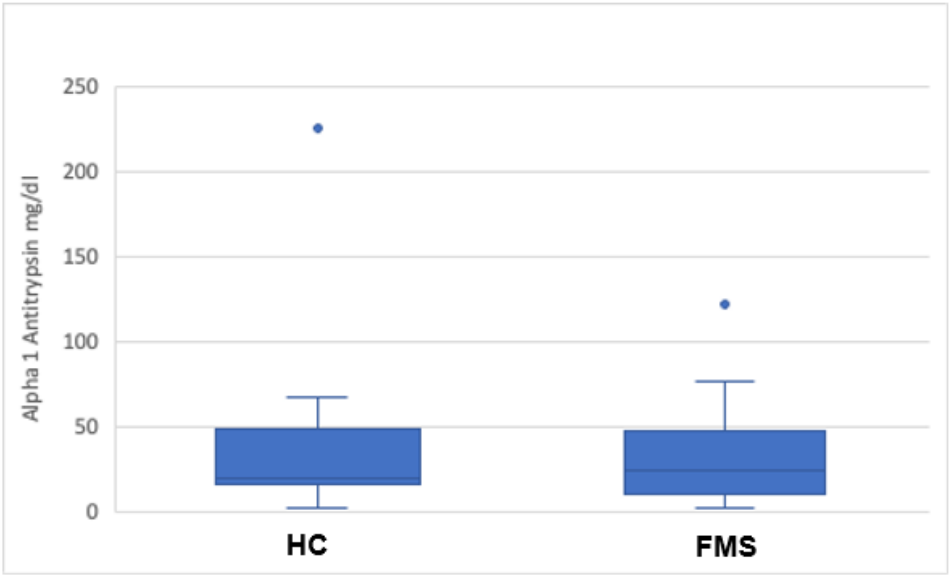


Figure 7: Statistical analysis of alpha 1 antitrypsin levels in stool samples from healthy controls (HC) (n=25) vs. FMS (n=24). Mann-Whitney-U Test was used to calculate the p-value (p= .83). Mean values of FMS vs. HC are 33.39 (SD ±29.60) and 36.07 (SD ±43.78), respectively. Physiological values are below 27.5.

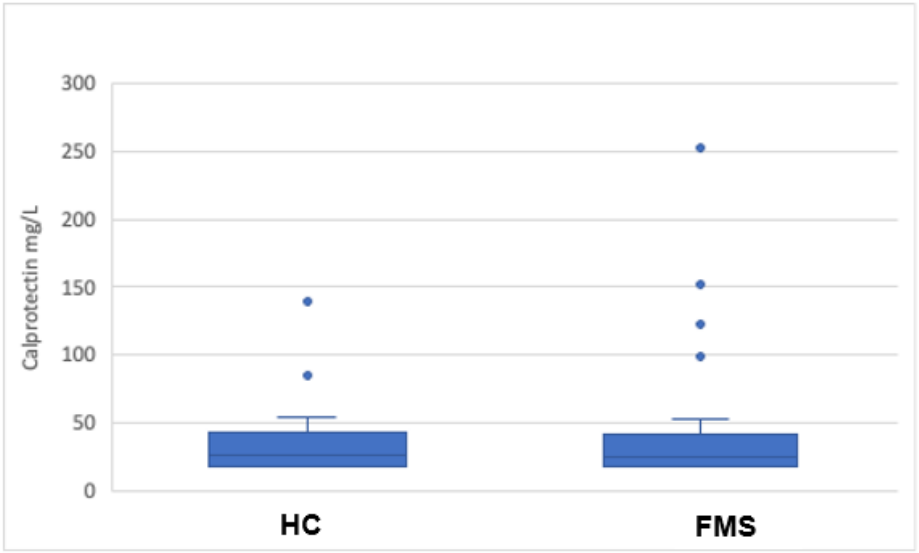


Figure 8: Statistical analysis of calprotectin levels in stool samples from healthy controls (HC) (n=25) vs. FMS (n=24). Mann-Whitney-U Test was used to calculate the p-value (p= .69), mean values of FMS vs. HC are 35.30 (SD ±26.86) and 48.71 (SD ±55.91), respectively. Physiological values are below 50.

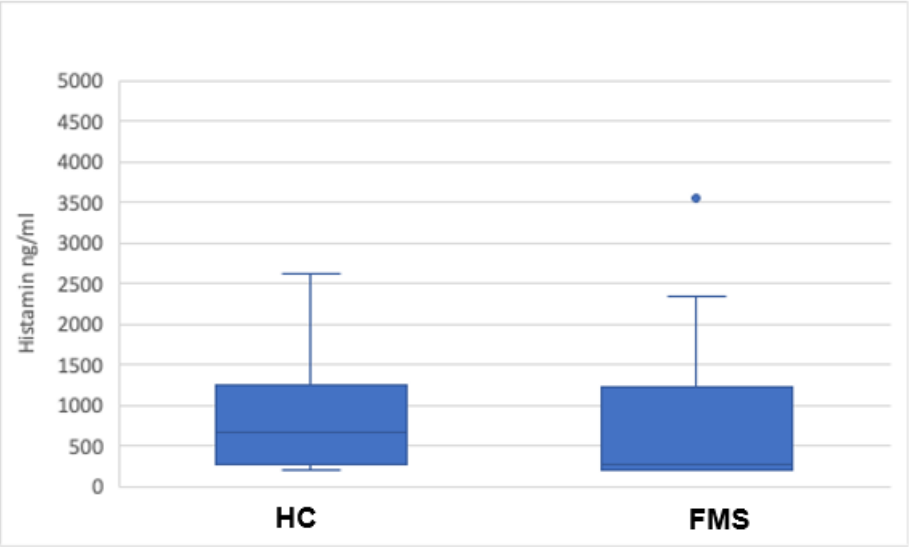


Figure 9: Statistical analysis of histamine levels in stool samples from healthy controls (HC) (n=25) vs. FMS (n=24). Mann-Whitney-U Test was used to calculate the p-value (p= .76). Mean values of FMS vs. HC are 1957.56 (SD ±4833.66) and 786.81 (SD ±895.41), respectively. Physiological values are below 959.

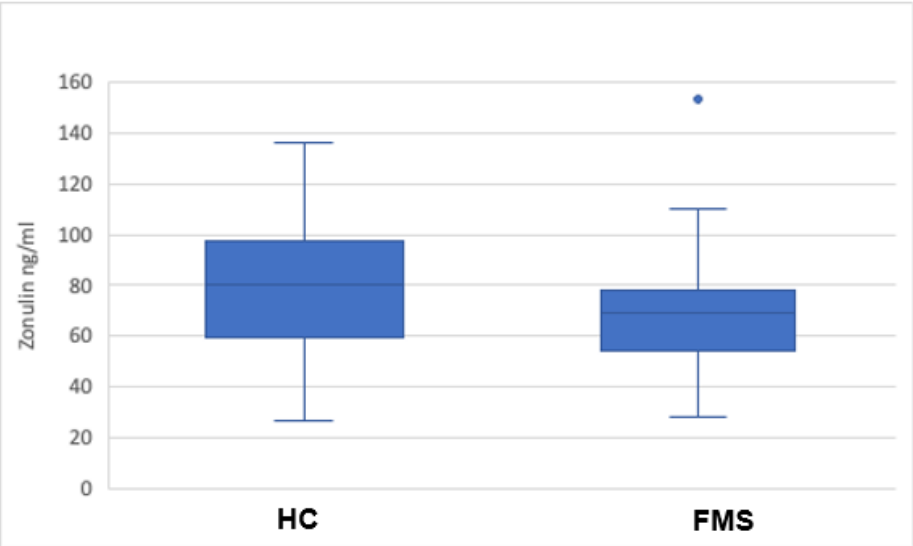


Figure 10: Statistical analysis of zonulin levels in stool samples from healthy controls (HC) (n=25) vs. FMS (n=24). ANOVA was used to calculate the p-value (p= .27). Mean values of FMS vs. HC are 78.26 (SD ±28.42) and 70.22 (SD ±26.68), respectively. Physiological values are below 55.

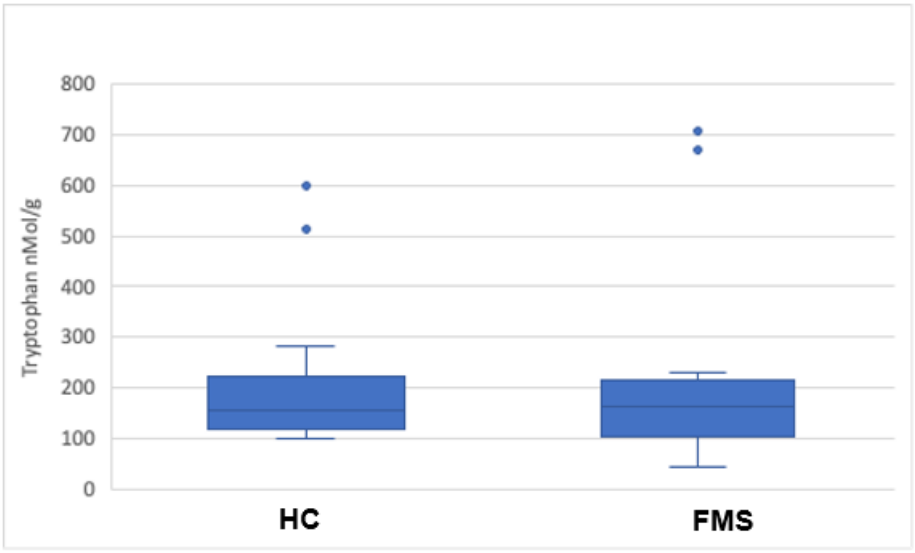


Figure 11: Statistical analysis of tryptophan levels in stool samples from healthy controls (HC) (n=25) vs. FMS (n=24). Mann-Whitney-U Test was used to calculate the p-value (p= .50). Mean values of FMS vs. HC are 193.03 (SD ±121.73) and 211.78 (SD ±181.63), respectively. Physiological values are above 80.

3.4 Immunological parameters

3.4.1 Neutrophilic/eosinophilic shape changes

Neutrophilic and eosinophilic shape changes stimulated by FMLP and LTB4, as displayed in Figures 12-15, were not significantly different between the two study groups.

The data were analyzed with ANOVA for repeated measurements, followed by Greenhouse-Geisser correction for significant spheres as the results displayed a normal distribution. The calculated p-values (as stated in each graph) did not reach statistical significance with regard to the concentration-dependent stimulation effects and the time-dependent neutrophilic/eosinophilic shape changes.

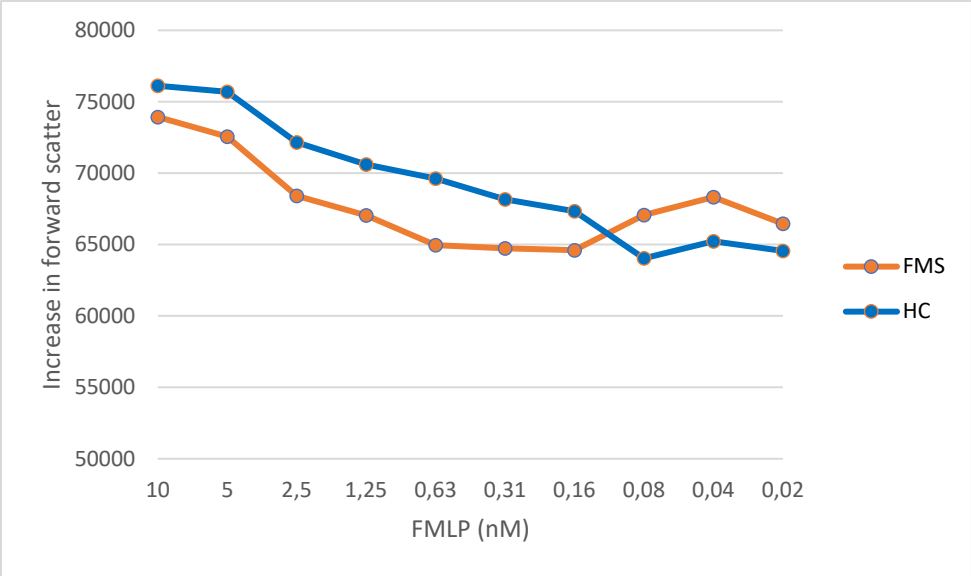


Figure 12: Stimulation of eosinophilic granulocytes with FMLP in a dilution row with diminishing concentrations. The values shown are geometric means of FMS (n=25) and HC (n=26).

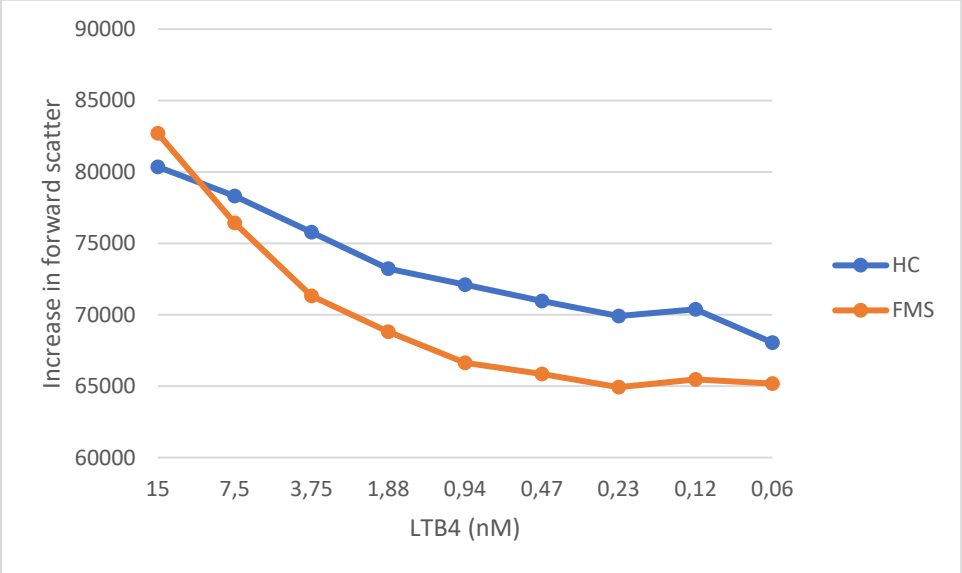


Figure 13: Stimulation of eosinophilic granulocytes with LTB4 in a dilution row with diminishing concentrations. The values shown are geometric means of FMS (n=25) and HC (n=26).

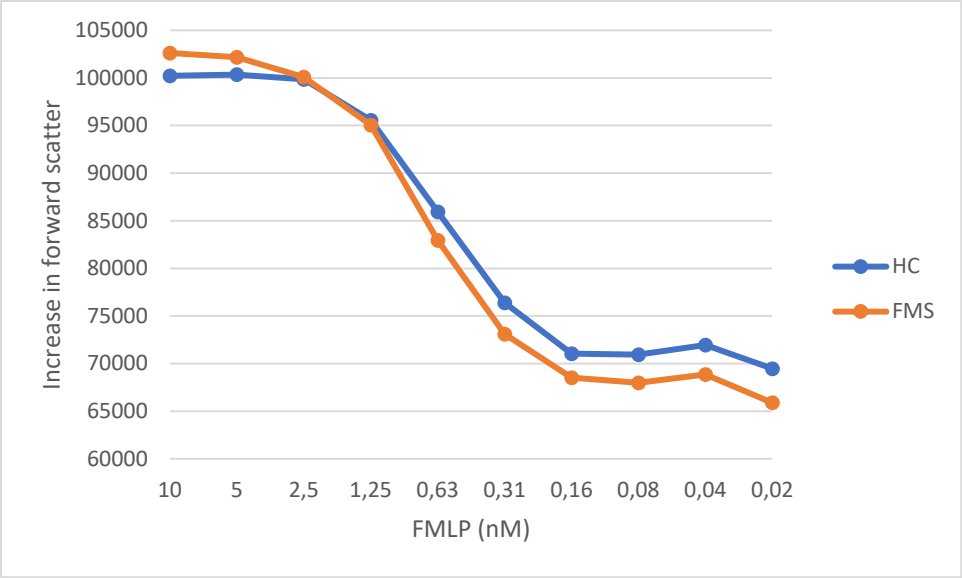


Figure 14: Stimulation of neutrophilic granulocytes with FMLP in a dilution row with diminishing concentrations. The values shown are geometric means of FMS (n=25) and HC (n=26).

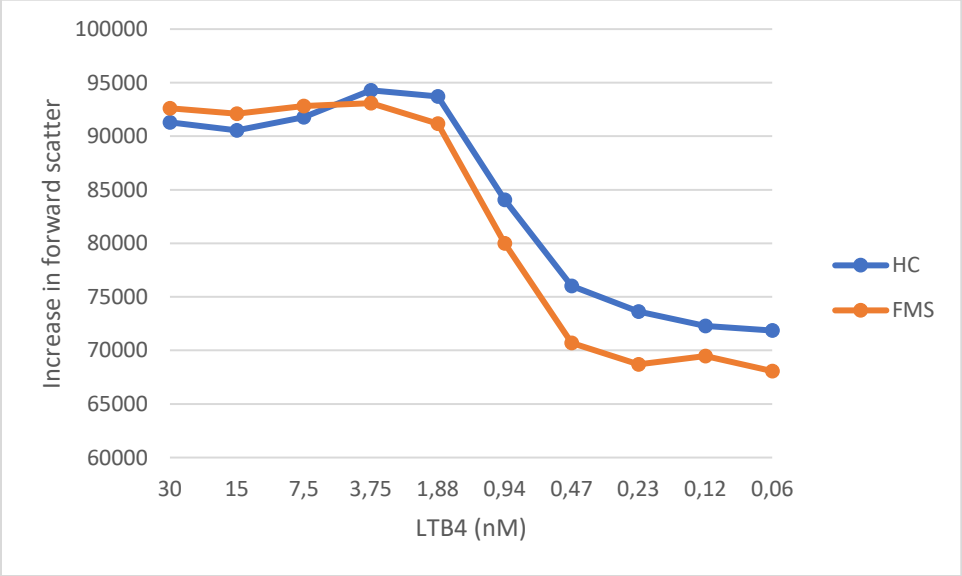


Figure 15: Stimulation of neutrophilic granulocytes with LTB4 in a dilution row with diminishing concentrations. The values shown are geometric means of FMS (n=25) and HC (n=26).

3.4.2. Stimulation of monocytes

The ability of monocytes to secrete PGE2 or TNF-alpha in response to LPS stimulation (Figures 16 and 17, respectively) tended to be increased in FMS but did not differ to a significant extent between the two study groups.

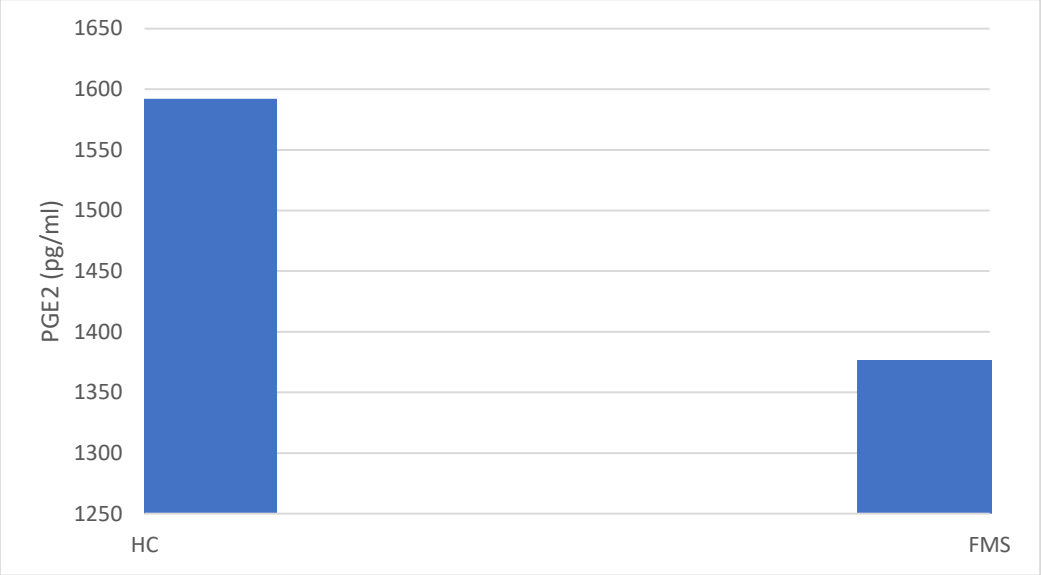


Figure 16: Secretion of PGE2 from monocytes stimulated by LPS. Statistical analysis of the normally distributed data points by two sample t test did not disclose any statistically significant difference (p-value of .523). The median for FMS (n=25) is 1377 pg/ml (SD ±202) and 1592 pg/ml (SD ±267) for HC (n=26).

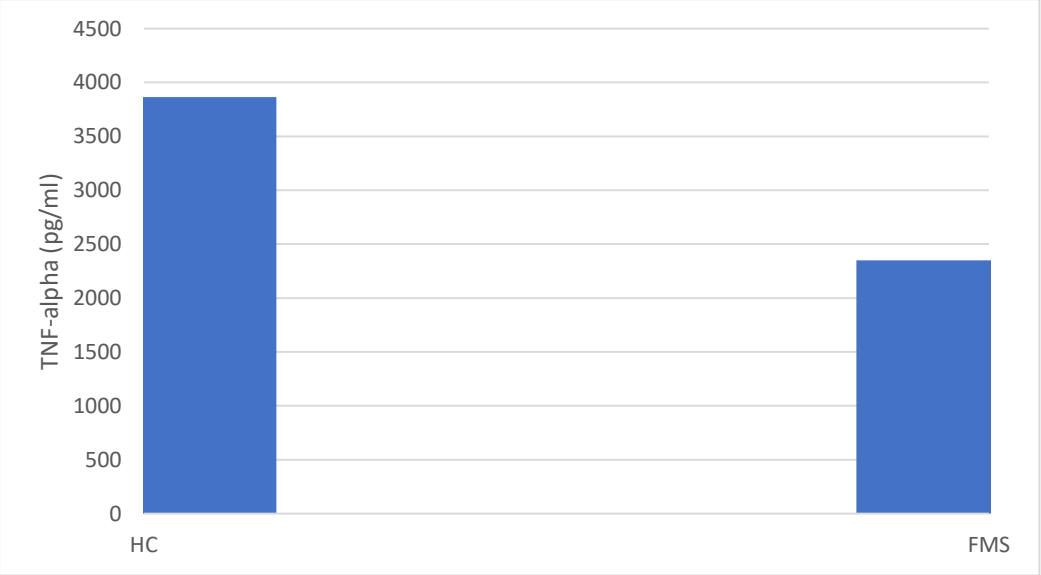


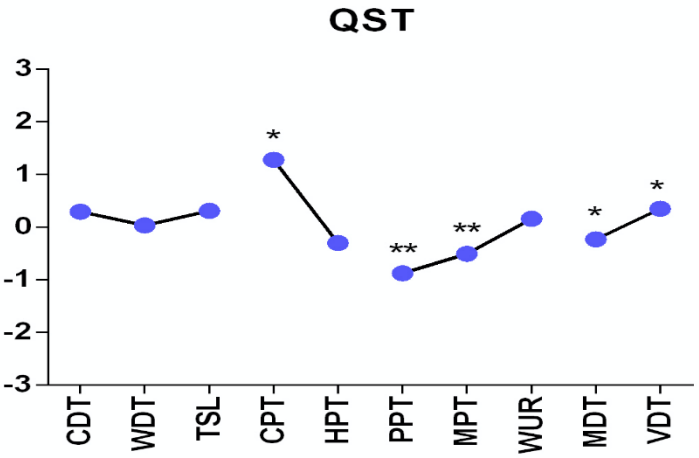
Figure 17: Secretion of TNF-alpha from monocytes stimulated by LPS. Statistical analysis of the non-parametrically distributed data points by the Mann-Whitney U test did not disclose any statistically significant difference (p-value of .426). The median for FMS (n=25) is 2350 pg/ml (SD ±272) and 3865 pg/ml (SD ±876) for HC (n=26).

3.5 Quantitative sensory testing

"QST was able to disclose significant differences in the cold pain threshold (p=.05), pressure pain threshold (p< .001), vibration detection threshold (p=.05), mechanical detection threshold (p=.05), mechanical pain threshold (p< .001) and dynamic mechanical allodynia (p< .001) between FMS patients and HC subjects (Table 14). To set all variables measured in the two populations in relation, I calculated z-values and display them in Figure 18." (12).

QST parameter	FMS (n=25)	HC (n=26)	p
CDT °C	30.82 (0.77)	30.59 (0.76)	.08
WDT °C	34.26 (1.50)	34.22 (1.17)	.85
CPT °C*	18.15 (8.91)	9.09 (6.71)	.001(*)
HPT °C	41.06 (3.46)	42.02 (3.15)	.51
TSL °C	33.44 (1.41)	33.06 (1.21)	.44
MDT mN	0.99 (0.69)	1.16 (0.74)	.48
MPT mN*	32.94 (36.28)	49.82 (51.63)	.04(*)
MPS PR*	5.78 (8.95)	1.10 (2.15)	.001(*)
DMA PR*	1.52 (1.36)	0.16 (1.29)	.002(*)
PHS n	0.12 (0.44)	0 (0)	.15
WUR PR	3.45 (3.38)	2.80 (1.17)	.91
PPT kPa*	2.08 (0.66)	2.91 (1.13)	.003(*)
VDT μm*	0.82 (0.74)	0.66 (0.47)	.04(*)

Table 14: Results of quantitative sensory testing (QST) of FMS patients vs healthy controls (HC, raw data). The values shown are means, with SD given in parenthesis; * indicates a significant p-value (ANOVA). CDT=cold detection threshold, WDT=warm detection threshold, CPT=cold pain threshold, HPT=heat pain threshold, TSL=temperature sensory limen, MDT=mechanical detection threshold, MPT=mechanical pain threshold, MPS=mechanical pain sensitivity, DMA=dynamic mechanical allodynia, PHS=paradoxical heat sensations, WUR=wind up ratio, PPT=pressure pain threshold, VDT=vibration detection threshold. The data have previously been published by Weber et al. (12).



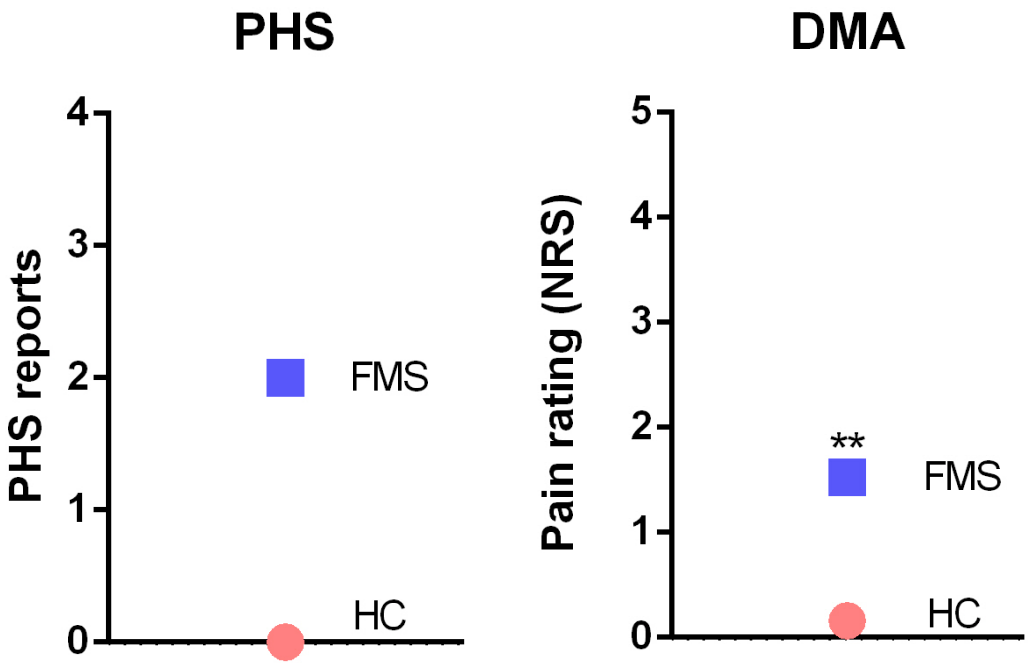


Figure 18: Z-adjusted values of the variables measured in quantitative sensory testing (QST) including singular reports for PHS and DMA (FMS vs HC with 1.52 (SD±1.36) and 0.16 (SD±1.26), respectively) in FMS patients (n=25) relative to HC (n=26). * p< .05, **p< .001 (ANOVA). CDT=cold detection threshold, WDT=warm detection threshold, TSL=temperature sensory limen, CPT=cold pain threshold, HPT=heat pain threshold, PPT=pressure pain threshold, MPT=mechanical pain threshold, WUR=wind up ratio, MDT=mechanical detection threshold, VDT=vibration detection threshold, PHS=paradoxical heat sensations, DMA=dynamic mechanical allodynia. Graph reproduced from Weber et al. (12).

4 DISCUSSION

In this section, wherever text from Weber et al. (12) is reused in unchanged form, it is written in italics and designated by quotation marks.

The main objective of this thesis was to explore novel mechanisms which could be relevant to the pathophysiology of FMS. The primary hypothesis addressed in my study was to examine whether the gut microbiome is altered in FMS patients. If so, this change could alter the delivery of immunostimulant factors such as LPS or FMLP from the gut microbiome to the immune system, especially under conditions of a leaky gut. LPS and FMLP stimulate monocytes and neutrophilic/eosinophilic granulocytes to release proinflammatory mediators. This process might lead to sensitization of nociceptive afferent neurons, on the one hand, and central sensitization, on the other hand, both processes contributing to hyperalgesia in FMS patients.

In the present study, however, I was unable to find any substantial evidence for a contribution of the human microbiome to the pathophysiology of FMS. Neither was the gut microbiome of FMS patients in its composition and diversity changed to any significant extent, nor did I see any evidence for an increase in mucosal permeability (leaky gut) as deduced from a number of markers analyzed in the stool of FMS patients relative to HC. Further on, the responsiveness of monocytes and neutrophil granulocytes to chemoattractant/immunostimulant factors was found unchanged in FMS patients as compared to healthy control subjects. These observations make it unlikely that a change in the gut microbiome and any immunological consequences arising from such a change play a significant role in the pathophysiology of FMS. In contrast, QST was clearly able to show peripheral as well as central pain sensitization in patients with FMS, these nociplastic alterations going along with enhanced scores of stress, depression and anxiety. In the following paragraphs I will discuss the findings of the thesis project in detail, elaborate the conclusions that can be drawn, consider limitations of my approach and point to further research in elucidating the pathophysiology of FMS.

4.1 Gut microbiome

The composition of the gut microbiome (alpha and beta diversity) in FMS patients did not significantly differ from that of HC subjects. Especially gram-negative bacteria, which are the major source of LPS, did not differ between the study groups. Likewise, analysis of potential differences in the microbial transcriptome via DeSeq2 failed to reveal any significant differences between FMS and healthy controls. Lastly, CCA was unable to disclose any particular pattern differences between the two study groups. Although a few studies have addressed the gut microbiome of FMS patients, there is currently no general consent on how

the gut microbiome is changed in FMS. Different workups of stool samples, different sizes of the study groups as well as differences in the analysis methods make it difficult to compare the results of the available studies with each other in a meaningful manner.

In a recent study, Minerbi et al. (28) evaluated the relationship between the gut microbiome and other possible factors that might interact in FMS pathology. In an elegant study design, they identified several positive correlations between bacterial strains and FMS pathologies. They used a LASSO machine learning algorithm, which was able to detect FMS with a high prediction accuracy only based on individual gut microbiome features. The authors (28) found 19 specific species that were differentially abundant: especially F. prausnitzii, B. uniformis, P. copri and Blautia faecis were well characterized. The abundance of F. prausnitzii was decreased in FMS (28), much as this bacterium is also depleted in other gastrointestinal disorders such as IBS (91). It is a producer of the SCFA butyrate which plays a key role in maintaining health of the colon. Specifically, it helps maintaining the integrity of the gut barrier and has anti-inflammatory effects. A deficiency of butyrate may lead to a leaky gut (22).

"While in the study of Minerbi et al. (28) the overall population structure and diversity of the microbiome in FMS patients was relatively similar to those in healthy reference subjects, the study Clos-Garcia et al. (27) revealed that larger cohorts of FMS patients are needed to detect FMS-related differences especially in the beta-diversity of the fecal microbiome and to identify bacterial taxa that are up- or down-regulated" (12).

In their study of the microbiome in FMS, Clos-Garcia et al. (27) also took a close look at neurotransmitter metabolism. They revealed significantly elevated serum levels of glutamate in FMS. This might be due to a depletion of Bifidobacteria and Lactobacillus in the gut microbiome, which are involved in transforming glutamate into gamma-aminobutyric acid (GABA) (92). Glutamate enters directly the blood stream probably because of an impaired intestinal barrier (leaky gut). Other studies have found increased levels of glutamate in the cerebrospinal fluid of FMS patients (93), which is of note because glutamate is thought to be involved in pain processing of FMS patients (93). However, the absolute levels of glutamate and GABA in brain tissue of FMS patients have been found unaltered (94). Nonetheless, there is experimental information that the gut microbiome can indirectly influence the GABA system in the brain (95). Thus, manipulation of the gut microbiome of mice alters the expression of different GABA receptors in various regions of the brain. The interaction between gut microbiome and brain is mediated by the vagal afferent neurons that project to the nucleus tractus solitarius in the brainstem, and this vagal afferent input is associated with changes in anxiety- and depression-related behavior (95). In addition, GABA in the nucleus tractus

solitarius can also stimulate output to the dorsal motor nucleus of the vagus, resulting in a decrease of activity in efferent vagal nerve fibers to the gut (96).

Further data are upcoming that suggest a potential implication of the gut microbiome in other chronic pain syndromes. For instance, a recent study by Freidin et al. (29) showed that a decreased alpha-diversity of the gut microbiome could be involved in the genesis of chronic widespread pain, just as in fibromyalgia, although the study was underpowered to show a causal relationship. Broadly speaking, there is a basic consensus that a higher diversity of the gut microbiome is beneficial for health although this generalization may not be universally applicable, depending on which specific microbial taxa are abundant. Accumulation of health deficits and the development of diseases may lead to a lower alpha-diversity of the gut microbiome, but a causal relationship has thus far been established only to a limited extent. Nevertheless, medical and non-medical interventions that directly or indirectly target the gut microbiome (eg diet, exercise, pro-biotics, drugs for microbiome targeting, fecal microbial transfer) are considered to have a beneficial health effect (97,98).

As stated earlier, FMS often coexists with IBS (59). In both disorders, inflammatory and immune factors are altered. The elevated levels of pro-inflammatory cytokines (20,26) might be related to alterations in the gut microbiome as in patients with IBS a lower diversity of the gut microbiome has been reported. Elevated levels of *Enterobacteriacae* (which are harmful) and lower levels of Lactobacilli and Bifidobacteria may lead to an increased intestinal permeability (99) and consequently to immune activation. Lastly, both disorders, FMS and IBS, are more prevalent in “families”, suggesting a genetic component. In FMS, for instance, the catechol-o-methyltransferase (COMT) gene and the serotonin (5-hydroxytryptamine) transporter (SERT) gene display symptom-related polymorphisms (100). In IBS, a polymorphism in the SERT gene likewise plays a role in the manifestation of the disorder (101). It awaits to be clarified to which extent these human gene polymorphisms have an impact on the gut microbiome. Currently, the FIDGIT study is running and the study protocol is published (102). It is the first study that aims at analyzing the relationship between FMS, gastrointestinal function (alterations without a structural basis), dietary intake and microbiome. It is performed as an observational case-control study with at least 100 participants (102). In a review envisaging the FIDGIT study, Erdrich et al. (103) stated that although some studies show subtle alterations in the microbial community of FMS patients, the gut microbiome remains a largely underexplored area in its relevance to chronic pain. The only problem, as suggested by Erdrich et al., is that many studies tend to regard IBS as a single entity without any subtypes, the most common symptoms being functional dyspepsia, functional constipation and diarrhea (103).

The microbiome results of my study tend to agree with the conclusion drawn by Erdrich et al. (103) and in addition may indicate that the gut microbiome is unlikely to be a particularly relevant factor in the manifestation of FMS, a conclusion that is also supported by the negative findings concerning an increase in mucosal permeability and any difference in the susceptibility of monocytes and neutrophilic granulocytes to chemoattractant/immunostimulant factors.

It should not go unnoticed, however, that this conclusion has a number of limitations because the composition and diversity of the gut microbiome can be modified by many endogenous and exogenous factors. Such influences may in part mask alterations of the microbiome associated with FMS and other pain-related syndromes. For instance, it is known that a number of drugs have a significant impact on the gut microbial community. Apart from antibiotics, NSAIDs (104), antidepressant drugs (105) and proton pump inhibitors (106), to name a few, can significantly alter the profile of the gastrointestinal microbiome. Many of the patients included in the current study reported a regular intake of at least one of these drugs that might interact with certain bacterial strains and thus obscure microbial alterations caused by the underlying disease.

Some 40% of the patients with FMS studied here confessed regular consumption of cannabis extracts, either via an oily solution (dronabinol) or smoked. There is some experimental evidence that THC and endocannabinoids have an effect on the gut microbiota (107-109) in animal studies. In addition, the administration of an endocannabinoid has been reported to blunt neuropathic pain which in an experimental model is associated with changes in gut microbial composition (109). However, the evidence for a role of the cannabinoid system in the interaction between microbial disturbances and pain is still scarce (110).

Cigarette smoking (tobacco) was also quite common in our study population (24% vs 0%, FMS vs HC, respectively). It is well known that smoking tobacco has an impact on intestinal disorders. For example, smoking can worsen Crohn's disease and plays a role in the development of peptic ulcer. On the other hand, it can improve symptoms of ulcerative colitis (111). Tobacco increases mucosal permeability and mucosal immune responses in the gut (111). A number of studies has also shown that tobacco smoking reduces the diversity of the gastrointestinal microbiota, although the results at the phylum and genus level are rather mixed and the pathophysiological relevance of the relationship between tobacco smoking and microbial disturbance is far from being fully understood (112).

Other environmental factors such as exercise might also be relevant, play a crucial role for the diversity of the gut microbiome and in this way have an impact on microbiota analysis in the current study. Although the study participants were not asked for such confounders, especially exercise is well known to have a major interaction potential with the gut microbiome (113-115).

Although moderate exercise is the primary therapeutic strategy for FMS, many of the patients do not follow these recommendations. Especially the FiQ asks for specific patterns of daily activities (66), but the present data reveal that many of the FMS patients studied here were unable to perform moderate exercise (3x30 min/week) on a routine basis.

Dietary habits, especially a very fat diet, have a specific impact on the gut microbiome. The classical Western diet, which is rich in fat and proteins and might include alcohol, has a severe influence on the microbiome (116,117). Specific bacterial strains (eg Bacteroides, Prevotella) have functional relevance regarding subjective well-being and co-diseases (IBS, diabetes mellitus type 2, depression, rheumatoid arthritis, to mention a few) (116). When the participants of the current study were asked for their dietary habits, no significant difference between the two study groups emerged. However, in valuing this observation a limitation of the method needs to be considered. Since all available questionnaires on dietary habits are quite long, I decided for the sake of better compliance to develop a shorter questionnaire together with Anna Eisenberger (chief dietician, University Hospital Graz). It refers to the most important parts of a healthy and balanced diet but has not yet been validated.

Clinical observations and recent studies suggest that paying attention to a healthy diet can strongly reduce symptoms in FMS. Dietary strategies that are suggested, but not limited to, are a healthy balanced diet (rich in fruits and vegetables), anti-inflammatory diet (rich in anti-oxidants like olive oil and whole grain), low FODMAP (fermentable oligo-di-mono-saccharides and polyols that are hard to digest) and gluten-free diet. Identifying food triggers (eg alcohol, sugar) and avoiding them might also help (118). It is likely that the dietary modifications work in part via interaction with gut microbiome-brain communication.

Infections are environmental factors that are highly relevant to several aspects of FMS including gut microbiome, neuroinflammation and pain. Infections are a known trigger for the development of chronic widespread pain. Specifically, Borrelia, mononucleosis as well as Covid-19 have been implicated in the development of chronic widespread pain and the chronic fatigue syndrome/myalgic encephalomyelitis (119,120). Many of the patients of the current study have a history of at least one infection (see Table 8). The detailed pathophysiology and contribution of infections to FMS are under current research, and there is considerable evidence that the innate immune system plays an important role in FMS etiology, a concept that was also pursued in the current study.

4.2 Gut mucosal permeability and responsiveness of monocytes/granulocytes to chemoattractants/LPS

Related to the primary hypothesis that the gut microbiome is altered in FMS patients, I reasoned that LPS or FMLP, two factors originating from the gut microbiome, might have entered the intestinal wall through a leaky intestinal mucosa to subsequently alter the local and systemic immune system (121). To first test this hypothesis, biochemical parameters that are increased under conditions of a leaky gut (alpha 1 antitrypsin, histamine, zonulin, calprotectin) were determined in the feces of the study participants.

Although I saw slight alterations in some of these biochemical parameters between FMS patients and HC subjects, I was unable to find a significant difference between the study groups. As a conclusion, these findings do not provide any evidence for a leaky gut in the FMS patients studied here. Although the patients reported on a variety of abdominal symptoms (constipation, diarrhea), I did not obtain consistent clinical evidence for a leaky gut either. The biochemical analyses were performed by a commercial laboratory, which may be seen as a limitation because no information on quality assurance was provided. This also applies to the lack of validated physiological reference values or ranges of the analytes, as the “normal values” of the analytes were provided by the laboratory without adequate reference to specific published literature. It is therefore difficult to attribute any relevance to the finding that the fecal zonulin levels were higher than standard in both study groups. Zonulin has been established as a regulator of epithelial barrier function, and loss of barrier function secondary to upregulation of zonulin leads to uncontrolled influx of microbial antigens and metabolites (122). In view of the current findings it may in the future be worthwhile to analyze factors other than zonulin that are involved in the regulation of intercellular tight junctions and gut mucosal permeability and might be altered in FMS. In addition, it needs to be considered whether parameters related to gut mucosal permeability should in addition be determined in serum, because the levels measured in the feces may be metabolically altered and thus not reflect the conditions prevailing at the intestinal mucosa in situ. Fecal histamine levels were found to be discretely, but not significantly, lower in FMS patients than in healthy controls. The relevance of this observation also remains unclear. Histamine as a marker for mast cell degranulation might be expected to be elevated in FMS, given that histamine intolerance appears be a factor contributing to some FMS symptoms (123).

As in both IBS and FMS (20,26) inflammatory and immune factors are altered, I originally hypothesized that elevated levels of pro-inflammatory cytokines could be found in the FMS patients under study and that these changes might result from alterations in the gut

microbiome. In IBS a lower diversity of the gut microbiome has been reported, and the change in the composition of the gut microbial community has been related to an increase of intestinal permeability (26) and subsequent immune activation. Related to the hypothesis of a leaky gut in FMS patients I reasoned that an increased influx of microbial metabolites such as FMLP and LPS might enhance the activity of particular cells of the local and systemic immune system. As a consequence, the immune cells might be primed to release an increased amount of proinflammatory and pronociceptive mediators, which leads to peripheral and central sensitization and hyperalgesia.

This question was investigated in neutrophilic and eosinophilic granulocytes as well as monocytes collected from FMS patients and HC. In the experiments, neutrophilic and eosinophilic granulocytes obtained from FMS patients and HC subjects did not differ in their reactivity (change in cell shape) to LTB4 acting via leukotriene receptors or FMLP acting via formyl peptide receptors, a class of pattern recognition receptors. Like FMLP which is derived from bacteria, LTB4 derived from leukocytes under conditions of inflammation causes chemotaxis and chemokinesis of immune cells (124,125). The current observations indicate that FMS does not alter the reactivity of granulocytes to these chemoattractant factors of exogenous and endogenous origin.

LPS is a cell wall constituent of gram-negative bacteria which stimulates monocytes via the pattern recognition receptor TLR4 to release proinflammatory and pronociceptive mediators such as PGE2 and TNF-alpha (126). Although the release of these two mediators was nominally elevated in patients with FMS vs. HC, the difference was statistically not significant. Although the results of the present study indicate that the reactivity of granulocytes and monocytes to microbiome-derived factors is not significantly altered in FMS, the virtually enhanced release of PGE2 and TNF-alpha from FMS-derived monocytes deserves further investigation in future studies. Such an investigation might include a larger cohort of study participants in which monocyte-derived mediators other than PGE2 and TNF-alpha are also analyzed, perhaps in a metabolomics approach. LPS is not necessarily derived from the gut microbiota only but may also come from other microbial sources in the body such as airways, urogenital tract and skin. The observation that plasma interleukin-6 is elevated in FMS patients relative to HC by Erdrich et al. (103) supports the need to a broader approach that goes beyond the gut and its microbiome.

4.3 Quantitative sensory testing

Patients with FMS present clinically with increased sensitivity to various forms of sensory stimuli including non-painful (eg sound) and painful stimuli (127). To confirm altered pain processing in the FMS patients under study and relate it to the other parameters measured in the study, QST was performed.

"The results of QST imply that both peripheral and central sensitization processes contribute to the symptoms of FMS. The lower thresholds for thermal and mechanical stimuli (CPT, MDT, MPT) together with allodynia (DMA) prove the contribution of unmyelinated C-fibers and thinly myelinated A-delta fibers to pain sensitization. Üceyler et al. (14) *suggested that a small fiber neuropathy might contribute to chronic widespread pain in FMS. I was able to show that sensory conduction by Aβ-fibers in the lemniscal pain pathway (fine touch, vibration, proprioception) is altered in FMS. Especially dynamic mechanical allodynia and vibration detection threshold (DMA, VDT) show positive z-values. As a conclusion, peripheral sensitization seems to make an important contribution to chronic widespread pain, but on the basis of these findings it stays unknown whether additional central sensitization develops as a result of altered peripheral input or vice-versa"* (12).

Several other studies have been performed to evaluate QST in FMS (127-130), although they are hard to compare because they were often performed with disparate test protocols. I performed the current QST analysis according to the German Network on Research of Neuropathic Pain (DFNS) protocol (86), which is most commonly used in the Middle European geographic region. Nevertheless, some common and some specific manifestations of sensory processing in FMS emerge from the various QST studies. In the study of Pickering et al. (130), 24 female patients with FMS and 24 female HC underwent QST. The authors found lower CDT, higher HDT, higher CPT-temperature and lower HPT in FMS patients vs HC subjects (130), which is in gross consensus with my current findings. In addition, diffuse noxious inhibitory controls as examined by conditioned pain modulation were found to be non-functional, which points to deficient spinal pain modulation in FMS (130).

Staud et al. (127) performed research on 23 patients with FMS and 28 healthy volunteers and found not only increased heat and mechanical pain sensitivity but also augmentation of sound perception. From these observations it was concluded that hypersensitivity mechanisms in FMS patients are operant at several levels of the central nervous system (127). While auditory augmentation is assumed to be processed in the brain, the hypersensitivity to painful stimuli is

hypothesized to take place at the levels of nociceptive afferent neurons, spinal cord and brain (127).
Using the DFNS protocol of QST, Fasolino et al. (128) tested 57 patients diagnosed with FMS and found various degrees of changes in the QST parameters at the feet and upper back region. The most clinically meaningful abnormality was increased mechanical pain sensitivity, which is compatible with central sensitization (128). Histologic analysis revealed that small fiber pathology in FMS patients does not significantly impact on somatosensory system function of FMS patients in this study (128).

QST is also able to differentiate between a regional musculoskeletal pain syndrome such as temporomandibular disorders (TMD) from a widespread pain syndrome such as FMS. In this context, Pfau et al. (129) compared 23 TMD, 18 FMS and 18 HC subjects with each other. The QST parameters were not only different between the three study groups but also with regard to the body region tested. In TMD only mechanical and cold hyperalgesia was found in restricted body regions while in FMS a more widespread hypersensitivity to cold and pressure as well as DMA were observed (129).

In this context it need also be mentioned that IBS, as stated earlier, often co-exists with FMS (59). Both conditions present with a dysfunction in central pain processing and both are believed to involve central sensitization and altered pain perception (allodynia, hyperalgesia). As discussed above, in the current study I was able to confirm peripheral and central hypersensitivity to pain with QST. Studies using QST in IBS patients have shown that conditioned pain modulation is diminished, which suggests that endogenous pain control by inhibitory descending pathways is impaired (131). A similar deficit in endogenous pain control has been described in FMS patients (2).

4.4 Psychopathological profile

Apart from the FMS-related alterations in pain processing as disclosed by QST, my study showed that FMS is associated with marked mental disturbances as deduced from the psychopathological profile of the patients. *"The current data clearly show that psychological stress but also the anxiety, depression and symptom severity scores are elevated in patients with FMS vs. HC subjects (DASS-S 11.2 vs. 2.45) and their quality of life is impaired"* (12).

Many FMS patients associate stressors with the onset or exacerbation of their condition, and the observation of elevated psychological stress in the present study is consistent with the evidence that FMS patients can present with reduced levels of resilience and a deficit in stress

coping strategies (2). The impaired capacity to cope with stress is frequently associated with a propensity to develop post-traumatic stress disorder (PTSD), anxiety and/or mood disorders (2). In this context it need also be mentioned that both FMS and IBS, which display a number of mechanistic and symptomatic similarities, also share a dysregulation of the stress-response system (2,101) in which the hypothalamic-pituitary-adrenal (HPA) axis plays a significant role (132). This system controls the reactions to stress and has a regulatory influence on digestion, immune system, mood and emotions, partly by a feedback mechanism to the brain from blood cortisol which is released from the adrenal cortex (132).

Depression is a common comorbidity in FMS as the lifetime prevalence of depressive disorders in FMS is 40-80%, which severely affects the quality of life (2,129). FMS patients with depression experience greater pain sensitivity than patients without such a disorder, a relationship that is referred to as cognitive–emotional sensitization to pain (2). The association of FMS and depressive disorders are also reflected by a number of similarities which may be due to common or related mechanisms:

- Similar symptoms like mood disturbances, sleep disorders and fatigue (2,129),
- Similar biological and/or genetic background: gene polymorphisms, as the two sets of disorders can occur in so called “depressive families” (2),
- Similar psychological background: eg PTSD which is common in both diseases (2).

The relationship between FMS and depression is bidirectional. Depressive mood disorders can induce central pain sensitization and lower nociceptive tresholds while chronic pain is a factor that causes mood changes which subsequently can develop to depressive disorders (2). Pharmacologically, therefore, both disorders are frequently treated in a similar way (eg with antidepressant drugs such as SNRIs).

Given the complexity of FMS and the insufficient knowledge of its underlying mechanisms in the peripheral and central nervous system, much research lies ahead to better understand the disorder in its pain and mental manifestations and develop more effective treatment strategies. Within the last 20 years, intensive research efforts have evaluated brain function and connectivity in FMS patients with various imaging techniques including functional magnetic resonance imaging (fMRI) which is a tool that measures brain activity by detecting changes associated with blood flow.

An early fMRI assessment of the cerebral response to painful pressure in FMS patients has already shown that brain activation is not only evident in somatosensory but also in emotion-related regions of the brain (133). A combination of several neuroimaging techniques, as reviewed by Gracely and Ambrose (134) and Napadow and Harris (135), has enabled

investigators to focus not only on the primary symptom of FMS, widespread pain, but also on the associated secondary symptoms including depression, catastrophizing, and cognitive dysfunction. Taken together, the studies provided a detailed overview of which regions (especially primary and secondary somatosensory cortex, insula and anterior cingulate cortex) are altered in their activity following a pain stimulus and how the activity and connectivity of these regions is disturbed in FMS patients.

The advances that have been achieved by these neuroimaging efforts have been published in numerous studies and summarized in reviews and meta-analyses. For instance, a fMRI study by Jensen et al. (136) involving 28 patients with FMS and 14 HC focused on pain dysregulation in FMS by examining the functional connectivity of the pain modulatory network in the brain. The study showed that the connectivity of the cerebral pain inhibitory network (rostral anterior cingulate cortex to amygdala, hippocampus, and brainstem, thalamus to orbitofrontal cortex) during calibrated pressure pain was much less in FMS patients than in HC (136). The authors concluded that the dysfunction of the descending pain modulatory network plays an important role in the maintenance of FM pain (136).

A meta-analysis of 1264 subjects from 37 studies published by Dehghan et al. (137) set out to explore common ground as to which brain areas known to be important for pain processing are altered in FMS. Despite the differences in stimulation paradigms, tasks, statistical evaluation of neuroimaging data and the high variability of findings, a number of commonalities could be identified. Thus, FMS patients differ from controls with regard to functional and structural changes in the insula, amygdala, anterior/mid cingulate cortex, superior temporal gyrus, primary and secondary somatosensory cortex, and lingual gyrus (137). As the authors point out, however, it is not clear whether the reported findings are associated with chronic pain in general or are unique features of patients with FMS (137).

4.5 Limitations

As already mentioned in some instances, the current study has some limitations that, on the one hand, need to be pointed out and, on the other hand, call for further studies whose direction might to some degree be guided by the limitations and failures of the current investigation. First, the number of participants in the study (25 FMS vs 26 HC) was small with a high drop-out rate of about 20%. This represents most probably a limitation in the analysis of the gut microbiome, given that a study with a larger study cohort (105 FMS vs 54 HC) was able to identify some discrete differences in the gut microbial community (27). In this respect, it needs also to be considered that the collection of bacterial 16S rRNA in stool samples does not reflect

the native microbial community residing in the mucosa of the gastrointestinal tract (138). In addition, analysis of the microbiome in the gut only provides a limited map of the microbial community colonizing the human body. Second, it is not known whether the collection of stool in tubes with preserving fluid impacts on the analysis relatively to other sampling methods. Third, the stool samples of two FMS patients could not be used for microbiome analysis due to the low quality of the PCR results. Fourth, 3 patients that recorded regular intake of antibiotics within the last three months were included in the study. Of course, this might lead to a considerable bias in microbiome analysis. All three subjects confirmed that they completed the antibiotic treatment at least 10 days before they collected the stool samples. In addition, as discussed elsewhere, other medications taken by the study participants may have had an impact on the microbiome and other variables measured in this study. However, these confounders are difficult to avoid, because it is considered unethical to deprive patients of the medications they benefit from. Fifth, several lifestyle factors such as exertive exercise may have had a relevant impact on gut microbiome.

4.6 Conclusions

Characterization of FMS patients relative to healthy controls by QST has convincingly shown that the cold pain threshold, pressure pain threshold, vibration detection threshold, mechanical detection threshold and mechanical pain threshold are lowered and dynamic mechanical allodynia is present.

These findings confirm the presence of peripheral sensitization of the sensory afferent system in FMS patients. In conjunction with the psychometric alterations seen in FMS it is very likely that central sensitization also contributes to the hyperalgesia in FMS.

Psychometric analysis of FMS patients relative to HC subjects disclosed multiple psychopathological alterations including psychological stress, anxiety, depression and elevated symptom severity along with an impairment of the quality of life.

The substantial alterations in the nociceptive and emotional-affective system of FMS patients calls for an analysis of the pathophysiological mechanisms underlying this complex pain and mental disorder.

Specific experiments conducted to this end do not support the hypothesis that a disturbance of the gut microbiome, an increase of gut mucosal permeability facilitating the influx of microbial metabolites and an increased reactivity of monocytes and granulocytes to microbial immunostimulants contribute to the etiology of FMS.

In summary, the current study provides important new insights and extends existing knowledge about specific alterations in the peripheral and central pain mechanisms and mental disturbances of FMS patients. Future analyses of the underlying pathomechanisms of this systems disorder should cover analyses of all microbial communities colonizing the human body (gut, urogenital tract, airways and skin) and a metabolomics analysis of potential mediators at all levels from the periphery to the brain.

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Appendix

FMS-Questionnaire

Universitätsklinik für Anästhesiologie und Intensivmedizin

Interdisziplinäre Schmerzambulanz

Medizinische Universität Graz

FMS Fragebogen

gemäß S3-Leitlinie 2017

I. Bitte geben Sie an, wie ausgeprägt die folgenden Beschwerden in der letzten Woche bei Ihnen waren, indem Sie das entsprechende Kästchen ankreuzen.

	nicht vorhanden	geringfügig oder mild ausgeprägt und/oder gelegentlich auftretend	mäßig oder deutlich ausgeprägt und/oder oft vorhanden	stark ausgeprägt: ständig vorhandene, lebensbeein-trächtigende Beschwerden
Tagesmüdigkeit	☐	☐	☐	☐
Probleme beim Denken oder Gedächtnis	☐	☐	☐	☐
Morgenmüdigkeit (nicht erholsamer Schlaf)	☐	☐	☐	☐

II. Wurden Sie in den letzten 6 Monaten durch eines der folgenden Symptome geplagt?

Schmerzen oder Krämpfe im Unterbauch: ☐ Ja ☐ Nein
Depression: ☐ Ja ☐ Nein
Kopfschmerz: ☐ Ja ☐ Nein

III. Bitte geben Sie an, ob Sie in den letzten 7 Tagen Schmerzen oder Berührungsempfindlichkeit in den unten aufgeführten Körperregionen hatten.

Bitte kreuzen Sie das jeweilige Kästchen an, wenn diese Körperregion schmerzhaft oder druckempfindlich ist.

☐ Kreuz ☐ Oberer Rücken (Brustwirbelsäule) ☐ Nacken ☐ Brustkorb ☐ Bauch	☐ Hüfte, links ☐ Hüfte, rechts ☐ Oberschenkel, links ☐ Oberschenkel, rechts ☐ Unterschenkel, links ☐ Unterschenkel, rechts	☐ Schulter, links ☐ Schulter, rechts ☐ Oberarm, links ☐ Oberarm, rechts ☐ Unterarm, links ☐ Unterarm, rechts	☐ Kiefer, links ☐ Kiefer, rechts
☐ in keiner der genannten Körperregionen Schmerzen			

IV. Waren die Beschwerden, die in den Fragen I-III aufgeführt sind, in der Regel in den letzten 3 Monaten vorhanden?

☐ Ja ☐ Nein

Diet Questionnaire

Wie oft haben Sie in den letzten 3 Monaten Milch (einschließlich Milch für Kaffee, Müsli) getrunken?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Milch trinken, wie viel trinken Sie davon meistens?

½ Glas (oder weniger)	
1 Glas (200 ml)	
2 Gläser	
3 Gläser	
4 Gläser (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Topfen, Joghurt, Buttermilch oder Kefir gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Topfen, Joghurt, Buttermilch oder Kefir essen, wie viel essen Sie davon meistens?

½ Becher (oder weniger)	
1 Becher (200 g)	
2 Becher	
3 Becher	
4 Becher	

Wie oft haben Sie in den letzten 3 Monaten zuckerhaltige Erfrischungsgetränke (z. B. Cola, Limonade, Eistee, Malzbier, Energiegetränke) getrunken? Nicht gemeint sind Light-Getränke.

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie zuckerhaltige Erfrischungsgetränke trinken, wie viel trinken Sie davon meistens?

½ Glas (oder weniger)	
1 Glas (200 ml)	
2 Gläser	
3 Gläser	
4 Gläser (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Bier/Wein getrunken?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Bier trinken, wie viel trinken Sie davon meistens?

½ Flasche (oder weniger)	
1 Flasche (330 ml)	
2 Flaschen	
3 Flaschen	
4 Flaschen (oder mehr)	

Wenn Sie Wein trinken, wie viel trinken Sie davon meistens?

1 Glas (125 ml)	
2 Gläser	
3 Gläser	
4 Gläser	
5 Gläser (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten hochprozentige alkoholische Getränke getrunken?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie hochprozentige alkoholische Getränke trinken, wie viel trinken Sie davon meistens?

½ Glas (oder weniger)	
1 Glas (2 cl)	
2 Gläser	
3 Gläser	
4 Gläser (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Vollkornbrot oder Vollkornbrötchen gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Vollkornbrot oder Vollkornbrötchen essen, wie viel essen Sie davon meistens?

½ Scheibe oder ½ Brötchen (oder weniger)	
1 Scheibe oder 1 Brötchen	
2 Scheiben oder 2 Brötchen	
3 Scheiben oder 3 Brötchen	
4 Scheiben (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Weißbrot oder Brötchen (auch Laugenbrötchen, Fladenbrot) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Weißbrot oder Brötchen essen, wie viel essen Sie davon meistens?

½ Scheibe oder ½ Brötchen (oder weniger)	
1 Scheibe oder 1 Brötchen	
2 Scheiben oder 2 Brötchen	
3 Scheiben oder 3 Brötchen	
4 Scheiben (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten rotes Fleisch (z.B. Hamburger, Steak) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie rotes Fleisch essen, wie viel essen Sie davon meistens?
Mit einer Portion ist etwa 1 Kotelett, 1 Steak oder 1 Schnitzel gemeint.

¼ Portion (oder weniger)	
½ Portion	
1 Portion	
2 Portionen	
3 Portionen (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Wurstprodukte (z.B. Bratwurst, Frankfurter, Käsewurst…) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Wurstprodukte essen, wie viel essen Sie davon meistens?

½ Scheibe	
1 Scheibe	
2 Scheiben	
3 Scheiben	
4 Scheiben (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Fisch (z. B. Seelachs, Forelle) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Fisch essen, wie viel essen Sie davon meistens?
Mit einer Portion sind 1 Fischfilet oder 4 Fischstäbchen gemeint.

¼ Portion (oder weniger)	
½ Portion	
1 Portion	
2 Portionen	
3 Portionen (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Obst und Gemüse (z. B. Apfel, Kompott, Salat, gemischtes Gemüse) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Obst und Gemüse essen, wie viel essen Sie davon meistens?
Mit einer Portion ist 1 handvoll Beilage, 1 Apfel, 1 Banane, 1 kleiner Beilagensalat gemeint.

½ Portion (oder weniger)	
1 Portion	
2 Portionen	
3 Portionen	
4 Portionen (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten frittierte Lebensmittel (z.B. Pommes Frites) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	

3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie frittierte Lebensmittel essen, wie viel essen Sie davon meistens?
Mit einer Portion ist etwa 1 durchschnittliche Portion Pommes, 1 Portion Kartoffelwedges gemeint.

¼ Portion (oder weniger)	
½ Portion	
1 Portion	
2 Portionen	
3 Portionen (oder mehr)	

Wie oft haben Sie in den letzten 3 Monaten Kuchen, Torten oder süße Backwaren (auch Muffins, Apfeltaschen, Sachertorte) gegessen?

Nie		1 Mal am Tag	
1 Mal im Monat		2 Mal am Tag	
2–3 Mal im Monat		3 Mal am Tag	
1–2 Mal pro Woche		4–5 Mal am Tag	
3–4 Mal pro Woche		Öfter als 5 Mal am Tag	
5–6 Mal pro Woche			

Wenn Sie Kuchen, Torten oder süße Backwaren essen, wie viel essen Sie davon meistens?

½ Stück (oder weniger)	
1 Stück	
2 Stück	
3 Stück	
4 Stück (oder mehr)	

Ernähren Sie sich ausschließlich vegetarisch (ausschließlich Nahrungsmittel pflanzlichen Ursprungs bzw. Produkte, die von lebenden Tieren stammen)?

Ja		Nein	

Ernähren Sie sich ausschließlich vegan (Verzicht auf sämtliche Nahrungsmittel tierischen Ursprungs)?

Ja		Nein	